Bioorganic & Medicinal Chemistry Letters 24 (2014) 2090–2093

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Discovery of two aminoglycoside antibiotics as inhibitors targeting the menin–mixed lineage leukaemia interface

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ARTICLE INFO

Article history:
Received 26 December 2013
Revised 13 March 2014
Accepted 17 March 2014
Available online 26 March 2014

Keywords:
Menin
Mixed lineage leukaemia
Pharmacophore-based virtual screening
Inhibitors

ABSTRACT

Menin functions as an oncogenic cofactor of mixed lineage leukaemia (MLL) fusion proteins in leukaemogenesis. The menin–MLL interface is a potential therapeutic target in acute leukaemia cases. In this study, approximately 900 clinical compounds were evaluated and ranked using pharmacophore-based virtual screening, the top 29 hits were further evaluated by biochemical analysis to discover the inhibitors that target the menin–MLL interface. Two aminoglycoside antibiotics, neomycin and tobramycin, were identified as menin–MLL inhibitors with binding affinities of 18.8 and 59.9 μM, respectively. The results of thermal shift assay validated the direct interactions between the two antibiotics and menin. The results of isothermal titration calorimetry showed that the equilibrium dissociation constant between menin and neomycin was approximately 15.6 μM. We also predicted the binding modes of inhibitors at the menin–MLL interface through molecular docking analysis. The results indicated that neomycin and tobramycin competitively occupy the binding site of MLL. This study has shed light on the development of powerful probes and new therapies for MLL-mediated leukaemogenesis.

Mixed-lineage leukaemia (MLL) protein, the human homolog of trithorax in *Drosophila*, is required to maintain the expression of homeobox (*Hox*) family genes which are important for normal haematopoiesis.[1,2] MLL functions as an H3K4 methyltransferase through the C-terminal SET domain in the form of a large multiprotein complex comprising MLL, RbBP5, Ash2L and WDR5.[3,4] Dysfunction of MLL by chromosomal translocation up-regulates the expression levels of *Hoxa7*, *Hoxa9* and *Meis1*, thereby enhancing cell proliferation, blocking haematopoietic differentiation and eventually leading to acute leukaemia in adults and children.[5–9] Leukaemia patients harbouring MLL gene abnormality have very poor prognosis under current medical conditions that mainly use conventional chemotherapy and stem cell transplantation.[5,10] Therefore, new therapeutic methods are urgently needed.

The leukaemogenic activity of mutant proteins is critically dependent on their interactions with menin,[11,12] the product of the multiple endocrine neoplasia type 1 (MEN1) tumour suppressor gene.[13] MEN1 mutation leads to the formation of tumour in endocrine organs, such as parathyroids, enteropancreatic endocrine tissues and the anterior pituitary.[14] Menin has significant functions in suppressing hyperplasia or tumour in other organs, including the lungs[15] and the prostate.[16] This protein also regulates gene transcription. Recruitment of menin by c-Myc, a transcription factor leading to leukaemia, promotes the expression of several genes, such as *Hox*, *Meis1* and *Ezh2*, along with MLL and LEDGF.[4,17,18] The menin–MLL interface is a candidate therapeutic target for novel drugs for acute leukaemia with MLL rearrangements. MLL interacts with menin through two N-terminal motifs, the high-affinity motif menin-binding motif 1 (MBM1) and the low-affinity motif MBM2. MBM1 mutation may significantly disturb the interaction between MLL and menin.[19] The cocrystal structure of menin complexed with MLL peptide (PDB code 4GQ6) reveals that $Pro10^{MLL}$ occupies the hydrophobic pocket surrounded by $Ala242^{menin}$, $Phe238^{menin}$, $Tyr276^{menin}$ and $Met278^{menin}$; hydrogen bonds are located between $Ala11^{MLL}$ and $Tyr323^{menin}$, $Arg12^{MLL}$ and $Glu359^{menin}$ or $Glu363^{menin}$.[20]

One potential therapeutic strategy for MLL-rearranged leukaemia is to block the interaction between menin and MLL. In 2012, the first small-molecule inhibitor[21] and the second-generation inhibitor with high affinity[20] can reverse the oncogenic activity of MLL fusion proteins in leukaemia by targeting the menin–MLL interface. These inhibitors share the same scaffold and function in

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http://dx.doi.org/10.1016/j.bmcl.2014.03.055
0960-894X/

distinct binding modes compared with MLL. Another macrocyclic peptidomimetic inhibitor was subsequently synthesised and optimised.[22] However, peptidometics with large molecular weights are less likely to penetrate the cell membranes;[23] thus, turning these inhibitors into therapeutically useful compounds is challenging.

Identifying inhibitors from existing drugs avoids substantial risks because these drugs have well-known safety and pharmacokinetic profiles.[24] This process also skips the stages of chemical optimisation, toxicology and formulation development. In the present study, we screened a collection of clinical compounds to identify the inhibitors that target the menin–MLL interface. Unlike small-molecule-binding sites in enzymes, protein–protein interfaces (PPI) are typically flat and lack deep cavities.[25] These interfaces provide large binding surfaces;[26] hence, finding small molecules that target PPI is challenging. Structure-based drug design is an effective, inexpensive and time-saving method that facilitates the identification of lead compounds.[27,28] In particular, pharmacophore modelling, which aims to describe the necessary molecular features of a ligand to effectively bind a receptor, is a well-accepted technique in high-throughput virtual screening.[29] Along with the increase in high-resolution protein structures, structure-based pharmacophore has become increasingly important. This method is useful in situations where the number of biologically active molecules is limited, which is the requirement for ligand-based pharmacophores. In the present study, we built an in-house library comprising 900 compounds from existing drugs and applied these compounds to screen inhibitors that target the menin–MLL interface.

Several crystal structures of menin are available in the Protein Data Bank,[20,22,30,31] which provides a prerequisite for structure-based pharmacophore modelling. We selected menin in the complex with the high-affinity motif (MBM1) of MLL with a resolution of 1.55 Å (PDB code 4GQ6)[20] to generate pharmacophore models comprising characteristic interaction patterns between these segments. Considering predefined features (e.g., hydrogen bond acceptor, hydrogen bond donor and hydrophobic) and particular shape constraints (e.g., maximum hydrogen bond distance), we generated the 10 best pharmacophore models sorted by an internal scoring function using Discovery Studio 3.0 (Accelrys Inc., San Diego, CA). Excluded volumes were also added based on the locations of atoms in the protein. The same crystal structure was used to predict the hot spots on the binding surface of menin using HSPred methods.[32] Hot spots are specific amino acid residues on the PPI having a marked increase in binding free energy greater than 4.0 kcal/mol if mutated to alanine.[33] Hot spots are critical for protein–protein interactions. One model with two hydrophobic groups and a hydrogen bond acceptor was selected based on the combination of fitness score and hot spot analysis and then used as queries against the database.

A set of 29 compounds were selected by the pharmacophore model to conduct fluorescence polarisation (FP) competition assay (Supplementary data).[21] FITC-MBM1 at 15 nM and menin at 2 μM in FP buffer (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM DTT and 0.1 mg/mL BSA) were incubated for 1 h in the dark at room temperature. For primary screening, each compound stock with a final concentration of 200 μM was added to the protein–peptide mixture and incubated for another hour. Then, 0.1% DMSO and 40 μM MBM1 were used as negative and positive controls, respectively. Fluorescence polarisation signal was monitored on a POLARstar Omega microplate reader (BMG) and then used to calculate the

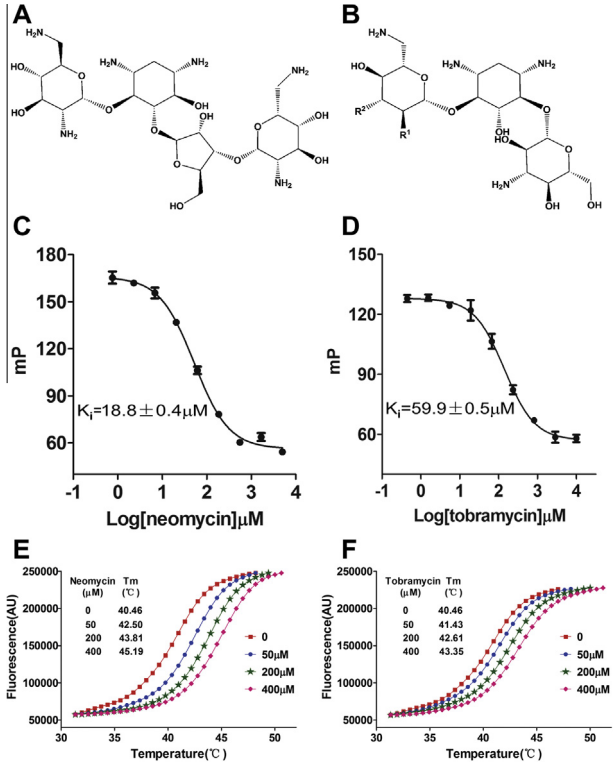

Figure 1. Neomycin and tobramycin compete with MBM1 binding to menin. (A and B) Structures of neomycin (A), tobramycin and kanamycin (B). Tobramycin: R^1-NH_2, R^2-H; kanamycin: R^1-OH, R^2-OH. (C and D) Neomycin and tobramycin inhibit FITC-MBM1 binding to menin with K_i of 18.8 and 59.9 μM, respectively; data represent mean values for triplicates ± SD (E and F) thermal stability of menin is enhanced by the presence of excessive amounts of neomycin or tobramycin, indicating the direct interaction between menin and neomycin or tobramycin. Data represent mean values for triplicates.

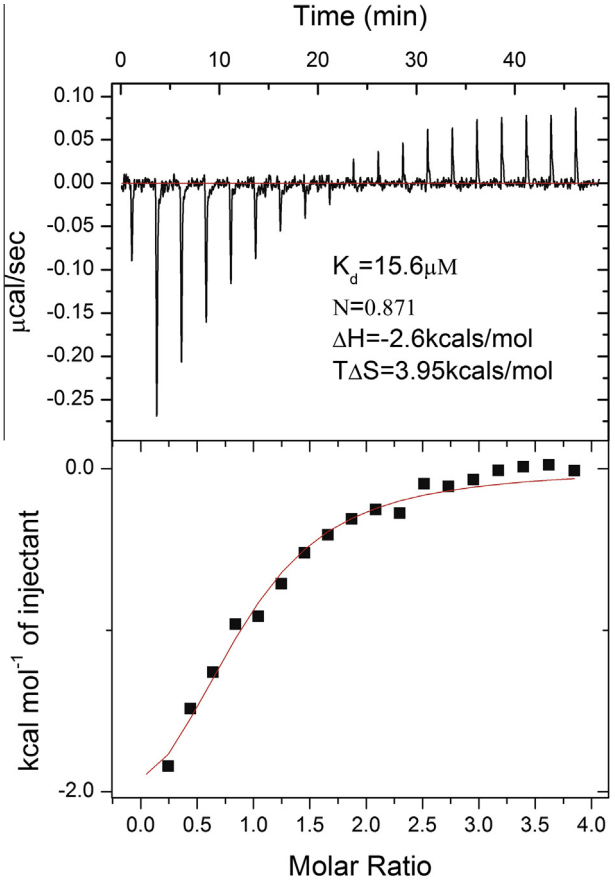

Figure 2. Isothermal titration calorimetry experiment confirms the interaction between neomycin and menin with 1:1 stoichiometry.