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Identification of KPNB1 as a Cellular Target of Aminothiazole Derivatives with Anticancer Activity

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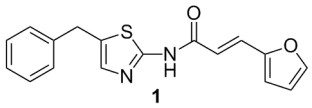
We found that aminothiazole derivative (*E*)-*N*-(5-benzylthiazol-2-yl)-3-(furan-2-yl)acrylamide (**1**) has strong anticancer activity, and undertook proteomics approaches to identify the target protein of compound **1**, importin β1 (KPNB1). A competitive binding assay using fluorescein-labeled **1** showed that **1** has strong binding affinity for KPNB1 (K_d: ~20 nM). Furthermore, through western blotting assays for KPNB1, KPNA2, EGFR, ErbB2, and STAT3, we confirmed that **1** has inhibitory effects on the importin pathway. KPBN1 appears to be overexpressed in several cancer cells, and siRNA-induced inhibition of KPNB1 shows significant inhibition of cancer cell proliferation, while leaving non-cancerous cells unaffected. Therefore, compound **1** is a promising new lead for the development of KPNB1-targeted anticancer agents. Fluorescein-labeled **1** could be a useful quantitative probe for the development of novel KPNB1 inhibitors.

Many 2-aminothiazoles exert their antitumor activity through the inhibition of various kinases.[1] In the search for new compounds with anticancer activity, we prepared aminothiazole derivatives by replacing the 2-amino position and the 4,5-substituted groups (Supporting Information). We examined the cytotoxicities of the resulting several hundred aminothiazole compounds against tumor cells; (*E*)-*N*-(5-benzylthiazol-2-yl)-3-(furan-2-yl)acrylamide (**1**) showed strong antiproliferative effects in the nanomolar concentration range, and the potency of compound **1** against ovarian (SK-OV3) and colon (HCT15) cancer cells was found to be respectively ~50- and 7-fold higher than that of doxorubicin (Table 1). A number of 2-aminothiazoles were also prepared in order to investigate the structure–activity relationship (SAR) of the inhibitory potency of compound **1**. In evaluating the antiproliferative effects of the compounds toward the SK-OV3 cell line in a 48 h cytotoxicity assay, the 2-furylethenolyl group was found to clearly provide the best inhibitory potency among the various acyl groups, and the benzyl group provided better results than the other 5-substituted groups and 4-substituted benzyl group (Supporting Information). At a concentration of up to 1 μM, compound **1** had no (or only marginal) inhibitory effects on several normal cell lines such as *E. coli*, yeasts, fungi, and normal BJ cells. Our results are consistent with those reported in a previous study, in which it was found that similar 2-aminothiazole analogues have selective effects on carcinoma cells and exert antiproliferative effects specifically on DU-145 human prostate carcinoma cells.[2] Therefore, these results indicate that aminothiazoles may exert antiproliferative effects via a specific cellular target.

Table 1. IC_{50} values of compound **1** in various human cell lines.



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Compd	IC_{50} [nM][a]					
	A549	SK-OV3	SK-MEL2	HCT15	XF498	A431
1	38 ± 2	1.3 ± 0.6	11 ± 1	5.4 ± 0.5	22 ± 2	43 ± 2
Dox[b]	2 ± 1	52 ± 2	1.7 ± 0.5	36 ± 2	21 ± 1	2.0 ± 0.3

[a] Values are the means ± SEM of $n = 3$ experiments. [b] Reference compound doxorubicin.

Many 2-aminothiazole derivatives exert their inhibitory activity against kinases by competing for the ATP binding site; therefore, we examined the effects of **1** on a panel of cancer-related kinases using a kinase profiling service (www.emdmillipore.com/Signaling). However, at 10 μM, compound **1** showed no or < 50% inhibition of the 109 different protein kinases examined, indicating that the inhibitory activity of **1** on cancer cells is not due to the cytotoxicity caused by the inhibition of the protein kinases. Therefore, although these aminothiazole compounds are promising new leads for the development of novel anticancer agents, no studies have provided information about a molecular target.

It has been reported that immobilization of the widely used kinase inhibitor and optimized biochemical conditions for affinity chromatography permit the enrichment and identification of several unknown protein targets of the kinase inhibitor.[3] To enrich potential targets of **1**, we also performed affinity chromatography; the affinity and control columns were prepared by coupling compound **2** to epoxy-activated Sepharose 6B and incubation of the solid support with ethanolamine, respectively (Figure 1). Then, whole-cell lysates obtained from 2.5×10^8 HeLa and SK-OV3 cells used as starting materials were evenly loaded on the affinity matrix and the control matrix, and the binding proteins were separated under optimized adsorption and elution conditions as described in the Supporting Information. The affinity-purified proteins were identified using two proteomic approaches: 2D gel-based mass spectrometry and quantitative liquid chromatography tandem mass spectrometry

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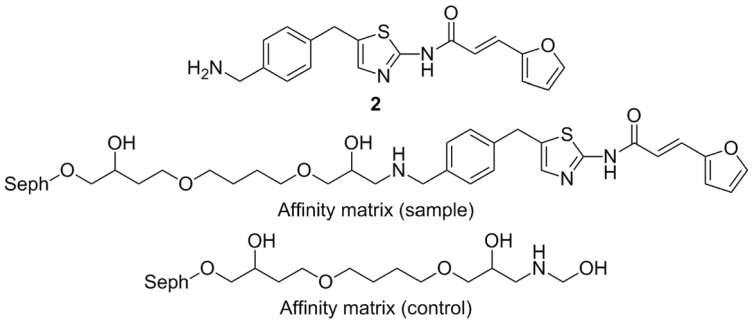
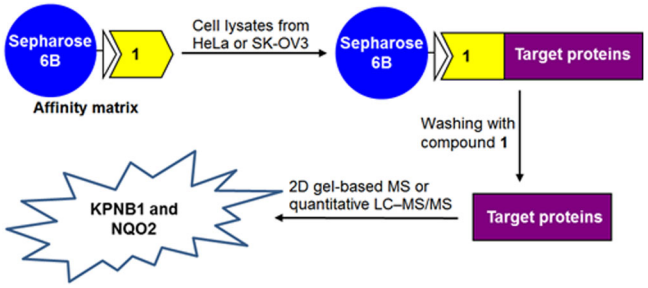


Figure 1. Structures of compound **2** and the affinity matrices.

(LC–MS/MS). 2D gel electrophoresis was performed under regular polyacrylamide gel electrophoresis (PAGE) conditions in the first dimension followed by sodium dodecyl sulfate (SDS)-PAGE in the second dimension. We identified 23 protein spots from the affinity-purified sample of HeLa cells, and 17 spots



from SK-OV3 cells (Figure S1, Supporting Information). To determine the extent of purification, we performed quantitative LC–MS/MS using an accurate and sensitive nanoLC-Orbitrap LTQ tandem mass spectrometer, which enables a repeat analysis of each sample of whole-cell lysates, affinity-purified proteins, and control eluents to statistically compare the peptide spectrum matches (PSMs) of the identified proteins, as described in the Supporting Information. After normalization of the PSMs of each sample, we calculated protein purification folds (Tables S1 and S2, Supporting Information). From the proteins highly purified using affinity chromatography, two compound-**1**-binding proteins, KPNB1 (importin β1) and NQO2 (NRH:quinone oxidoreductase 2), were found in HeLa and SK-OV3 cells; these were considered to be the target protein candidates of **1**.

The first candidate, NQO2, is known to reduce quinones and nitroaromatic compounds using a shortened nicotinamide derivative, nicotinamide riboside, which is not present in cells.[4] We examined the activity of a reconstituted NQO2 protein on the reduction of dichlorophenolindophenol (DCPIP) with NRH synthesized by the addition of calf intestinal alkaline phosphatase to nicotinamide mononucleotide in phosphate-buffered saline, pH 7.4, as described previously.[4] Reoxidation-reduced DCPIP occurred within a few minutes after saturation of the enzyme reaction; therefore, the concentration of NQO2 was limited to produce a 0.1-a.u. change in absorbance difference per minute. This reaction velocity was sufficient to show the concentration–inhibition curve of compound **1** that had little or no effect on NQO2 up to a concentration of 1 mM, which exceeded the solubility of the compound (Figure S2, Supporting Information). This inhibitory effect of **1** was even lower than that of dicoumarol, with a half-maximal inhibitory concentration (IC_{50}) of 708 μM, which is generally resistant with NQO2.[5] The concentration–inhibition curve indicated that NQO2 is not an effective target protein of **1**, although it effectively bound to a sample affinity resin and **1** eluent by the optimized buffer systems in the affinity purification procedure.

The other candidate, KPNB1, is involved in the nuclear translocation of proteins.[6] KPNB1 recognizes a nuclear localization sequence (NLS) on cargo proteins to be transported to the nucleus and interacts with KPNA (importin α1) to import proteins containing a classical NLS.[7] KPNB1 can also directly interact with the cargo proteins and transport them into the nucleus via a non-classical transport pathway.[8] In cancer cells, KPNB1 is reported to play a role in the nuclear translocation of phosphorylated STAT3 (pSTAT3), cell membrane receptors, and receptor tyrosine kinases (RTK) containing NLS sequences, such as ErbB2 and epidermal growth factor receptor (EGFR), from the cell surface to the nucleus.[9] Then, nuclear EGFR and ErbB2 interact with several proteins including cyclin D1, cyclooxygenase-2 (COX-2), aurora kinase A, c-Myc, breast cancer resistant protein (BCRP), STAT1, and B-Myb, activating their transcription, thus leading to accelerated cell-cycle progression.[10]

To confirm the inhibitory effects of **1** on the function of KPNB1, we performed western blotting using EGFR, ErbB2, STAT3, and pSTAT3 in whole-cell lysates and nuclear fractions of SK-OV3 treated with various concentrations of **1**. Whereas the nuclear levels of EGFR, ErbB2, STAT3, pSTAT3, KPNB1, and KPNA decreased in a concentration-dependent manner after treatment with **1**, their levels in whole-cell lysates were nearly constant (Figure 2). Results of western blotting showed that compound **1** inhibits the classical importin pathway of KPNB1 and KPNA, and in turn decreased the nuclear transport of EGFR, ErbB2, STAT3, and pSTAT3. The EGFR superfamily has a significant impact on tumor initiation, cell-cycle progression, tumor progression, and drug resistance. These membrane-bound proteins have been intensively studied to block RTK-centered signaling transduction networks, including the downstream pathways of STAT3. However, the internalization and nuclear translocation of these membrane receptors induced by specific agents highlight the need to modify the strategy for the discovery of new anticancer agents, considering the nuclear levels of RTKs as important markers in cancer cells.[11]

Regarding the binding effect of **1** on KPNB1, methods for determining small-molecule binding affinity and analyzing direct molecular interactions include surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). However, we were unable to obtain any information about binding affinity using these methods because these are easily influenced by exogenous factors. Therefore, we prepared fluorescein-labeled

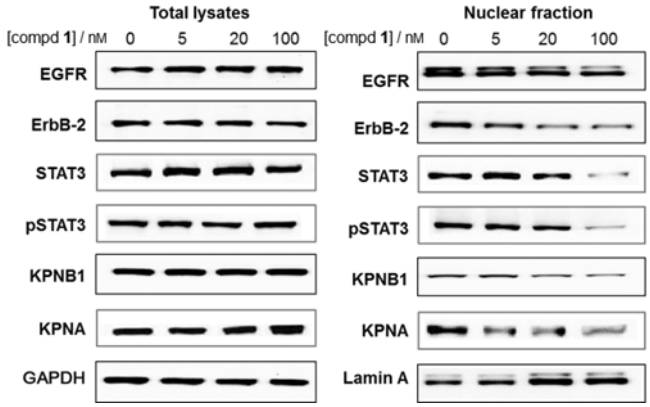


Figure 2. Effect of compound **1** on in vitro nuclear import in SK-OV3 cells: Cells were treated with compound **1** at various concentrations for 24 h at 37 °C, after which time nuclear protein extraction was performed using an NE-PER nuclear extraction reagent. Proteins from the nuclear fraction and total lysates were then analyzed by western blotting with antibodies to ERBB-2, EGFR, KPNB1, KPNA2, STAT3, pSTAT3, Lamin A, and GAPDH.

1 for competitive binding assays.[13] To determine the K_d value of the fluorophore probe, we incubated compound **1** (100 nM) for 30 min with increasing concentrations of KPNB1 (0–2 μM). The fluorescence intensity of fluorescein-**1** increased by ~50% upon addition of KPNB1 (Figure 3a). The affinity of fluorescein-**1** for KPNB1 was ~10 nM, as determined by nonlinear curve fit of data according to the 1:1 binding model (Figure 3b). We used a competition assay to determine the value of K_d for the **1**–KPNB1 complex by using the K_d value of the complex between fluorescein-**1** and KPNB1. Upon titrating the fluorescein-**1**–KPNB1 complex with various concentrations of **1**, the displacement of fluorescein-**1** by compound **1** was observed as a decrease in fluorescence (Figure 3c), giving a K_d value of ~20 nM.

Active site binding modes of compound **1** were then investigated using ICM-Pro 3.6-1d molecular docking software (Molsoft) to gain structural insight into mechanisms by which **1** inhibits KPNB1. As shown in Figure 4, the docking of compound **1** into the pocket around S476 has the strongest binding energy at 14.41 kcal mol^{-1}. This model has three hydrogen bonding sites between **1** and KPNB1, each of which have a distance within 2.6 Å (dotted lines in Figure 4b, S476···O: 2.58 Å, E479···O: 2.63 Å, and S475···O: 3.09 Å). Participating in hydrogen bonding are the amide oxygen atom and the hydroxy group of S476, the amide NH group of E479, and the furan ring oxygen atom and hydroxy group of S475. These three hydrogen bonding interactions fix the binding direction of **1** by placing the three positions in a nearly planar structure determined by central extended π bonding in compound **1**. Another weak interaction (3.54 Å) is present between the phenyl ring in compound **1** and the side chain of P440 through CH–π hydrogen bonds. This unconventional hydrogen bonding is known to be important in the recognition of proline residues.[12]

KPBN1 appears to be overexpressed in several cancer cell types, and siRNA-induced inhibition of KPNB1 shows significant inhibition of cancer cell proliferation, but does not affect normal cells.[13] Results from these studies indicate that KPNB1 is a potential target, and inhibition of KPNB1 can be used as a novel therapeutic approach for the treatment of cancer. A pyrrole compound, karyostatin 1A, importazole, peptides, and small protein-like peptidomimetics bind to KPNB1 and inhibit the classical importin pathway.[14] However, some inhibitors are not cell-permeable and their K_d values range single-digit micromolar to several hundred micromolar. Because its cytotoxicity

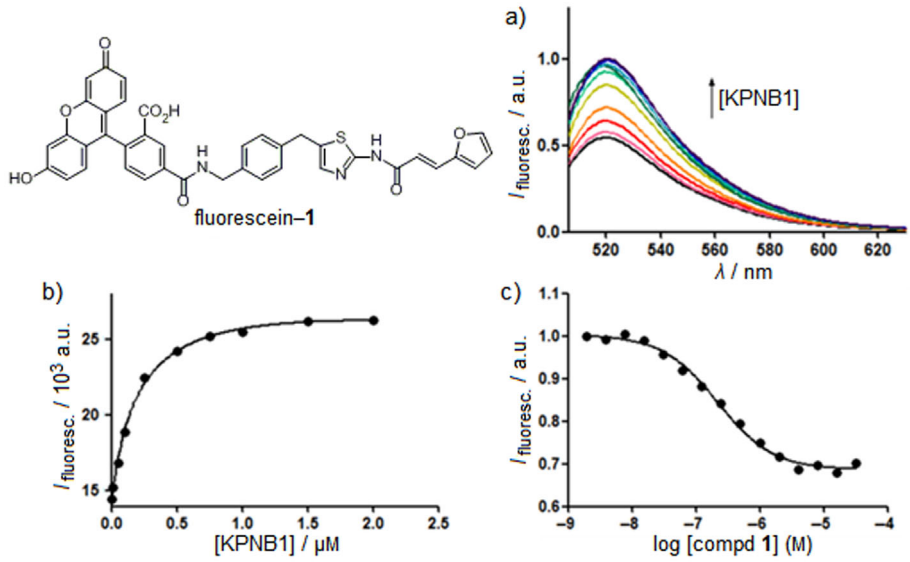


Figure 3. Competitive binding assays: a) Emission spectra of 100 nM fluorescein-**1** excited at 491 nm with the addition of 0–2 μM KPNB1. b) Graphical analysis of the fluorescence emission peak (520 nm) of fluorescein-**1** with increasing concentrations of KPNB1. c) Titration curve of the fluorescein-**1**–KPNB1 complex with various concentrations of compound **1**. The obtained polarization values were analyzed by a nonlinear regression fitting program using GraphPad Prism software (version 5.0).

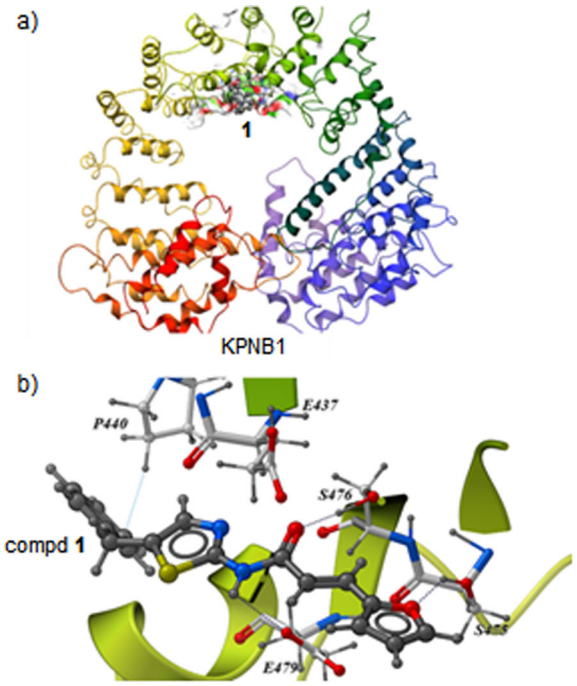


Figure 4. Binding modes of compound **1** in KPNB1. The initial structure of compound **1** in water was optimized with density functional theory (DFT) methods implemented in Gaussian 09. The flexible **1** was docked to a grid representation of KPNB1 (PDB ID: 2QNA) as receptor and assigned a score for complex quality according to the iterative conditional modes (ICM) method using ICM-Pro 3.6-1d docking software at Molsoft (www.molsoft.com). a) Binding position of **1** in KPNB1. b) Binding modes of **1** at the binding sites in KPNB1.

(~1–5 nM) is more potent than its binding affinity for KPNB1 (~20 nM), compound **1** may also elicit cytotoxicity through other targets. Nevertheless, our results indicate that **1** is a promising new lead compound with higher potency for the development of KPNB1-targeted anticancer agents. Moreover, fluorescein-**1** could be a useful quantitative probe for the identification of a novel KPNB1 inhibitor by competition assays with compound libraries.

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Keywords: anticancer agents · competitive binding · KPNB1 · LC–MS/MS · proteomics

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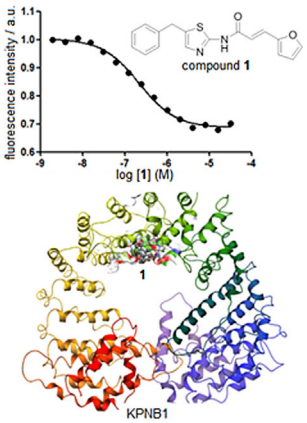
An *importin* discovery: A proteomics approach was used to identify the target protein (KPNB1) of compounds with anticancer activity. A competitive binding assay using a fluorescein-labeled compound showed that the compound has strong binding affinity for KPNB1, suggesting that the KPNB1-targeted anticancer agents and fluorescein-labeled compound could be useful quantitative probes for the development of novel KPNB1 inhibitors.



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