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In Vitro Selection of Anti-Akt2 Thioether-Macrocyclic Peptides Leading to Isoform-Selective Inhibitors

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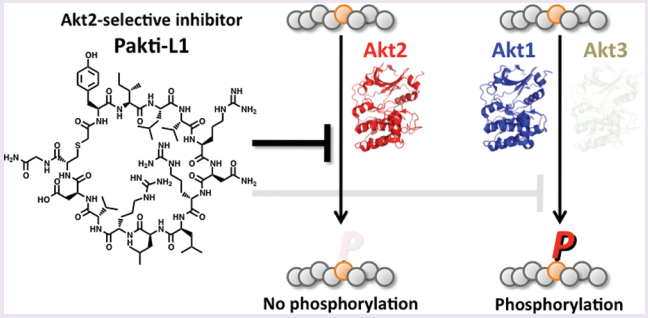
Supporting Information

ABSTRACT: The Akt kinase family, consisting of three isoforms in humans, is a well-validated class of drug target. Through various screening campaigns in academics and pharmaceutical industries, several promising inhibitors have been developed to date. However, due to the mechanistic and structural similarities of Akt kinases, it is yet a challenging task to discover selective inhibitors against a specific Akt isoform. We here report Akt-selective and also Akt2 isoform-selective inhibitors based on a thioether-macrocyclic peptide scaffold. Several anti-Akt2 peptides have been selected from a library by means of an *in vitro* display system, referred to as the RaPID (Random nonstandard Peptide Integrated Discovery) system. Remarkably, the majority of these "binding-active" anti-Akt2 peptides turned out to be "inhibitory active", exhibiting IC_{50} values of approximately 100 nM. Moreover, these peptides are not only selective to the Akt kinase family but also isoform-selective to Akt2. Particularly, one referred to as Pakti-L1 is able to discriminate Akt2 250- and 40-fold over Akt1 and Akt3, respectively. This proof-of-concept case study suggests that the RaPID system has a tremendous potential for the discovery of unique inhibitors with high family- and isoform-selectivity.



In human cells, the Akt family, belonging to the serine/threonine (S/T) kinase family, plays critical roles in regulating various signal transduction pathways.[1] Because misregulation of Akt causes alterations of apoptosis, cell proliferation, and metabolisms depending upon the Akt isoforms, it is of great interest for academics, as well as pharmaceutical industries, to develop inhibitors against this kinase family. Three isoforms, Akt1, Akt2, and Akt3, are known in the human Akt family. Akt1 activates the translocation of nuclear factors, such as NF-κB (nuclear factor of kappa light polypeptide gene enhancer in B-cells),[2] which suppress apoptosis in a transcription-independent manner; thereby it promotes cell survival. In fact, elevated expression and activation of Akt1 have been observed in human gastric, breast, prostate, and ovarian cancers, implying that it is a major factor in the cancer development, *i.e.*, Akt1 is an oncogene.[3,4] Similar events involving Akt2 have been also observed in various carcinomas,[5−7] consistent with a view that Akt2 plays a similar role as Akt1 in developing malignant phenotype of these cancers. However, data generated by experiments involving Akt1 or Akt2-deficient transgenic mice have suggested that Akt2 likely plays a specific role in the insulin receptor signal transduction, implying that Akt2 is an important drug target for controlling diabetes mellitus.[8] The function of Akt3 is least understood among the Akt family, but it is expressed predominantly in the brain.[9] Presumably, it contributes to the development of the brain through the activation of growth factor mediated signal transductions.[10]

Because of the therapeutic significance of Akt in cancers and other diseases, a number of screening campaigns of molecules have been conducted to yield potent Akt inhibitors (representative Akt inhibitors along with their potency and selectivity against a specific Akt isoform over other isoforms and kinase family are summarized in Supplementary Table 1). There are four classes of inhibitors: (I) those that compete for binding to the ATP-binding site, (II) those that bind to the pleckstrin homology domain (PH domain), (III) those that bind to an allosteric site(s), and (IV) those derived from substrate peptide sequences and others.

The class I inhibitors, represented by A674563 and GSK690693, have remarkable potencies against all Akt isoforms with low nanomolar IC_{50}'s.[11−15] However, since all kinase families have structurally similar ATP-binding sites, they generally show broad spectra of inhibitory activities against many S/T kinases with comparable potencies, thus interacting with various cellular kinases in a nonselective manner. On the other hand, since the PH domain of the Akt family is unique over other kinases, the class II inhibitors are inherently Akt-selective, but because all Akt members have nearly identical PH domains, they are so far unable to distinguish a specific isoform from other isoforms.[16−21] Representative class III inhibitors are

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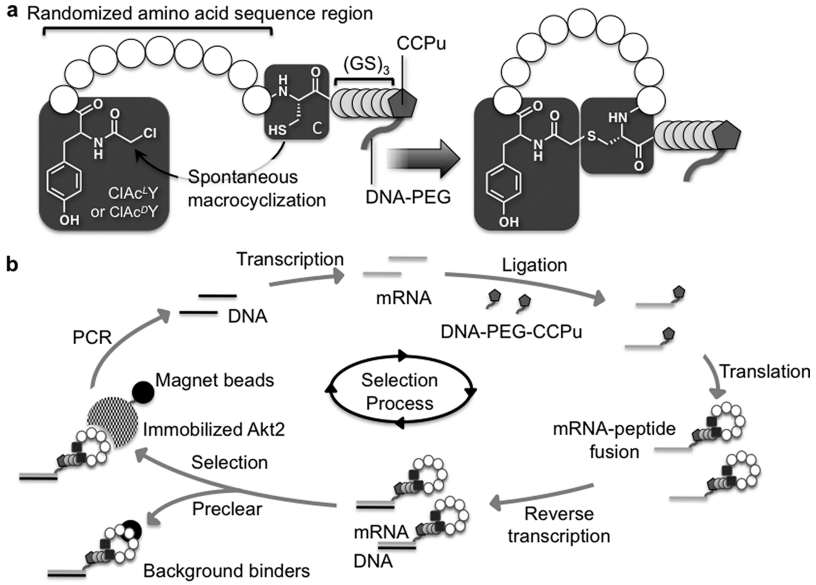

Figure 1. Schematic presentation of the macrocyclic peptide library and RaPID selection. (a) A macrocyclic peptide library closed by a thioether bond. The randomized amino acid sequence region (white circles) consists of 4−12 amino acid residues. Spontaneous cyclization takes place between ClAc^{L}Y or ClAc^{D}Y initiator and sulfhydryl group of a cysteine (C) residue, both shown in dark gray boxes. Although the C residue is designated right after the random region in each peptide, a C residue or residues can also be appeared from the random region to form a smaller ring sized head and a tail containing the unreacted cysteine residue(s). The $(GS)_3$-linker peptide is shown in gray circles. 5′-CCPu-3′ and DNA-PEG regions in DNA-PEG-CCPu are shown in a dark gray pentagon and a dark gray line, respectively. (b) Selection using RaPID system. DNA and RNA libraries are shown in black and gray lines, respectively. The full-length Akt2 protein and Ni^{2+}-NTA magnetic beads are shown in a black-dotted circle and black circles, respectively.

MK2206, Akti-1, Akti-2, and Akti-1/2 (Akt inhibitor VIII), all of which are categorized as allosteric inhibitors.[22−25] Among them, Akti-1/2 has been extensively studied by the X-ray structure complexed with Akt1.[26] This inhibitor binds to the interface between "N-lobe" and "C-lobe" in the kinase domain and further interacts with the PH domain, resulting in stabilization of the ternary interactions. This ternary complex, referred to as a "PH-in" form, prevents phosphorylation of T308 and S473, inhibiting activation of the kinase domain. The IC_{50} value of Akti-1/2 is 58 nM against Akt1 compared with 4- and 40-fold elevated IC_{50} values against Akt2 and Akt3, respectively. On the other hand, Akti-2, which is structurally similar to Akti-1/2, exhibits a greater isoform-selectivity (70-fold against Akt1 over Akt2), but its potency dropped to 325 nM IC_{50}. The class IV inhibitors competitively bind to the protein substrate-binding site of Akt.[27−30] Although the isoform selectivity of this class of inhibitors is unknown in literature, they display marginal inhibitory potencies even *in vitro*.

Even though simultaneous inhibition of all members of the Akt family by ATP-competitive inhibitors gave a maximal efficacy for caspase-3/7 activation,[31] these inhibitors may have a risk of side effects caused by undesired inhibitions of non-Akt kinases. An approach to devise inhibitors binding to the PH domain has yielded Akt-selective inhibitors, but it has been yet difficult to install an isoform-selectivity into the inhibitors. Allosteric inhibitors have given the Akt- and isoform-selective properties, but it remains difficult to achieve high selectivity and potency simultaneously against a specific isoform. Therefore, it is still a difficult challenge to devise Akt- and isoform-selective inhibitors.

We here report a potent Akt2 isoform-selective inhibitor with a 100 nM IC_{50} discovered from a library of thioether-macrocyclic peptides with a complexity of 10^{12}, by means of an *in vitro* display system, referred to as RaPID (Random nonstandard Peptide Integrated Discovery) system (Yamagishi *et al. Chem. Biol.*, in press).

RESULTS AND DISCUSSION

Thioether-Macrocyclic Peptide Libraries. In 2008, we reported a new strategy to ribosomally express thioether-macrocyclic peptides involving an initiation codon reprogramming. To facilitate such a codon reprogramming for this study, we have devised a FIT (Flexible In vitro Translation) system where a custom-made *in vitro* translation system lacking methionine (M) is supplied with *N*-(2-chloroacetyl)-aminoacyl-tRNA$^{fMet}_{CAU}$ (ClAc-aa-tRNA$^{fMet}_{CAU}$) prepared by a flexizyme (flexible tRNA acylation ribozyme),[32−35] resulting in a reassignment of AUG initiator codon from M to the ClAc-aa. In this setup, the peptide translation of mRNA is initiated by the ClAc-aa, and a cysteine (C) residue installed at a downstream position in the sequence reacts with the 2-chloroacetyl group, giving macrocyclic peptide closed by a thioether bond (Figure 1a).[36,37] The cyclization takes place spontaneously without an additional reagent, yielding a clean desired product almost regardless of the length and sequence composition. In fact, we were able to prepare a macrocyclic peptide library with various lengths from random sequences of mRNAs.

In the present work, we constructed two thioether-macrocyclic peptide libraries initiated by either L or D isomers of *N*-(2-chloroacetyl)-tyrosine (ClAc^{L}Y or ClAc^{D}Y, respectively). To construct each peptide library, mRNA template libraries were designed to have AUG-$(NNK)_n$-UGC, where AUG and UGC assign ClAc^{L}Y or ClAc^{D}Y and C, respectively, which would undergo the thioether bond formation for cyclization; also where $(NNK)_n$ (N and K represent any of four bases and U or G, respectively) assign all possible 20 amino acids with a range of lengths between 4 and 12, *i.e.*, a mixture of peptides with a

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Table 1. Selected Peptides with Frequency of Appearance and Their IC_{50} Values against Akt Isoforms[b]

Peptide	Sequence	Frequency	IC_{50} [nM]		
			Akt2	Akt1	Akt3
Pakti-L1	Ac-^{L}YILVRNRLLRVDC-G-NH_2	28/37	110	>25,000	4,200
Pakti-L2	Ac-^{L}YWILITWPLVRRKC-G-NH_2	2/37	120	~1,000[a]	~1,000[a]
Pakti-L3	Ac-^{L}YWIVLTWPIVTRRC-G-NH_2	2/37	92	~1,000[a]	~1,000[a]
Pakti-L4	Ac-^{L}YTYWFVSMIC-G-NH_2	1/37	inactive	N.D.	N.D.
Pakti-L5	Ac-^{L}YIRRPWVPIMYLGC-G-NH_2	3/37	active	N.D.	N.D.
Pakti-L6	Ac-^{L}YILVRNRPLRVDC-G-NH_2	1/37	active	N.D.	N.D.
Pakti-D1	Ac-^{D}YAVRILGHYLQVGC-G-NH_2	35/37	active	N.D.	N.D.
Pakti-D2	Ac-^{D}YLSRRHGLLFLIRC-G-NH_2	1/37	inactive	N.D.	N.D.
Pakti-D3	Ac-^{D}YLSREFNLLFLVRC-G-NH_2	1/37	active	N.D.	N.D.

[a] IC_{50} values were estimated by the graph shown in Figure.2b and c [b] Peptides shown in this table were synthesized by standard solid-phase synthesis and applied to the inhibition assay of kinase activities. IC_{50} values were determined by the plot shown in Figure 3. Pakti-L5, L6, D1, and D3 inhibited Akt2 kinase activity (the accurate IC_{50} value was undetermined), whereas Pakti-L4 and D2 were inactive by a preliminary assay. N.D. denotes "not determined" in this study.

random region of these lengths would be expressed. It should be noted that the thioether-macrocyclic peptide library initiated with ClAc^{D}Y would cover different conformational sequence space from that initiated with ClAc^{L}Y since a single D-isomer in the macrocyclic scaffold would potentially alter the tertiary structural conformers from those in all-L-macrocyclic peptides. We referred the respective libraries to as ^{L}Y- and ^{D}Y-libraries for our convenience in discussion below.

To perform the selection of active species, these libraries were integrated with an *in vitro* display method, so-called mRNA display,[38,39] where puromycin (Pu) was ligated to the 3′-end of a synthetic hybrid oligonucleotide (DNA-PEG-CCPu; the DNA sequence is complementary to the 3′-end sequence of mRNA, followed by a poly(ethylene glycol) linker, deoxyribonucleotides CC, and puromycin), which allows for fusion with the peptide C-terminus (Supplementary Table 2). To facilitate the integration, we designed the above mRNA libraries to have three repeats of (GGC)(AGC) encoding $(GS)_3$ (G, glycine; S, serine) after UGC (encoding a cysteine residue), UAG stop codon, followed by a short stretch of RNA that hybridizes the DNA-PEG-CCPu. It should be noted that our FIT system using this display experiment did not contain release factor-1 (RF1), which usually terminates the elongation at UAG stop codon; instead, ribosome would be stalled at UAG codon and the efficiency of puromycin-peptide fusion should be enhanced. By means of this system, the estimated complexity generated by the $(NNK)_n$ library transcribed from the corresponding DNA library was approximately 10^{14}, and the efficiency of the peptide-puromycin fusion was generally more than 30% in our hands for representative model peptides with the range of lengths and degenerated sequences used in the library (data not shown). Therefore, we confidently estimated the diversity of the initial display library to be 10^{12} or more. We referred to this system enabling display of nonstandard macrocyclic peptide libraries as the RaPID (Random nonstandard Peptide Integrated Discovery) system (Yamagishi *et al. Chem. Biol.*, in press). Significantly, the C-terminal end of each thioether-macrocyclic peptide in the library is covalently linked to its encoding mRNA *via* Pu, and thus active species of peptide based on their binding capability against a therapeutic target could be enriched by RT-PCR (reverse transcription polymerase chain reaction). Therefore, the RaPID selection process can be repeated until an active population emerges in the selected pool.

Selection of Anti-Akt2 Macrocyclic Peptides. We performed *in vitro* selection of the nonstandard macrocyclic peptides against Akt2 (Figure 1b). The ^{L}Y- and ^{D}Y-libraries were independently applied to the RaPID selection against a full-length Akt2 that was immobilized on Ni^{2+}-NTA magnetic beads *via* the interaction with hexa-His residues at the Akt2's N-terminus. Prior to the selection against Akt2-beads, the respective libraries were treated with the Ni^{2+}-NTA magnetic beads (up to 12 times) to remove undesired background-binding peptide species in the pool, and the peptide fraction unbound to the beads was then applied to the selection against Akt2-immobilized beads. At the sixth round, we observed an appreciable enrichment of active population in the both pools monitored by the recovery amount of selected cDNA by RT-PCR, while the background binding to the beads was significantly suppressed (Supplementary Figure 1).

The enriched pool was cloned and individual colonies were arbitrarily picked for sequencing, yielding a total of 37 DNA sequences from each of the ^{L}Y- and ^{D}Y-libraries (Table 1). The most abundant ^{L}Y-clone, referred to as Pakti-L1, was found 28 times in 37 clones, and shared the common sequence with Pakti-L6 found once. Pakti-L1 consists of 11 residues originating from the random sequence region, giving a total body length of a 13-mer macrocyclic peptide without the linker peptide region. Two clones, Pakti-L2 and 3, were each found twice, and shared a common sequence motif, consisting of 12 residues originating from the random region (a total of 14-mer body length). Pakti-L4 and L5 were independently found once and thrice, respectively. On the other hand, the ^{D}Y-library was dominated by a single kind of a peptide; Pakti-D1 was found 35 times. Pakti-D2 and D3 were found once, respectively, and shared the common sequence motif. Interestingly, no Pakti-D peptides had sequence similarity to any Pakti-L peptides. This implied that, even though the random region originated from

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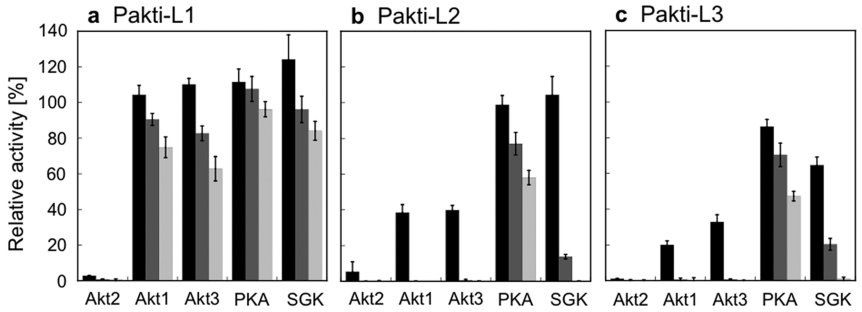

Figure 2. Inhibitory activities of Pakti-L1−L3 (a−c) against Akt isoforms and other kinases. (a) Akt- and isoform-selectivity of Pakti-L1. Black, gray, and light gray bars show observed activities of the kinase at the peptide concentrations of 1, 5, and 10 μM, respectively. The respective activity was defined as an average of activities of the kinase in triplicate in the presence of inhibitor relative to those in the absence of inhibitor. Error bars are shown as the standard deviations of the relative activities in triplicate. (b) Akt- and isoform-selectivity of Pakti-L2. (c) Akt- and isoform-selectivity of Pakti-L3.

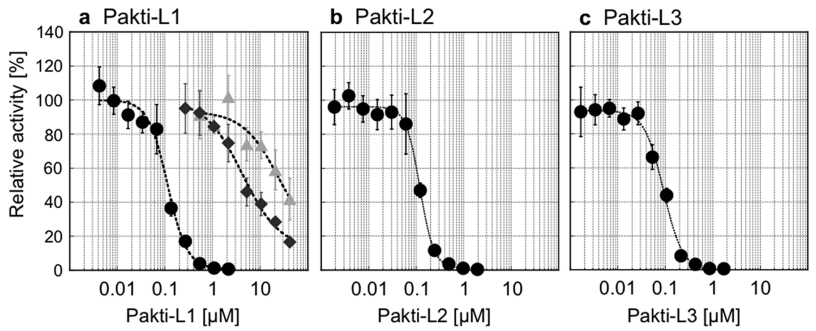

Figure 3. Titration of kinase activities as a function of Pakti-L inhibitor concentrations. (a) Inhibitory titration of Pakti-L1. Data plot in black circles are against Akt2. Those in light gray triangles and gray diamonds are against Akt1 and Akt3, respectively. IC_{50} values were determined by a curve fitting to a Hill equation generated by KaleidaGraph (Hulinks Inc.). (b) Inhibitory titration of Pakti-L2. (c) Inhibitory titration of Pakti-L3.

the same pool of mRNA sequences, the library composed of ^{L}Y-initiator covered different conformational sequence space from that of ^{D}Y-initiator.

Anti-Akt2 Macrocyclic Peptides Are Inhibitory Active. All peptides from the Pakti-L and -D families were chemically synthesized using a standard solid-phase method with an additional glycine-carboxamide at the C-terminus. The individual peptides were then subjected to kinase inhibitory screening using a universal S/T kinase assay system composed of biotin-labeled peptide substrates (HTRF KinEASE STK2 and STK3, the appropriate one of which was treated with the corresponding kinase). After the phosphorylation, the biotin in the substrate was captured with XL665-labeled streptavidin, and a phospho-specific monoclonal antibody labeled with Eu^{3+}-cryptate was used to detect the signal of time-resolved-fluorescence resonance energy transfer (TR-FRET). In this study, we used enzymatically active GST-Akt#-ΔPH (Carna Biosciences Inc.) in which GST, #, and ΔPH denoted glutathione *S*-transferase as a N-terminal tag, isoform number, and deletion of PH domain, respectively, and the respective Akt isoforms were phosphorylated at threonine/serine residues essential for the activation of kinase domain. Our preliminary activity screening for inhibition using Pakti-L1−L6 and Pakti-D1−D3 revealed that the Pakti peptides isolated in this study, except for Pakti-L4 and D2, were active inhibitors against Akt2 (Table 1). On the basis of this preliminary data, we chose three peptides from the Pakti-L family, Pakti-L1−L3, for further isoform-selectivity studies. In parallel, other representative S/T kinases, PKA (protein kinase A) and SGK (serum- and glucocorticoid-regulated protein kinase), were also tested for inhibition by these peptides (Figure 2a−c).

Akt- and Isoform-Selectivity of Pakti-L Peptides. The concentrations of each peptide were set at 1, 5, and 10 μM for the inhibitory test against Akt1−3, PKA, and SGK. Remarkably, under any of the above concentrations, Pakti-L1 completely shut down the kinase activity of Akt2, but all other kinases tested in this study retained activity over 50% even at 10 μM (Figure 2a). This result clearly shows a high isoform selectivity of Pakti-L1. Likewise, Pakti-L2 and L3 shut down the kinase activity of Akt2, but they also inhibited the kinase activity of both Akt1 and Akt3 to some extent (Figure 2b and c); at their 1 μM concentration, both Akt's gave an approximately 40% activity, and thus their IC_{50} values were estimated to be ∼1 μM (Table 1). Similar to Pakti-L1, Pakti-L2 and L3 displayed very weak inhibitory activities against PKA and SGK (IC_{50} values could be <10 μM). Thus, Pakti-L2 and L3 are Akt2-selective but less isoform- and Akt-selective compared with Pakti-L1. Even though the *in vitro* selection of nonstandard macrocyclic peptides against Akt2 from the ^{L}Y- and ^{D}Y-libraries was based on their binding ability leading to enrichment, it has successfully yielded potent inhibitors from both libraries.

To assess more accurate IC_{50} values of the Akt2-inhibitors, we directly monitored the kinase activity of Akt2 by a classical filter-binding assay of ^{32}P-phosphorylated substrate peptide on a phosphocellulose paper.[40] For this experiment, we modified a known cross-reactive substrate, so-called crosstide, to generate a crosstide-KK in which two additional lysine residues were added to its C-terminus.[40,41] The phosphorylation activity was titrated in the presence of various concentrations of each peptide ranging from 2 to 2,100 nM against Akt2 (Figure 3a−c). All Pakti-L1−L3 displayed nearly the same inhibitory potencies against Akt2 with IC_{50} values of 100 nM (Table 1).

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Because Pakti-L1 appears to exhibit a high selectivity to Akt2 according to the screening result (Figure 2a), we also attempted to determine the IC_{50} values against Akt1 and Akt3 (Figure 3a). The titration of Pakti-L1 in a range of 270−42,000 nM against Akt1 and Akt3 has revealed estimated IC_{50} values of 25 and 4.2 μM (Figure 3a and Table 1). It should be noted that the inhibitory activities of Pakti-L1 against Akt1 and Akt3 were intrinsically very weak, and therefore we were unable to completely titrate the inhibitory activity with 42 μM Pakti-L1, meaning that the above IC_{50} values were yet estimates. Nevertheless, the isoform selectivity of Pakti-L1 to Akt2 over Akt1 and Akt3 was 250- and 40-fold, respectively.

In summary, Pakti-L1, generated from ^{L}Y-library and studied in depth in the present work, displays an isoform-selective inhibitory activity against Akt2 over Akt1 and Akt3. It also exhibits nearly no activity against other families of S/T kinases. Thus, Pakti-L1 is a remarkable Akt-selective and Akt2 isoform-selective inhibitor. The other class of thioether-macrocyclic peptides, Pakti-L2 and L3, display potent inhibitory activity against Akt2, modest activities against Akt1/3, and very weak activity against other families of S/T kinases. Although they are weakly isoform-selective, they are highly Akt-selective inhibitors.

Discussion. The full-length of Akt2 used for the selection was an enzymatically inactive construct, *i.e.*, a nonphosphorylated form. Therefore, it is quite surprising that the majority of selected peptides found in ^{L}Y-library were able to show inhibitory potencies with a range of 100 nM IC_{50} against active Akt2. Moreover, all inhibitors found in this study inhibit the kinase activity of Akt2-ΔPH, suggesting that their binding site(s) should reside in the kinase domain of Akt2. The most intriguing question is how these macrocyclic peptides inhibit the kinase activity of Akt. Particularly, Pakti-L1 exhibits a high isoform-selective activity, so the questions where it binds in the Akt kinase domain and how it discriminates Akt2 over Akt1/3 are of greatest interest.

Since we have tertiary structural information of neither Pakti-L1 nor its complex with Akt2, we are only able to provide a speculative discussion for possible mechanisms of its isoform-selectivity, but it would be worthy to discuss such at this point. We propose two hypotheses as follows. The first hypothesis is that Pakti-L1 interacts with the substrate-binding domain and competitively inhibits Akt2 activity. It is known that a generic substrate sequence, such as crosstide, contains a RXRXX(S/T) motif (the phosphorylating S/T is assigned to position 0, and its N-terminal region is assigned to −1 to −5) where the arginine residues at positions −3 and −5, interact with the conserved glutamate residues (E236, E279, and E342) in Akt (Supplementary Figure 2a).[42] It turns out that Pakti-L1 has a RNR motif embedded in the middle of sequence (Table 1), and therefore this motif may be able to position Pakti-L1 into the active site. On the other hand, all Akt kinases share the same amino acid residues that interact with the substrate backbone amides or side chains, and unique residues in Akt2 differing from those in Akt1/3 are scattered outside of active site in the structure (see Supplementary Figure 2b). Therefore, if this hypothesis were correct, Pakti-L1 could use a unique mechanism, such as induced fit, to gain specific interactions, enabling the discrimination of Akt2 against Akt1/3.

The second hypothesis is that Pakti-L1 acts as an allosteric inhibitor. It may have a mechanism similar to Akti-1/2 where Pakti-L1 stabilizes the interaction between the kinase and PH domains, resulting in stabilization of the kinase-inactive "PH-in" form.[26] However, since Pakti-L1 is structurally bigger than Akti-1/2, the binding mode of Pakti-L1 should be largely different from that seen in Akti-1/2. Alternatively, Pakti-L1 interacts with a unique short sequence clustered in the region of 455−466 and allosterically inhibits the kinase activity (Supplementary Figure 2a and b). Unfortunately, this region is invisible in any of available X-ray structures of Akt, suggesting that this region may be largely unstructured. It is known that the downstream sequence, F470−Y475, docks on the "N-lobe" region when S474 is phosphorylated, resulting in activation of the kinase function.[42] Thus, if Pakti-L1 binds to this unique region and prevents the F470−Y475 region from docking, the kinase activity can be allosterically inhibited.

Since we do not have any data that enables us to rule out either hypothesis, we would like to leave open questions for the future investigations. Presumably, structural studies of the binary complex of the kinase domain with Pakti-L1 or the ternary complex with PH domain will be critical to reveal the exact inhibitory mechanism of Pakti-L1.

Conclusion. Here, we have reported thioether-macrocyclic peptide inhibitors with high Akt-selectivity generated by RaPID system. One of the inhibitors, Pakti-L1, was studied in depth and also showed a high isoform-selectivity against Akt2 over other isoforms. It is quite remarkable that even though the selection was performed against only Akt2, the selected peptides possessed isoform-selective properties. This suggests that the RaPID system could have a potential to yield isoform-selective inhibitors against various therapeutic targets of which isoform-selectivity is a strong requisite for the development of therapeutic agents. More extensive attempts by means of RaPID system against other therapeutic targets are currently underway in our laboratory.

METHODS

Preparation of ClAc^{L}Y and ^{D}Y Peptide Libraries. The mRNAs with the 4−12 repeated NNK sequences for the random region of amino acid sequence were prepared by *in vitro* transcription of their template cDNA amplified by PCR, respectively, using the primers P1, PNNK4−PNNK12, and P2 (Supplementary Table 2). The mRNA library was prepared by mixing the respective mRNAs with the 4−12 repeated NNK sequences with the following ratio, $(NNK)_4$:$(NNK)_5$:$(NNK)_6$:$(NNK)_7$:$(NNK)_8$:$(NNK)_9$:$(NNK)_{10}$:$(NNK)_{11}$:$(NNK)_{12}$ = 20^{-3}:20^{-2}:20^{-1}:1:10:10:10:10:10:10, adjusted to 10 μM total concentration of mRNA and denoted as a mRNA library. Two samples of 1 μM mRNA library were ligated with 1.5 μM of DNA-PEG-CCPu (Supplementary Table 2) by T4 RNA ligase at a scale of 200 μL total volume and incubated at RT for 30 min. The ligated libraries were purified by phenol-chloroform treatment and ethanol precipitation. The mRNA libraries fused with DNA-PEG-CCPu were translated in the Met-deficient FIT system at a scale of 150 μL total volume containing 50 μM ClAc^{L}Y-tRNA$^{fMet}_{CAU}$ or ClAc^{D}Y-tRNA$^{fMet}_{CAU}$,[36,37] prepared as described in Supporting Information, for 30 min at 37 °C and an additional 12 min at RT to enhance the mRNA-peptide conjugation efficiency. To quench the reaction, the samples were mixed with 15 μL of 200 mM EDTA (pH 7.5) and incubated at 37 °C for 30 min. For desalting, the samples were gel-filtered through 700 μL of fresh cross-linked dextran polymer beads, Sephadex G-25 Fine (GE Healthcare), equilibrated with PBST (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, and 0.05% (v/v) Tween20 adjusted to pH 7.6 with HCl) twice. Preparation of the two libraries, ClAc^{L}Y (^{L}Y) library and ClAc^{D}Y (^{D}Y) library, was completed by adding an equivalent volume of 2× blocking buffer (1 M NaCl and 0.2% (w/v) acetyl-BSA in PBST) into the filtrates.

Selection Procedure by RaPID System. The following enrichment process was independently performed with the respective ^{L}Y and ^{D}Y libraries. At the first round, the ^{L}Y and ^{D}Y libraries were applied to

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no protein-immobilized Ni^{2+}-NTA magnetic beads (Life Technologies, Cat. No. 10104D) twice to remove all the components tagged with the hexahistidine in the Met-deficient FIT system and undesired background beads binders from the libraries (this process is referred to as preclear below), then applied to $(His)_6$-Akt2-immobilized Ni^{2+}-NTA magnetic beads, and incubated at 4 °C for 30 min (this process is referred to as positive selection). After the incubation, the beads were washed with 500 μL of PBST four times. The washed beads were resuspended in 40 μL of reverse-transcription reaction mixture. The mRNAs remaining on the Akt2-immobilized beads were reverse-transcribed by MMLV reverse-transcriptase (Promega, Cat. No. M1705) at 42 °C for 1 h using the primer P2. The resultant cDNAs on the beads were eluted by being mixed with 200 μL of 1× PCR reaction buffer and heated at 95 °C for 5 min, with immediate separation of the supernatant from the beads. The amounts of the eluted cDNAs from the beads in the respective libraries were measured by qPCR, and the eluted cDNAs were amplified by PCR using the primer P1 and P2 for the preparation of the mRNA libraries for the next round. The aforementioned processes of (selected) library preparation, preclear, and positive selection constitute one cycle of the enrichment process.

From the second round, in the enrichment process, the reverse-transcription of mRNA to prepare the cDNAs was performed prior to the selection and the selection was performed by autodispenser machine (Nikkyo Technos Co., Ltd.). At the second round, the individual peptide libraries were prepared by *in vitro* transcription of the amplified cDNAs, ligation of the mRNA libraries with DNA-PEG-CCPu, *in vitro* translation, and reverse transcription. The libraries prepared in 5 μL of the translation scale and diluted to 100 μL with blocking buffer and PBST, the fresh magnetic beads, the wash buffer (PBST), inactive Akt2 protein, and H_2O were set in the appropriate wells of the 96-well plate. The machine was programmed to run the following processes, immobilization of Akt2 protein to Ni^{2+}-NTA magnetic beads, washing the extra Akt2 protein from the beads, and applying the respective library to the preclear and selection processes. After iterative preclear processes up to 6−12 times depending upon the rounds, the respective libraries were applied to the Akt2-immobilized beads for the selection and incubated at about 10 °C for 15−30 min. The beads were washed with 100 μL of PBST three times and resuspended with 40 μL of H_2O, and then the machine program was executed to completion. The beads were mixed with 50 μL of 2× PCR buffer and adjusted to 100 μL total volume with H_2O. The eluted cDNAs were recovered by heating the sample at 95 °C for 5 min and separating the supernatant from the beads. The recovered cDNAs were amplified by PCR and then transcribed to prepare mRNA libraries for the next round. The enrichment process was completed at the sixth round, where appreciable enrichment of the recovery rate was observed.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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