ACS Medicinal Chemistry Letters

Letter

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Single Diastereomer of a Macrolactam Core Binds Specifically to Myeloid Cell Leukemia 1 (MCL1)

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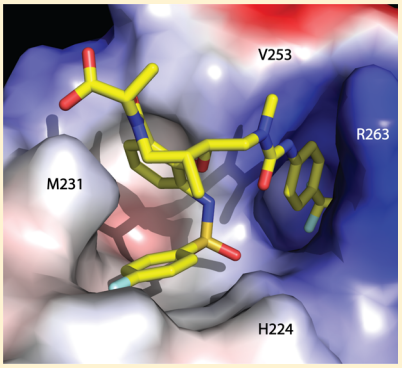
Supporting Information

ABSTRACT: A direct binding screen of 100 000 sp^3-rich molecules identified a single diastereomer of a macrolactam core that binds specifically to myeloid cell leukemia 1 (MCL1). A comprehensive toolbox of biophysical methods was applied to validate the original hit and subsequent analogues and also established a binding mode competitive with NOXA BH3 peptide. X-ray crystallography of ligand bound to MCL1 reveals a remarkable ligand/protein shape complementarity that diverges from previously disclosed MCL1 inhibitor costructures.



KEYWORDS: *MCL1, myeloid cell leukemia 1, sp^3-rich, biophysical validation*

Evasion of programmed cell death, or apoptosis, is a hallmark of cancer that allows tumor cells to survive stresses that would kill a normal cell. Specifically, cell death-inducing mitochondrial permeabilization is prevented by tight sequestration of membrane-localized proteins by antiapoptotic members of the BCL-2 family, which include BCL-2, BCL-XL, BCL-W, A1, and MCL1.[1−7] Human genetics points to a selective advantage of MCL1-amplified tumor cells. The analysis of over 3000 diverse human tumors indicates that *MCL1* is among the top 10 most frequently amplified genes in human cancer.[8,9]

Consistent with its frequent amplification, *MCL1* is highly expressed in many tumor types, and high expression levels of *MCL1* contribute to tumor development and resistance to chemotherapy.[10,11] As a result, there have been considerable efforts to develop small molecule therapeutics that target the function of MCL1 protein (Figure 1).[12−16]

To identify chemical starting points for targeting MCL1, we conducted a direct binding screen of 100 000 molecules synthesized at the Broad Institute.[17−20] This chemical library contains a high degree of stereochemical and skeletal diversity that augments traditional screening collections. The enumeration of nonplanar, sp^3-rich cores spanning four-membered ring azetidines to 14-membered macrocycles provides a rich source of three-dimensional space to sample for suitable protein/ligand shape complementarity (average core F_{sp^3} = 0.60).[21] A particular strength of this library is the elaboration of individual diastereomers in optically enriched form, thereby allowing rapid stereochemical deconvolution of identified hits.

Differential scanning fluorimetry[22,23] (DSF, or thermal shift assay) was selected as the biophysical method to detect ligand binding to MCL1. This direct binding technique is amenable to high-throughput screening formats and requires lower concentrations of protein relative to other methods (typically < 500 μg/mL). By screening for direct binders of MCL1, as compared to traditional peptide competition screens, we allow discovery of novel inhibitor binding modes (i.e., competitive and noncompetitive with BH3 peptide).

After screening over 13 000 stereochemically defined scaffolds, the macrolactam **5** emerged as a hit (ΔT_m = +0.3 °C at 25 μM ligand and 100 μg/mL MCL1 protein). The synthetic pathway to assemble this macrolactam has been described previously.[18] Importantly, only 1 of the 8 diastereomers that were screened caused a significant shift of

Received: September 23, 2014
Accepted: November 2, 2014
Published: November 11, 2014

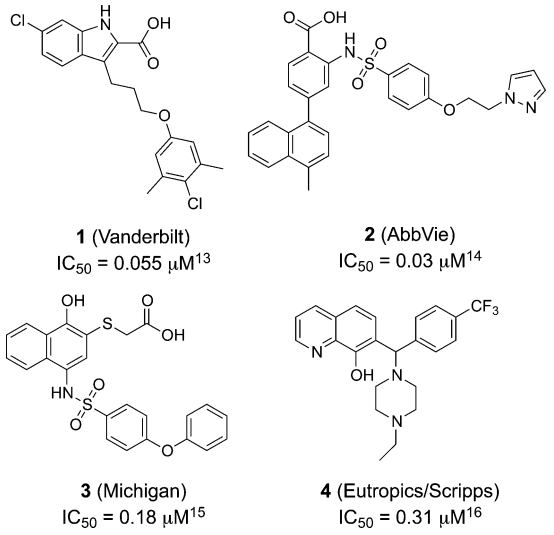


Figure 1. Representative MCL1 inhibitors.

MCL1 T_m, and it had the 5*R*,6*S* configuration in the macrolactam core (Figure 2). The strong preference for the

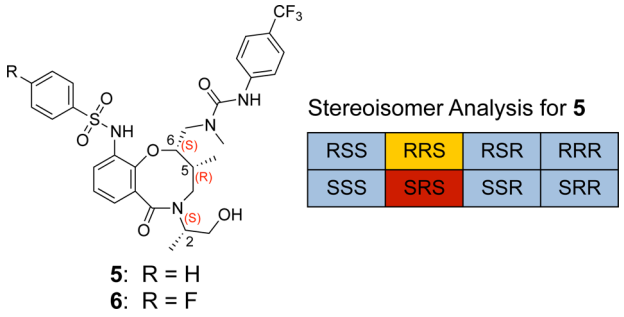


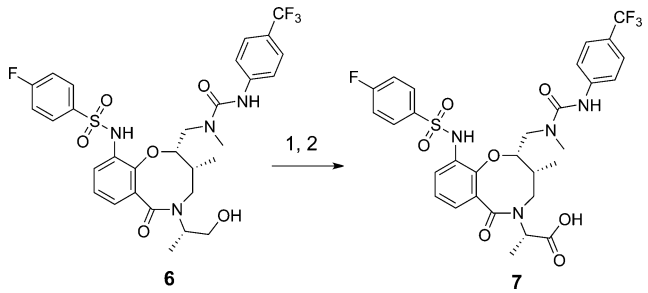
Figure 2. Identification of macrolactam hit **5** from DSF screening (stereochemistry labeling as C2C5C6; red, binding hit; blue, no binding; yellow, inconclusive).

5*R*,6*S* core configuration suggests a highly specific binding event of **5** to MCL1. Meanwhile, identification of additional 5*R*,6*S* congeners revealed no consistent absolute configuration at C2 of the exocyclic side chain (see Supporting Information Figure SI-1), implying that this portion of the molecule does not directly engage the MCL1 protein surface. Molecular modeling of all eight diastereomers confirms that the C5 and C6 stereogenic centers largely dictate the spatial presentation of the sulfonamide and urea side chains, independent of the C2 stereochemistry (see Supporting Information Figure SI-2 for modeling data).

Several sulfonamide variants of the 5*R*,6*S* macrolactam core were identified as hits in the primary screen, with the 4-fluorophenyl sulfonamide **6** displaying the largest thermal shift (ΔT_m = +0.6 °C at 25 μM ligand, 100 μg/mL MCL1 protein). To rule out HTS-derived assay artifacts, we investigated the binding of **6** using orthogonal biophysical methods. Differential scanning calorimetry (DSC) relies on a similar physical principle as DSF to determine the effect of ligand binding but directly measures the enthalpy of protein unfolding during a controlled temperature ramp without the use of a fluorescent reporter dye.[24,25] When macrolactam **6** was tested for binding to MCL1 by DSC, a ΔT_m = +1.9 °C was observed at 100 μM of ligand. Importantly, we again observed no binding of the other core stereoisomers by this second biophysical technique (see Supporting Information Figure SI-3).

A third approach to validate binding of putative hits is isothermal titration calorimetry (ITC).[26−28] This method provides a direct measurement of binding enthalpy and also delineates entropic contributions to binding. In our initial experiments with **6**, we were unable to detect binding at 800 μM ligand and 25 μM protein. We suspected that low aqueous solubility of **6** limited our ability to measure binding, even in the presence of 4% DMSO. To improve aqueous solubility, the exocyclic alcohol of **6** was oxidized to the corresponding acid **7** in two steps, with Dess−Martin oxidation conditions being optimal to minimize C2 epimerization (Scheme 1).[29] With the

Scheme 1. Oxidation of Exocyclic Alcohol to Afford Ligand 7[a]



[a]Reaction conditions: 1. Dess−Martin periodinane, DCM, 88%; 2. $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene, *t*BuOH/CH_3CN, 65%.

more soluble macrolactam **7**, we measured an MCL1 K_D = 1.9 μM by ITC. Interestingly, the measured ΔH (enthalpy, −5.6 kcal/mol) and ΔS (entropy, −3.2 cal/mol/K) upon binding of macrolactam **7** were different from previously described MCL1 inhibitors. The indole carboxylate **1** reported by Fesik,[13] for instance, derives much of its binding energy from enthalpic interactions with Arg263 of MCL1, as evidenced by the ΔH = −9.4 kcal/mol (ΔS for **1** = 1.7 cal/mol/K). The lower enthalpy of binding for macrolactam **7** suggests that the appended carboxylic acid does not engage the MCL1 protein and instead serves solely as a solubility handle.

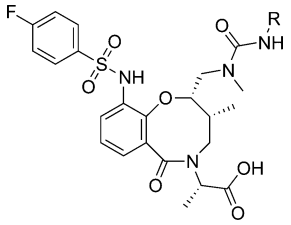
The 4-fluoro and 4-trifluoromethyl groups on the sulfonamide and urea side chains offer a ligand-based method to further confirm binding of macrolactam **7** to MCL1. Specifically, ^{19}F NMR can be used to detect the binding of fluorinated ligands without requiring isotopically labeled protein.[30,31] The ^{19}F NMR resonance of the CF_3 group, in particular, was significantly shifted in the presence of MCL1 ($\Delta\nu$ = 5.4 Hz at 60 μM ligand, 10 μM protein), with significant line-broadening as expected for a slower-tumbling protein−ligand complex. Using ^{19}F NMR detection, we measured an intrinsic K_D = 6 μM for macrolactam **7** binding to MCL1, which is in good agreement with affinity measured by ITC.

To establish the binding mode for **7**, we employed a fluorescence polarization (FP) assay that measures the ability of ligands to competitively bind in the presence of NOXA BH3 peptide. Macrolactam **7** inhibits BH3 peptide binding to MCL1 with IC_{50} = 4.5 μM. Importantly, compound **7** does not interfere with binding of BH3 peptides to antiapoptotic family members Bcl-2 and Bcl-xL (see Supporting Information Figure SI-4). Thus, this direct binding discovery effort has identified a novel macrolactam, which selectively binds in the hydrophobic BH3 binding groove of MCL1.

dx.doi.org/10.1021/ml500388q | *ACS Med. Chem. Lett.* 2014, 5, 1308−1312

Having thus validated the binding of macrolactam **7** by several additional biophysical methods (DSC, ITC, ^{19}F NMR, and FP), we were encouraged to explore the structure-binding relationships of this novel scaffold. Several analogues were synthesized in straightforward fashion to probe both the urea and sulfonamide portions of **7** (Tables 1 and 2). Phenyl ureas

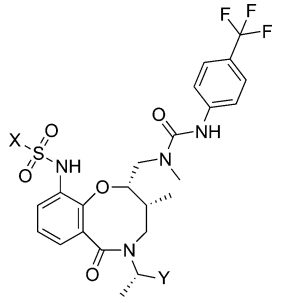
Table 1. Structure–Activity Relationship (SAR) on the Urea Domain of 7



compd	R	DSC ΔT_m (°C)[a]	FP IC_{50} (μM)[b]
7	*p*-$CF_3C_6H_4$	2.9	4.5
8	*m*-$CF_3C_6H_4$	0.2	>25
9	*o*-$CF_3C_6H_4$	0.2	>50
10	Ph	0.2	>50
11	*p*-$MeOC_6H_4$	0.2	>50
12	1-Np	−0.1	>50
13	2-Np	0.8	>25

[a]Differential scanning calorimetry (DSC) assay: 250 μM compound and 25 μM MCL1 (173–329) in 25 mM Hepes, pH 7.4, 100 mM NaCl, 0.1 mM TCEP, and 4% DMSO. [b]FP assay: Serial dilutions of compound, with a top concentration of 50 μM, were preincubated for 30 min with 200 nM MCL1 and 25 nM TAMRA-Noxa in 25 mM Hepes, pH 7.4, 100 mM NaCl, 0.005% Tween 20, and 2% DMSO.

Table 2. SAR on the Sulfonamide Domain of 7



compd	X	Y	DSC ΔT_m (°C)	FP IC_{50} (μM)
7	*p*-F-C_6H_4	CO_2H	2.9	4.5
14	*m*-F-C_6H_4	CO_2H	3.4	4.1
15	*o*-F-C_6H_4	CO_2H	1.9	21.3
16	Ph	CO_2H	2.8	10
17	$PhCH_2$	CH_2OH	1.4	>50
18	$PhCH_2CH_2$	CH_2OH	0.4	>50

lacking an electron-withdrawing group (**10**) or bearing an electron-donating −OMe group (**11**) failed to bind MCL1. The *para*-CF_3 phenyl substituent of the urea in **7** proved to be crucial for binding, as the *ortho*- or *meta*-CF_3 analogues displayed no binding by DSC and FP (Table 1, compounds **7**–**9**). The unique binding of the *para*-CF_3 analogue suggests a specific lipophilic interaction, and not a general electronic effect, is required for binding to MCL1. Other lipophilic side chains including 1- and 2-naphthyl urea groups are apparently too large to effectively bind MCL1, as both showed a smaller thermal shift (ΔT_m = −0.1 °C for **12**; ΔT_m = +0.8 °C for **13**).

A wider range of sulfonamide substitution patterns retained binding to MCL1 (Table 2). In contrast to the strong dependence on para substitution pattern observed in the urea portion, all three F-phenyl sulfonamide regioisomers (**7**, **14**, and **15**) and the unsubstituted phenyl analogue **16** bind MCL1 with similar degree of thermal shift and inhibition of BH3 peptide binding. Chain extension, meanwhile, appears to disfavor ligand binding, as both the benzyl (**17**) and phenethyl (**18**) sulfonamide show a smaller ΔT_m and no inhibition of BH3 peptide binding.

We have recently designed an MCL1 protein construct that can be readily crystallized in the presence and absence of well-validated MCL1 ligands.[32] We were able to cocrystallize macrolactam **7** with this construct, and X-ray diffraction was used to generate a high-resolution (1.8 Å) structure of how this novel scaffold binds to MCL1 (Figure 3, PDB entry 4WGI; Supporting Information Table SI-1). We anticipated that the *p*-CF_3-phenyl group of **7** would engage the large hydrophobic pocket near L246 seen in previous MCL1 structures.[13−16] However, as **7** did not make a direct interaction with R263 of MCL1, both the arginine side chain, F254, and the surrounding residues 247–260 shift and reveal a new hydrophobic binding pocket (Figure 3). This marks the first cocrystallized ligand that binds MCL1 without engaging R263 via an ionic interaction and reveals a distinct binding mode relative to ligands such as **1**. Any interaction with R263 with **7** is more subtle, with a C_δ–H interaction with the *p*-CF_3-phenyl ring potentially contributing to binding. The movement of R263 and the 80 degree rotation of F254 (relative to 4HW2) also demonstrate the conformational flexibility of the MCL1 protein in response to rigid small molecule ligands.

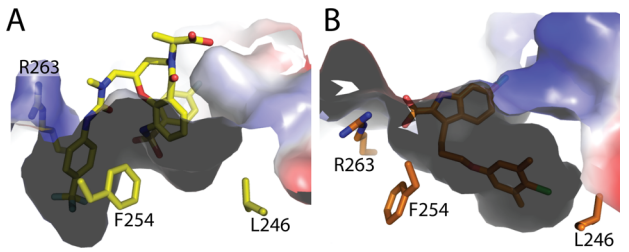


Figure 3. Structure of **7** and MCL1 at 1.8 Å (A) and its comparison to 4HW2 (B) reveals a new hydrophobic pocket. Superposition RMSD of MCL1 and PDB ID 4HW2 chain A, 0.58 Å overall, calculated on all common Cα.

The novel hydrophobic pocket easily accommodates a CF_3 substitution at the *para* position of the phenyl ring; however, substitutions at the *meta* or *ortho* positions encounter steric clash with the main chain of MCL1, which explains the lack of binding for **8** and **9** as described in Table 1. The remaining portions of **7** interact with the binding pocket of MCL1 by shape complementarity, making no specific hydrogen bond or π-stacking interactions. This binding mode is consistent with the relative lack of binding enthalpy observed in ITC experiments. Interestingly, the conformation of macrolactam **7** when bound to MCL1 agrees well with the modeled conformation of free ligand, which suggests a low entropic penalty upon binding of **7** within the hydrophobic groove of MCL1.

dx.doi.org/10.1021/ml500388q | ACS Med. Chem. Lett. 2014, 5, 1308–1312

The competitive binding behavior of compound **7** with regards to BH3 peptide can be examined in more detail by alignment with the NOXA-bound structure PDB ID 2NLA (Figure 4A). The hydrophobic binding pocket induced by the

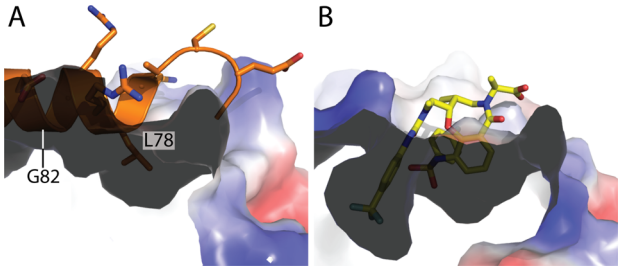
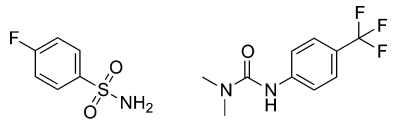


Figure 4. Structure of MCL1 bound to **7** at 1.8 Å aligned to MCL1 bound to the NOXA peptide (PDB ID 2NLA).

p-CF_3 phenyl urea aligns with G82 of NOXA, while the *p*-F phenyl substituent occupies the space filled by L78 (H2 residue) of NOXA. The displacement of BH3 peptides by compound **7** can thus be explained by at least two perturbations: (1) insertion of a hydrophobic moiety into a region where only glycine is presented by BH3 binding partners; and (2) the mimicry of a BH3 hydrophobic residue by the *p*-F phenyl substituent of **7**.

Meanwhile, the lipophilicity of macrolactam **7** (clogP = 4.25)[33] prompted us to identify the minimal structural elements required for binding to MCL1. The urea and sulfonamide side chains were tested for their ability to bind MCL1 (**19** and **20**, Figure 5). Surprisingly, even at ligand concentrations up to



19 **20**

Figure 5. Side chain fragments derived from **7** that fail to bind MCL1.

1000 μM, we were unable to detect measurable binding of either fragment to MCL1 (see Supporting Information Figure SI-5). Our inability to measure binding of these side chains to MCL1 may be attributed to at least two factors: (1) insufficient sensitivity of our biophysical techniques with respect to the intrinsic solubility of these fragments and/or (2) the requisite display of these side chains from a stereochemically unique, rigid core that enabled their discovery. Thus, scaffold-based display of potential binding elements offers a complementary approach to fragment-based discovery methods.

In summary, we have discovered a novel macrolactam that binds to MCL1 in a manner distinct from previously described chemotypes. A suite of biophysical methods reveal the largely entropic mode of binding, which was ultimately confirmed by X-ray crystallography. The selective binding of a single core diastereomer highlights the importance of screening with stereochemically diverse scaffolds to adequately sample three-dimensional space while also suggesting a necessary complement to fragment-based screening methods for tackling difficult biological targets.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis and characterization data for all new compounds; biological and computational methods to establish ligand binding. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was conducted as part of the Slim Initiative for Genomic Medicine, a project funded by the Carlos Slim Health Institute in Mexico. Additional support was provided by the Robertson Foundation.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to acknowledge Acme Biopharma for preparing a key intermediate for macrolactam assembly.

■ ABBREVIATIONS

MCL1, myeloid cell leukemia 1; DSF, differential scanning fluorimetry; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; FP, fluorescence polarization

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