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Inhibitors of Cell Migration that Inhibit Intracellular Paxillin/α4 Binding: A Well-Documented Use of Positional Scanning Libraries

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Summary

Screening combinatorial libraries for inhibition of Paxillin binding to the cytoplasmic tail of the integrin α4 provided the first inhibitors of this protein-protein interaction implicated in enhanced rates of cell migration and chronic inflammation. The preparation of substructure analogs of the lead identified features required for activity, those available for modification, and those that may be removed. The most potent lead structure was shown to inhibit $\alpha_4\beta_1$-mediated human Jurkat T cell migration in a dose-dependent manner, validating the intracellular Paxillin/α4 interaction as a useful and unique target for therapeutic intervention. Moreover, the lead structure emerged from a library that was prepared in two formats: (1) a traditional small mixture format composed of 100 mixtures of 10 compounds and (2) a positional scanning library. Their parallel testing provided the rare opportunity to critically compare two approaches.

Introduction

The integrin $\alpha_4\beta_1$ (also know as very late antigen 4 [VLA4]) is a cell surface receptor that plays an important role in embryogenesis, hematopoiesis, and the immune response [1, 2]. It binds natural ligands including vascular cell adhesion molecule 1 (VCAM-1) and an alternatively spliced connecting segment (CS-1) from the extracellular matrix protein fibronectin. It mediates cellular adhesion and activation through a variety of cell-cell and cell-matrix interactions that regulate leukocyte migration into tissues during inflammatory responses and lymphocyte trafficking [3, 4]. This integrin is believed to regulate cellular functions differently from other integrins because the α4 cytoplasmic tail binds tightly to the signaling adaptor protein Paxillin through a short conserved sequence motif dominated by two residues (Glu^{983} and Tyr^{991}) [5, 6]. The α4/Paxillin interaction leads to enhanced rates of cell migration and reduced rates of cell spreading, focal adhesion, and stress fiber formation (Figure 1) [7]. These biological responses to integrin-mediated cell adhesion contribute to leukocyte migration and changes in gene expression important in chronic inflammation. While extracellular inhibitors for this class of integrins are under development for the treatment of asthma and multiple sclerosis [8–13], inhibitors targeting the unique cytoplasmic Paxillin/α4 interaction have not yet been described or explored. Such intracellular versus extracellular inhibitors offer a potential opportunity for the development of compounds with distinct therapeutic profiles, would constitute a rare example of small molecule therapeutic intervention through disruption of a protein-protein interaction, and could ultimately lead to new treatments for diseases including asthma, multiple sclerosis, and rheumatoid arthritis.

Since the structural basis of the Paxillin/α4 interaction is unknown, a combinatorial chemistry approach was undertaken in search of small molecule antagonists of this protein-protein interaction [14, 15]. Our efforts in this area have focused on the exploitation of a technically nondemanding solution-phase strategy that dependably delivers pure individual compounds or small to large combinatorial mixtures [16, 17]. We recently reported a number of such libraries [18–20], including the solution-phase preparation of a 1000 membered library assembled in two distinct formats: (1) a traditional library composed of 100 mixtures of 10 compounds [21], and (2) a technically less demanding positional scanning library [22]. The synthesis of positional scanning libraries [23–26] represents one of the most useful protocols for mixture synthesis, but can only be conducted with solution-phase techniques and is not easily adapted to solid-phase synthesis. Not only is it much less time intensive than the parallel synthesis of individual compounds or small mixtures and technically less demanding than spatially arrayed [27] or tagged split-and-mix library synthesis [28–35], but it produces depository libraries for use in multiple screens and capable of immediate deconvolution [36–44]. Thus, unlike other mixture deconvolution protocols, positional scanning libraries can provide lead identities in a single round of testing. Despite these attributes, it is not clear how well such libraries may perform in screens for inhibition of protein-protein interactions. Herein, we present the results of the screening of our libraries enlisting an ELISA assay using the immobilized α4 cytoplasmic tail and examining the inhibition of soluble recombinant Paxillin binding that led to the discovery of the first class of agents that can disrupt α4/Paxillin binding. These agents came from the library that had been prepared in two formats and represents a rare case for which the parallel screening of a traditional library of 100 mixtures of 10 compounds was conducted alongside the related positional scanning library such that the results could be compared [44]. Both led to the identification of the same lead compound, demonstrating the power of the positional scanning strategy. In addition, structural features contributing to this inhibition were clear from the initial screening results, and the subsequent examination of key partial structures of the initial leads define a class of potent Paxillin/α4 antagonists. The potent lead structure (IC_{50} = 300 nM) was shown to decrease $\alpha_4\beta_1$-mediated human Jurkat T

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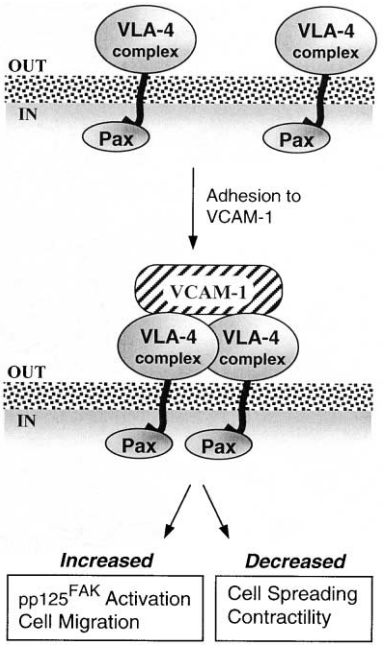


Figure 1. Overview of Paxillin/α4 Binding-Mediated Enhancement of Cell Migration

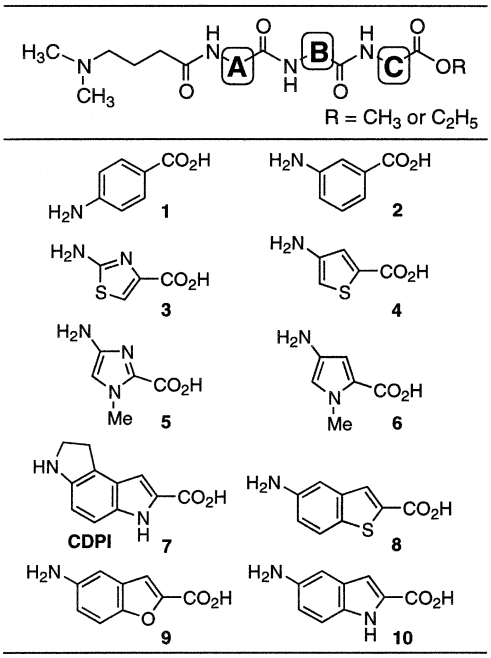
Paxillin binds to the α4 cytoplasmic tail of VLA-4 ($\alpha_4\beta_1$ integrin). Dimerization of two Paxillin/VLA-4 complexes occurs through binding to VCAM-1 in the extracellular compartment and triggers a cascade of events and the gene expression responsible for cell migration.

cell migration in a dose-dependent manner, validating this new therapeutic target.

Results and Discussion

Library Composition

We recently reported the preparation of the library of 1000 compounds from which the lead emerged in a traditional small mixture format [21]. The structure of the library components share an identical scaffold composed of three subunits (A, B, and C) linked by amide bonds, and a basic side chain 4-(dimethylamino)butyric acid (DMABA) linked to the A subunit (Figure 2). Using 10 different aromatic amino acids (Figure 2), the library was prepared by parallel synthesis of the 100 individual compounds constituting all possible B-C combinations followed by their coupling with the **A1–A10** mixture. This provided a 1000-member library in a format of 100 mixtures of 10 compounds (Figure 2, **11-AByCz**). We also reported the solution-phase synthesis of the positional scanning library that contained the same compounds but arranged differently [22]. The positional scanning library consists of 30 sublibraries that are divided into three sets. Each set is defined by a fixed position of a monomer subunit within the tripeptide. Within set 1 (Figure 2, **12-AxBC**, "A-Scan"), each subunit (**1–10**) is individually present at position A, and a full mixture of **1–10** is present at positions B and C. In set 2 (**13-AByC**, B-Scan), the B position is defined with a single subunit, but A and C are undefined (full mixture). In set 3 (**14-ABCz**, "C-Scan"), the C position is defined with a single subunit, but A and B are undefined. The C termini of the library compounds were capped as methyl or ethyl esters, and the N termini were acylated with 4-(dimethylamino)butyric acid (DMABA).



10-member mixture library:

11-AByCz

XB_1C_1, XB_1C_2, ---------, XB_1C_{10}

XB_2C_1, XB_2C_2, ---------, XB_2C_{10}

$XB_{10}C_1$, $XB_{10}C_2$, --------, $XB_{10}C_{10}$

Positional Scanning libraries:

Set 1, 12-AxBC (A-Scan):

A_1XX, A_2XX, ----------, $A_{10}XX$

Set 2, 13-AByC (B-Scan):

XB_1X, XB_2X, ----------, $XB_{10}X$

Set 3, 14-ABCz (C-Scan):

XXC_1, XXC_2, ----------, XXC_{10}

Figure 2. General Structure of the Library, Structure of the Ten Aromatic Amino Acid Subunits Used in the Library, and Library Composition of the Mixture and Positional Scanning Libraries

Screening Results

Initially, roughly 40,000 compounds were screened in approximately 2000 wells (most as a mixture of 10 compounds, 50 μM total concentration) in an ELISA assay using an immobilized His-tagged α4 tail [45] and examining the binding inhibition of soluble recombinant Paxillin

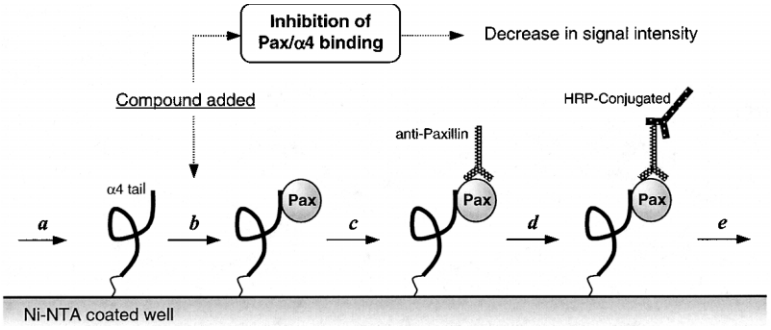


Figure 3. Description of the High-Throughput Screening (ELISA)

α4 tail is coated onto a Ni-NTA microtiter plate (*a*), Paxillin is allowed to bind to α4 (*b*), the first antibody is added (anti-Paxillin) (*c*), the second antibody is added (HRP-conjugated anti-mouse) (*d*), and Paxillin/α4 binding efficiency is measured by detection at 490 nm (*e*). For binding inhibition, compounds were added during Paxillin incubation (step *b*). 100% inhibition (background) was measured from incubation with no Paxillin and no compound, 0% inhibition was measured from incubation with Paxillin and no compound.

[46] (Figure 3). From this screen, mixture library **11-AByCz** (50 μM; data not shown) exhibited numerous mixture hits. This library of 1000 compounds was retested at lower concentrations (5 μM total compound, 0.5 μM per component), and the results are reported as percent inhibition versus no compound in Table 1A. Mixtures **11-AB7C6**, **11-AB7C7**, **11-AB7C10**, and **11-AB10C9** showed exceptional and consistent activity in this concentration range exhibiting ~80% inhibition. In nearly each case, mixture B7 and to a lesser extent C7 mixtures, exhibited the most potent inhibition, followed by B10 or C10 mixtures. The presence of subunits **7** or **10** (two very close structures, see Figure 2) in each of the four most active mixtures suggested a specific structure-related inhibition. Consequently, we resynthesized each individual compound of the four most potent mixtures from the archived Boc-B7C6, Boc-B7C7, Boc-B7C10, and Boc-B10C9 precursors using the route previously disclosed [21]. The forty individual compounds were tested at 1 μM, and the results (% inhibition) are reported in Table 1B. Impressively, every molecule derived from mixture **11-AB7C7** was found to be active at this concentration. Except for the closely related **11-A7B10C9**, the leads share an identical scaffold composed of two CDPI subunits (**11-AxB7C7** or **11-A7B7Cz**) and led to the conclusion that the $CDPI_2$ motif is key to Paxillin/α4 binding inhibition. We also established that none of the lead compounds exhibited their activity by disrupting the binding of α4 to the Ni-NTA well. We measured (anti-α4) the concentration of α4 integrin bound to the plate in the presence or absence of compound and found that none of the lead compounds destabilized the bound α4.

At the time the initial screening was conducted, the Boc-protected precursors [21] to the **11-AByCz** library (1000 compounds) lacking the DMABA side chain were also examined as identical mixtures of 10 compounds (50 and 5 μM). Although none of these derivatives was as active as the DMABA derivatives, they did display analogous trends, with B7 and C7 mixtures exhibiting the greatest binding inhibition (data not shown). These results suggested that either the DMABA basic side

Table 1A. Inhibition (%) of Paxillin/α4 Binding by Mixtures **11-AByCz** (5 μM Total Compound)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
11-AB1Cn	17	8	21	15	14	22	27	6	2	18
11-AB2Cn	6	11	12	6	23	23	32	9	7	6
11-AB3Cn	2	14	9	17	3	15	24	17	12	21
11-AB4Cn	25	6	4	21	8	8	6	15	6	13
11-AB5Cn	24	3	15	19	11	16	19	8	16	12
11-AB6Cn	19	18	14	11	3	6	9	15	11	16
11-AB7Cn	53	61	60	58	28	*78*	*88*	72	71	*80*
11-AB8Cn	3	0	2	5	16	5	36	0	8	14
11-AB9Cn	25	17	19	22	16	32	38	19	32	45
11-AB10Cn	21	26	24	23	12	31	16	21	*75*	23

Table 1B. Inhibition (%) of Paxillin/α4 Binding by Deconvoluted Samples of **11-AxByCz** (1 μM)

	A1	A2	A3	A4	A5	A6	*A7*	A8	A9	A10
11-AnB7C6	31	−8	29	31	47	−11	*74*	60	60	54
11-AnB7C7	*70*	*58*	*80*	*76*	*68*	*66*	*76*	*74*	*81*	*63*
11-AnB7C10	40	4	54	54	27	29	*77*	59	67	54
11-AnB10C9	29	−9	72	−3	2	−23	*63*	48	−10	35

Table 1C. Inhibition of Paxillin/α4 Binding by Deconvoluted Samples of **11-AxByCz** (IC_{50} in μM)

	A1	A2	A3	A4	A5	A6	*A7*	A8	A9	A10
11-AnB7C6	–	–	–	–	–	–	*0.5*	–	–	–
11-AnB7C7	*0.6*	*1.4*	*0.4*	*0.4*	*0.5*	*2.2*	*0.3*	*0.7*	*0.4*	*4.8*
11-AnB7C10	–	–	–	–	–	–	*0.4*	–	–	–
11-AnB10C9	–	–	–	–	–	–	*2.2*	–	–	–

chain was contributing productively to the binding inhibition or that an *N*-terminus Boc group was detrimental. This point will be discussed in more detail below.

We further tested the thirteen individual compounds in italics in Table 1B at a range of concentrations, and the results are reported as IC_{50} in Table 1C. Compound **11-A7B7C7**, containing three CDPI subunits, was the most effective inhibitor of Paxillin/α4 binding, with a IC_{50} of 300 nM. However, the fact that every component of mixture **11-AB7C7** (**11-A1B7C7** to **11-A10B7C7**) exhibited such similar activity suggests either that the A subunit is unnecessary or that the binding site is able to accommodate a wide variety of groups at position A. We will return to this point later.

An important feature in this work rested with the parallel screening of the related positional scanning library for which every component contained in the traditional compound library (**11-AByCz**) was also present, but assembled such that immediate deconvolution is possible. This library was initially screened at the 50 μM total compound concentration and, like the traditional library, displayed numerous active mixtures. Consequently, they were rescreened at 5, 10, and 20 μM total compound concentrations. Thus, scanning for the best subunit at position A by screening sublibrary **12-AxBC** showed that the greatest inhibition was observed for mixture **12-A7BC** at each concentration, identifying the CDPI subunit as the best subunit at position A (Figure 4). Using the same procedure, CDPI was also identified as the most effective subunit at positions B and C (Figure 4). Immediate deconvolution of the results identifies the **A7B7C7** combination as a potent Paxillin/α4 binding antagonist. Thus, the same lead compound (**11-A7B7C7**) was identified from the two different combinatorial strategies. This success must be tempered by the fact that the positional scanning library screening and direct deconvolution do not identify as candidate inhibitors **11-A3B7C7**, **11-A9B7C7**, or **11-A7B7C6**, which were identified from the traditional library. This is a natural consequence of testing larger 100-compound mixtures and the relative insensitivity of the assay to the contribution of any single, uniquely acting compound in the mixture. Thus, the global observations were effectively detected with the positional scanning library, and a useful lead structure with defined properties was identified. However, more subtle discoveries within the library were not identified. Thus, the disadvantages associated with the loss of their detection and this information contained within the library must be balanced against the advantages of the ease of synthesis of the parent libraries and judged in light of the objectives of the library screening. As in this case, the positional scanning libraries typically would be most effective for lead identification and would be less suitable for lead optimization.

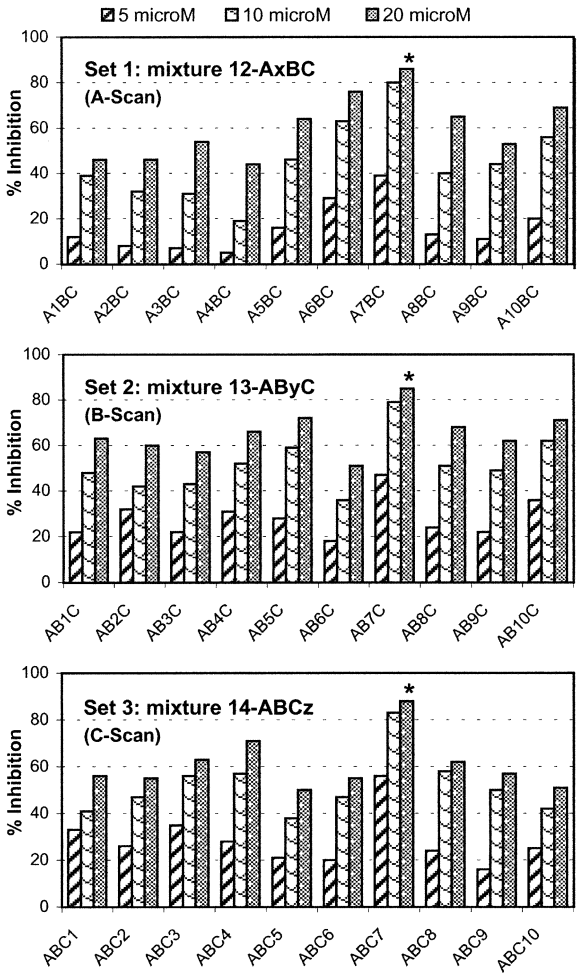


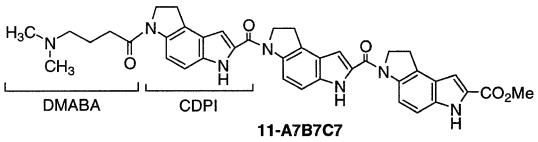
Figure 4. Inhibition of Paxillin/α4 Binding by the Positional Scanning Libraries **12-AxBC**, **13-AByC**, and **14-ABCz**

Each mixture was tested in triplicate at 5, 10, and 20 μM (total compound), and the results are reported as percent inhibition of Paxillin/α4 binding.

Further Structure-Activity Studies

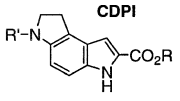
Following the identification of **11-A7B7C7** by two different combinatorial strategies, the structural features contributing to its properties were defined by examining key partial structures (minimal structure scanning). Notably, a first level structure-activity relationship study was available from the original library screening, albeit conducted on mixtures, indicating that the CDPI subunit (**7**) was more effective than the closely related benzothiophene, benzofuran, or indole subunits (**8–10**), with the more closely related indole **10** typically being better than subunits **8** or **9** (i.e., typically: **7** > **10** > **9** > **8**). Consequently, the subsequent studies focused on 13 additional CDPI derivatives containing one, two, or three CDPI subunits. Within each series, the new analogs contained either a methyl ester or a free acid at C terminal position, and either a dimethylaminobutyric acid (DMABA), a Boc, or a free amine at N terminal position. Previously unreported analogs [47] that contain the DMABA side chain were prepared using traditional Boc-deprotection (4 N HCl-dioxane) and peptide coupling with 4-(dimethylamino)butyric acid effected by EDCI/DMAP (DMF). Each analog was tested for Paxillin/α4 inhibition at a single concentration (1 μM), and the most active compounds were further characterized by measuring their IC_{50} for Paxillin/α4 binding inhibition. The results are summarized in Table 2. Compounds with one CDPI subunit (**15–19**) or two CDPI subunits (**20–23**) were not active or were only weakly active at the concentra-

Table 2. Inhibition of Paxillin/α4 Binding by Key Substructure Analogs of **11-A7B7C7**

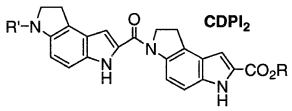


11-A7B7C7

Compound	Inhibition of Paxillin/α4 binding (% at 1 μM)	Inhibition of Paxillin/α4 binding (IC_{50} in μM)
Boc-CDPI-OMe, **15**	3	nd
H-CDPI-OMe, **16**	19	nd
H-CDPI-OH, **17**	11	nd
Boc-CDPI-OH, **18**	6	nd
DMABA-CDPI-OMe, **19**	−3	nd
Boc-$CDPI_2$-OMe, **20**	13	nd
H-$CDPI_2$-OMe, **21**	14	nd
DMABA-$CDPI_2$-OMe, **22**	36	nd
H-$CDPI_2$-OH, **23**	27	nd
Boc-$CDPI_3$-OMe, **24**	25	nd
H-$CDPI_3$-OMe, **25**	**73**	0.3
DMABA-$CDPI_3$-OMe, **11-A7B7C7**	**78**	0.3
H-$CDPI_3$-OH, **26**	**68**	0.5



	R	R′
15	Me	Boc
16	Me	H
17	H	H
18	H	Boc
19	Me	DMABA



	R	R′
20	Me	Boc
21	Me	H
22	Me	DMABA
23	H	H

tion tested, and only DMABA-$CDPI_2$-OMe (**22**) exhibited moderate activity (36% inhibition at 1 μM). Moreover, the activity smoothly increased as one progressed through the series $CDPI_1 < CDPI_2 < CDPI_3$. Thus, although the nature of the A subunit is not critical to the observed activity, its presence substantially potentiates it. Retesting compound **11-A7B7C7** confirmed its activity (IC_{50} = 300 nM), and analog **25**, lacking the basic DMABA side chain, exhibited a similar potency (**25**, IC_{50} = 300 nM), indicating that the DMABA group is not necessary for activity. Replacing the DMABA side chain with a Boc group (Boc-$CDPI_3$-OMe, **24**) led to a substantial loss of activity. This observation confirms, as suggested earlier, that the presence of an N-terminal Boc group is detrimental to the inhibition properties. Just as interestingly, analog **27**, lacking both the DMABA group at the N terminus and the methyl ester at the C terminus, exhibited only a slight reduction in potency against Paxillin/α4 binding. This result not only indicates that the methyl ester is dispensable, but that the in vivo activity of the leads against the target protein-protein interaction will remain relatively unchanged even upon ester hydrolysis.

Affinity Chromatography Binding Experiments

In order to confirm the effectiveness and selectivity of **11-A7B7C7** at inhibiting Paxillin/α4 binding, we conducted a series of binding experiments using affinity chromatography techniques [7, 45]. Recombinant GST-Paxillin (100 nM) was allowed to bind to a Ni^{2+}-charged resin loaded with α4 tail protein in the absence or presence of compound **11-A7B7C7** or in the presence of compound **11-A6B6C6** that was found to be inactive for Paxillin/α4 binding inhibition in the initial assay (data not shown). Bound protein was collected and separated by electrophoresis and analyzed by immunoblotting. Compound **11-A7B7C7** markedly reduced the binding of Paxillin to the α4 tail protein (Figure 5A). In contrast, compound **11-A6B6C6** showed no inhibitory capacity at 25 μM. Quantification of bound Paxillin showed that **11-A7B7C7** reduced Paxillin/α4 binding to near background levels at concentrations as low as 5 μM in this assay and confirmed the selectivity and effectiveness of **11-A7B7C7** at inhibiting Paxillin/α4 binding.

Inhibition of Cell Migration

The functional biological activity of **11-A7B7C7** was established by examining its effects on integrin $\alpha_4\beta_1$-mediated cell migration in Jurkat T cells [48]. Compound **11-A7B7C7** efficiently blocked cell migration in a dose-dependent manner ($IC_{50} \sim 10$ μM), validating the Paxillin/α4 target for therapeutic intervention. In contrast, a compound that failed to inhibit the α4/Paxillin interaction, **11-A6B6C6**, had no effect at concentrations up to 15 μM (Figure 5B), the maximal concentration achievable in 1% DMSO. **11-A7B7C7** did not cause leakage of cytoplasmic lactate dehydrogenase from the cells at the concentrations up to 15 μM, indicating that the inhibition of migration was not due to cytotoxicity. Furthermore, at the highest possible dose (15 μM), inhibition appeared

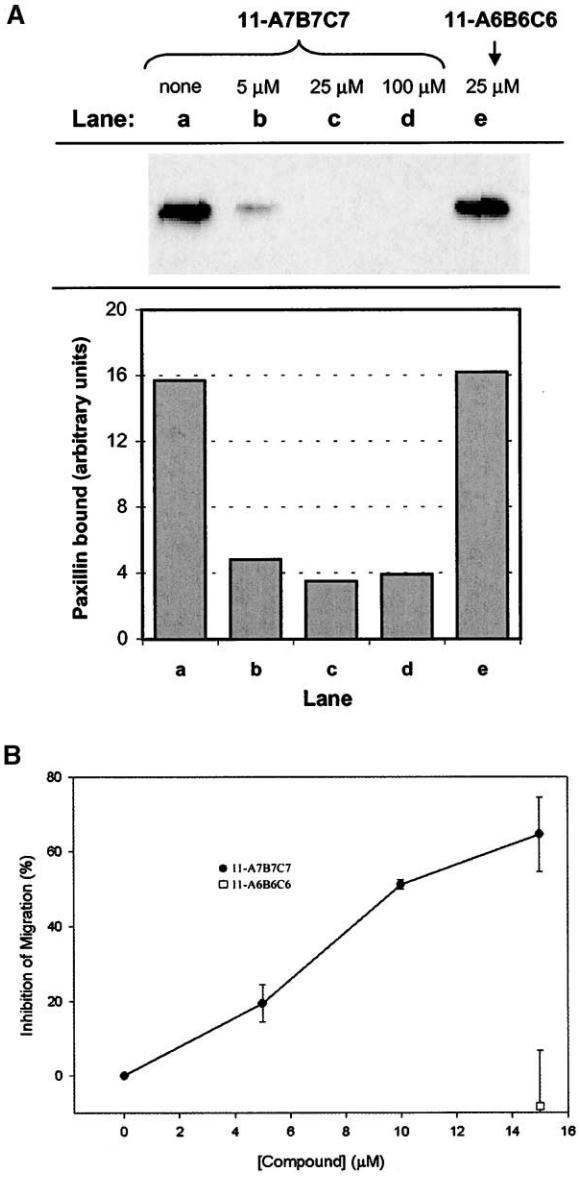


Figure 5. Affinity Chromatography and Cell Migration Assays

(A) Affinity chromatography binding experiment. Upper panel: recombinant HA-tagged GST-Paxillin (100 nM) was added to Ni^{2+}-charged resin loaded with α4 tail protein in the absence (lane a) or presence of compound **11-A7B7C7** (lane b, 5 μM; lane c, 25 μM; lane d, 100 μM) or compound **11-A6B6C6** (lane e, 25 μM). Bound protein was collected and separated by SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane, and stained with HA-tag-specific antibody, 12CA5. Lower panel: bound Paxillin was quantified by scanning densitometry of these immunoblots using the NIH Image program. Depicted is a representative result from two experiments.

(B) Effect of **11-A7B7C7** on integrin $\alpha_4\beta_1$-mediated migration of Jurkat T cells. Cell migration was assayed in a modified Boyden chamber assay system as previously described [48]. In this system, migration is specific to the α4 integrin, as it is completely inhibited by function-blocking anti-α4 antibodies [48]. Transwells (Costar, Corning) polycarbonate membranes containing 3.0 μm pores were coated with 5 μg/ml recombinant soluble VCAM-1. Membranes were blocked with 2% BSA in PBS for 30 min at room temperature. 2.0×10^5 cells in RPMI-1640 containing the indicated concentration of the compound and a final concentration of 0.1% DMSO were added to the top chamber. SDF-1α (R&D Systems) at a final concentration of 15 ng/ml was added to the bottom chamber. Cells were allowed to migrate for 4 hr at 37°C. Cells in the bottom chamber were enumerated with a hemocytometer, and data are expressed as percent inhibition of migration. Depicted are the mean and range of duplicate determinations from one of two experiments with similar results.

to approach a maximum at ~70%, similar to the degree of inhibition produced by mutations in α4 that disrupt Paxillin binding.

Significance

Screening for inhibition of Paxillin binding to the integrin α4 cytoplasmic tail provided the first inhibitors of this protein-protein interaction. The lead structure emerged from a library of 1000 compounds that was prepared in two formats: (1) a traditional small mixture format composed of 100 mixtures of 10 compounds, and (2) a less traditional positional scanning library composed of larger mixtures (100 compounds/mixture). Parallel testing of both libraries led to the identification of the same potent lead structure and provided the opportunity to critically compare the results derived from both approaches. The deconvolution of the traditional library required resynthesis of the individual compounds from each active mixture from archived samples of the library precursors, while the active lead structure was deduced directly from the positional scanning testing results. The traditional library provided more and subtle SAR information regarding the Paxillin/α4 inhibition, whereas the positional scanning libraries provided the lead structure with considerably less synthetic and screening effort. Thus, both approaches served the purposes intended, subject to their individual limitations.

Subsequent substructure analogs of 11-A7B7C7 identified structural features required for activity, those available for modification (A subunit), and those that may be removed (DMABA side chain) or modified (ester) without impacting the activity. The functional activity of 11-A7B7C7 was established with its dose-dependent inhibition (IC_{50} ~10 μM) of $\alpha_4\beta_1$-mediated cell migration in Jurkat T cells. Thus, complementary to $\alpha_4\beta_1$ antagonists that function extracellularly by inhibiting the binding of VCAM-1 or fibronectin, the intracellular inhibition of Paxillin/α4 binding also disrupts cell migration, offering an alternative target for therapeutic intervention by a rare example of a small molecule disruption of an intracellular protein-protein interaction [15, 49–54]. Such target validation is often first established with monoclonal antibodies or peptide consensus sequences derived from endogenous ligands. It is noteworthy that the Paxillin/α4 target validation came from this exploration of small molecule libraries.

Experimental Procedures

Production and Purification of α4 Tail

The design and production of recombinant cytoplasmic α4 tail have been described [45]. Briefly, polymerase chain reaction was used to generate a HindIII-BamHI fragment for each wild-type or mutant integrin cytoplasmic domain. Each polymerase chain reaction prod-

uct was ligated into the pCR vector using a TA cloning kit (Invitrogen). After cDNA sequencing, each fragment was ligated into HindIII-BamHI sites of the modified pET15b vector described before [45]. α4 tail was expressed in BL21(DE3)pLysS cells (Novagen), isolated by Ni^{2+}-charged resins, and further purified to >90% homogeneity using a reverse-phase C18 HPLC column (Vydac).

Production and Purification of Paxillin

The expression and isolation of recombinant glutathione S transferase (GST)-Paxillin have been described [48].

Immobilized Paxillin/α4 Binding Assays

Paxillin/α4 binding assays were performed as follows. Ni-NTA His-Sorb microtiter strips (Qiagen) were coated overnight at 4°C with purified α4 tail integrin (5 μg/ml, 100 μl per well) in PBS buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 135 mM NaCl, 2.5 mM KCl [pH 7.4]) supplemented with heat-denatured BSA (0.2%). The plates were washed three times with PBS buffer to remove unbound integrin, blocked with 150 μl of heat-denatured 1% BSA (denatured at 85°C for 30 min and supplemented with 0.05% sodium azide) for 1 hr at room temperature, and washed again three times with PBS buffer. Purified Paxillin in binding buffer (10% DMSO, 0.2% BSA in PBS buffer) was added to the wells at a concentration of 5 μg/ml in the presence or absence of compounds (1 mM DMSO stock solutions). During incubation, DMSO concentration never exceeded 0.5% (up to 10% DMSO alone was found to have no effect on Paxillin/α4 binding efficiency). As a control, blocked wells without integrin were examined for binding. After 1 hr at room temperature, the wells were washed three times with PBS buffer and incubated with the first antibody (12CA5 ascites, 1/10000 in PBS plus 1% BSA, 100 μL) for 1 hr at room temperature and then with the second antibody (100 μl HRP-conjugated anti-mouse [Biosource] in PBS buffer plus 1% BSA) for 1 hr at room temperature. Each well was treated with 50 μl of substrate solution (4 mg *o*-phenylenediamine, 4 μl of 30% H_2O_2, 80 mM citrate phosphate [pH 5]) and stopped after 10 min at room temperature with 50 μl H_2SO_4 (2 N). Binding was quantitated on a v_{max} kinetic microplate reader (Molecular Devices) at 490 nm. 0% inhibition control was measured with Paxillin and no compound, and 100% inhibition control (background) was measured with no Paxillin and no compound. Each well was duplicated, and percent inhibition was averaged.

Affinity Chromatography Assay

Integrin tail affinity chromatography was performed as described [48]. Briefly, 1 mg of α4 integrin cytoplasmic domain was dissolved in 1 ml of 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 135 mM NaCl, and 2.5 mM KCl (pH 7.4) (binding buffer) and was bound to 50 μl of Ni^{2+}-charged His-Bind resin (Novagen) at 4°C overnight. The resin was then washed four times with binding buffer (1 ml) and stored in 1 ml of binding buffer at 4°C. Soluble recombinant HA-tagged Paxillin-GST fusion protein was then added (0.5 μg) to 100 μl of integrin tail-coated resins in the presence or absence of compounds (1 mM DMSO stock solutions). The mixture was incubated at 4°C with rotation for 1 hr. Resins were washed five times with 1 ml binding buffer. Bound proteins were extracted with 50 μl of reducing SDS sample buffer, separated on 4%–20% SDS-polyacrylamide gels (PAGE), transferred onto a nitrocellulose membrane, and analyzed by immunoblotting (anti-HA antibody [12CA5] ascites, 1/2000 for 2 hr).

Cell Migration Assay

The Jurkat E6-1 T leukemic cell line was purchased from American Type Culture Collection (ATCC), Rockville, MD and cultured in RPMI-1640 (Biowhitaker Inc, Walkersville, MD) supplemented with 10% FCS (Biowhitaker Inc), 1% glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (Sigma Chemical, St Louis, MO).

Cell migration was assayed in a modified Boyden chamber assay system. Transwells (Costar, Corning) polycarbonate membranes containing 3.0 μm pores were incubated with VCAM-1 in 0.1 M $NaHCO_3$ (pH 8.0) overnight at 4°C. Membranes were blocked with 2% BSA in PBS for 30 min at room temperature. 2.0×10^5 cells in RPMI-1640 with 10% FCS, 1% DMSO containing the indicated concentration of compound were added to the top chamber. SDF-1α (R&D Systems) at a final concentration of 15 ng/ml was added to the bottom chamber. Cells were allowed to migrate for 4 hr at 37°C. Cells in the bottom chamber were enumerated with a hemocytometer.

Supplemental Data

General procedures for the preparation of the 40 individual compounds derived from **11-AB7C6**, **11-AB7C7**, **11-AB7C10**, and **11-AB10C9**, characterization data for the four lead structures **11-A3B7C7**, **11-A7B7C7**, **11-A9B7C7**, and **11-A7B7C6**, and experimental and characterization data for **19**, **21–26**, and [^{14}C]-**11-A7B7C7** are provided. Please write to chembiol@cell.com for a PDF.

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