NMR structural studies of protein-small molecule interactions

Shah, D.M.

Chapter 2

Discovery of small molecule fragments that inhibit the DNA binding of the TEL-ETS domain

Dipen M. Shah,[‡,†] Eiso AB,[†] Ruta K. Nachane,[†] Johan G. Hollander, [†] David A. Baker,[§] and Gregg Siegal [‡,†]*

[‡] Leiden Institute of Chemistry, Leiden University, Leiden 2300RA, the Netherlands.

[†] ZoBio BV, Leiden 2300RA, the Netherlands.

[§] Leiden University Medical Center (LUMC), Section of Growth Control and Transcription, Department of Molecular Cell Biology, 2300 RC Leiden, the Netherlands.

Manuscript to be submitted to the PLOS One journal

Abstract

The discovery of small molecule ligands help in the design of inhibitors against a protein target involved in a particular disease. Here, we present a study in which we have applied a fragment based screen against a potential tumor angiogenesis target, TEL. TEL and its associated network proteins are involved in control of the development of blood circulatory system. TEL is a transcription repressor that functions through bind of its C-terminal ETS domain to DNA which sterically blocks the access of transcription factors to the promotor. The development of specific inhibitors which would disrupt DNA binding is desirable as this would lead to loss of downstream signaling cascade. Here, we have characterized the DNA binding of TEL-ETS domain using protein-observed NMR, gel retardation and surface plasmon resonance. Of a number of fragment hits, three were further validated using protein-observed NMR. Chemical shift mapping revealed that these fragments share the same binding interface as the DNA. Furthermore, we see that the presence of fragments at a high concentration in the gel retardation assay disrupts the DNA binding capability of TEL. These fragments provide valuable starting entities to develop potent compounds against TEL with a novel mechanism which in turn could lead to loss of formation of blood vessels in tumors.

Introduction

Activation and repression of the transcription of eukaryotic genes during cellular development and/or response to extracellular stimuli, is a highly organized event involving assembly of many protein complexes at promoters and enhancers.[1] Amongst various signal dependent transcriptional regulators, the ETS-family of proteins is one of the largest known involved in proliferation, differentiation and tumorigenesis.[2] ETS1 is the founding member of this family and the name "ETS" is derived from the avian erythroblastosis virus E26, which carried "*v-ets' (E twenty-six)* oncogene.[3] Many of the members are known to activate transcription but the ETS family also includes transcriptional repressors.[2] One of the known repressors is TEL which was identified due to its fusion to the 3' -half of the gene encoding the platelet derived growth factor β receptor in chronic myelomonocytic leukemia.[4] The transcriptional repression may involve one of the following mechanisms. The repressor may act by 1] directly binding to the activation domain of a transcriptional activator, 2] by displacing an activator from activation site, 3] indirectly by altering the chromatin structure and accessibility of a region on the DNA or 4] interfering with the basic transcriptional machinery.[2]

Structurally, TEL possesses an evolutionarily conserved ETS-domain which binds DNA sequences with a GGAA/T core motif.[5] In fact, most of the ETS proteins recognize a consensus 5' -GGAA/T-3' motif within the context of a 9- to 10-bp DNA sequence. [3,6,7] TEL also carries a PNT (Pointed or SAM) domain at the N-terminus which is observed to influence not only the DNA binding but its interaction with other regulatory proteins. The PNT domain of TEL is capable of forming a stable head to tail polymer and hence most likely the full length TEL also has the ability to polymerize. This ability of the PNT domain to cause self-association of the protein

also results in the constitutive activation of the tyrosine kinase activity of fusion protein TEL-ABL, TEL-platelet derived growth factor β receptor and TEL-JAK2, a property that is essential to their transforming and leukemogenic properties.[4,8-11] On other hand, the role of the ETS-domain of TEL, here after refered as TEL_{ETS}, in DNA binding is influenced by an autoinhibitory mechanism in which a flanking C-terminal helix sterically blocks the monomeric ETS-domain. A conformational change is then likely to be necessary in order to carry out DNA binding. It has also been proposed that TEL displays a conformational equilibrium between two structural states 1] where the C-terminal helix is in the inhibitory conformation and 2] where it is in an uninhibited conformation thus not interfering with the DNA binding.[4]

In addition to structural features, the functional importance of TEL is realized by the fact that it is also a therapeutic target to inhibit tumor angiogenesis. TEL is indispensable for endothelial sprouting and normal development of the *Danio rerio* blood circulatory system. TEL acts by regulating the transcription of various angiogenesis inhibitors and executes its function in conjunction with CtBP (Carboxy terminal binding protein). The complex of TEL-CtBP helps in the conditioning of the endothelial cells for angiogenesis. This conditioning is achieved by regulating the balance between stimulatory and antagonistic sprouting cues. One of the plausible hypotheses is also considered where the interaction of CtBP with the PxEIM motif of C-terminal helix of TEL leads to increased DNA binding affinity of TEL. By this way, TEL is able to block the transcription of other essential proteins thought to control the development of blood vessels to tumorigenic tissues. Clearly, the development of specific inhibitors that would either disrupt the DNA or CtBP binding of TEL would open the way for the development of therapeutic strategies to inhibit pathological angiogenesis.[12]

In this work we have demonstrated the binding of the TEL_{ETS} domain to DNA by protein observed NMR, a gel retardation assay and surface plasmon resonance. With a view to find small molecule inhibitors for TEL_{ETS} that would potentially disrupt the DNA binding and hence downstream signaling cascade, we have applied a fragment based screen to generate a set of primary hits. In fragment based drug discovery (FBDD) approach, a larger portion of chemical structure space is explored with a smaller number of compounds. Since the fragments are small and much simpler than drug-like molecules, the fragment methodology enables to discover molecules which bind more efficiently on the protein surface. A fragment based screen was carried out using TINS screening of a fragment library consisting of commercially available compounds.[13-16] Three fragments hits from the screen were further validated using a protein observed NMR approach and gel retardation assay. Using chemical shift mapping through protein-observed NMR experiments, we were able to obtain the binding site of the fragments. Interestingly, the binding surface for the DNA and fragments mapped onto the 3D structure of TEL_{ETS} available in the protein data bank (PDB 2DAO) revealed that the fragments bind at a similar binding interface as the DNA. Furthermore, we have shown that the presence of high concentration of the fragments in the gel retardation assay disrupts the DNA binding capability of TEL_{ETS} domain.[13]

Results

Target immobilized NMR screening of TEL-ETS domain:

We employed TINS (Target Immobilized NMR screening) screening as a primary screen for hit generation on TEL_{ETS} domain.[14] TEL_{ETS} and the reference protein, Akt PH domain were purified and immobilized *via* primary amine coupling as described in the experimental section. The PH domain of Akt was chosen as the reference protein

because it represents a typical protein surface, yet devoid of any specific small molecule binding sites. Further, the use of reference protein in TINS eliminates very weak and non-specific binders which otherwise might lead to false positives in the hit identification process. The functionality of immobilized TEL was determined by repetitive injection of a fragment that appeared as a hit during initial part of the screening procedure.[15]

A fragment library consisting of 1364 commercially available fragments was screened against TEL_{ETS}.[16] The fragment library was screened by repeated cycles of injection of various mixes of fragments, which consisted of 3 to 5 compounds per mix, into both the cells of a dual-cell packed with immobilized TEL_{ETS} domain and the reference protein. After a mix was injected, the flow was stopped and the NMR data was acquired. TINS NMR experiments consisted of using spatially selective Hadamard pulse sequences and the results were analyzed as described.[14,15] The fragments were washed out prior to the next injection of a new fragment mix. The binding of a fragment to a protein molecule immobilized on the solid support causes broadening of the resonances from that fragment. As a result a fragment binding specifically to TEL_{ETS} domain could be detected by simple reduction in the height of all the NMR signals from that fragment in the presence of TEL_{ETS} relative to that in the presence of a reference protein (Figure S1). The fragment screen resulted in the identification of 103 unique hits for TEL_{ETS} domain resulting in a hit rate of 7.5%.

Sequential Backbone resonance assignments

The sequential backbone assignment for the TEL_{ETS} was achieved by acquiring standard triple resonance NMR experiments. Although the solution structure of the TEL_{ETS} domain is deposited in the pdb (Figure S2), there are no published resonance

assignments that are available. The sequential backbone assignments were obtained by correlation of Cα and Cβ chemical shifts of i and i-1 residues to the amide ^{1}H and ^{15}N resonances using HNCACB, CBCAcoNH, HNCA and HNcoCA spectra. Overall, the spectra were of good quality and 82% of the backbone sequential assignment was obtained.

Characterization of TEL-DNA binding

The DNA binding of TEL_{ETS} was characterized using three different approaches. We have used protein-observed NMR, gel-retardation and surface plasmon resonance.

DNA binding by Protein-observed NMR

We employed a frequently used protein observed 2D-heteronuclear single quantum coherence (HSQC) NMR method wherein increasing concentrations of the compound is titrated into uniformly ^{15}N labeled protein and changes in the NMR spectra are followed in presence and absence of the compounds.[17,18] In order to assess the DNA binding capability of TEL_{ETS} , an oligonucleotide consisting of 10 base pairs containing the TEL_{ETS} recognition site was titrated into the ^{15}N isotopically labelled TEL_{ETS}. A short oligo nucleotide sequence (5'-ACA**GGA**AGTG-3') was purposely chosen for the NMR study. The oligonucleotide was titrated at three protein to oligo ratios of 1:2, 1:4 and 1:6. Significant chemical shift perturbations (CSPs) are observed in the 2D [^{1}H,^{15}N] HSQC NMR spectrum upon addition of the DNA as seen in Figure 1.[17,18] It is also observed that at a protein to oligo ratio of 1:4, the binding is saturated as the position of the CSP does not change beyond that concentration. The upper limit for the binding affinity is estimated to be around 80-100 μM. The availability of the sequential backbone assignment of the protein allowed us to map the specific residues involved in DNA binding onto the available solution structure of the protein (PDB 2DAO). Figure 2 shows the DNA binding

interface of TEL_{ETS} domain based on the observed CSPs. Figure 2A shows the binding map of all the residues that show significant chemical shift perturbations in the presence of DNA. The key protein residues involved in the binding are I40, L46, G51, K54, S65, R66, Y71, Y72, R84, F87, T101, L112 and S113. The numbering of the residues here is same as PDB 2DAO. Overall, the protein-DNA complex seems to be in the fast exchange regime on an NMR time scale. Some broadening of the resonance peaks is observed when concentration of DNA is gradually increased.

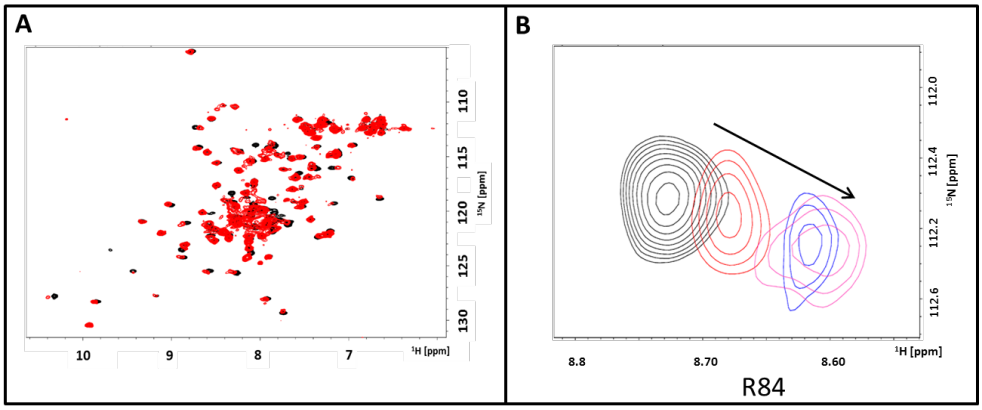

Figure 1. The characterization of DNA binding using protein-observed NMR. [A] The overlay of the 2D [^{1}H, ^{15}N] HSQC spectra of TEL_{ETS} in the absence of DNA (black) and in the presence of the DNA (red). The concentration of the protein used was 60 μM. [B] The concentration dependent chemical shift perturbation observed for residue R84 in the presence of the increasing protein to DNA ratios is shown. In black is the free protein. The red, blue and pink represent protein to DNA ratios of 1:2, 1:4 and 1:6 respectively.

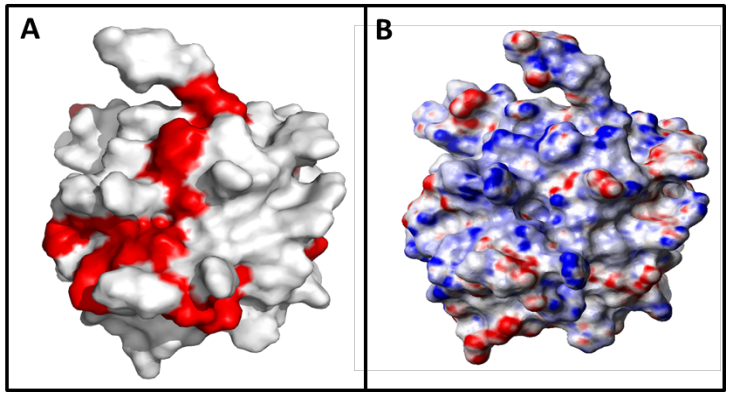

Figure 2. The determination of protein-DNA binding interface. A] Chemical shift mapping of the residues involved in the DNA binding as obtained by titration of increasing concentrations ($\Delta\delta$, ($CSP_{difference}$) > two times standard deviation + $\Delta\delta_{avg}$) of the DNA into ^{15}N labeled TEL and observed by 2D HSQC NMR. The mapping of the residues was performed using the available structure of TEL (2DAO) B] The electrostatic surface potential (blue-positive charge, red- negative charge) of the protein. The figure was created in PyMOL [19]

Gel retardation assay

The DNA binding to TEL_{ETS} was characterized using a gel retardation assay. To investigate DNA binding using gel retardation, increasing amounts of the protein were titrated into a ^{32}P-labelled- DNA oligonucleotide containing three ETS-binding sites (5'-AAACA**GGA**AGTGAGAACA**GGA**AGTGAGAACA**GGA**AGTGG-3'). Subsequently, DNA binding was analyzed using a gel retardation assay. As seen in Figure 3, the migration of the labeled oligonucleotide was retarded indicating that the protein binds to the DNA. The DNA binding of the protein is only visible upon titrating higher concentration of protein indicating that TEL_{ETS} on its own binds comparatively more weakly than the full length TEL harboring the N-terminal PNT-domain.[4] It is also observed in Figure 3 that two bands (a lower band representing unbound oligonucleotide and a higher band representing protein-DNA complex) are seen for DNA to protein ratios between 1:40 to 1:200. To quantitate the binding affinity of the protein-oligonucloetide interaction, we further characterized the complex by SPR

(Surface Plasmon Resonance) assay.

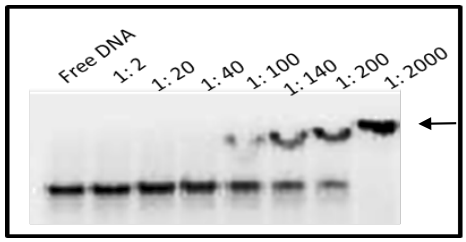

Figure 3. The DNA binding by TEL_{ETS} using gel retardation assay. TEL_{ETS} was titrated into a constant amount of ^{32}P labeled oligonucleotide at indicated ratios. The position of the DNA-protein complex is denoted by arrow. The retardation of the DNA can be clearly seen upon titrating higher concentration of the protein, indicative of the complex formation.

Surface Plasmon Resonance

The characterization and affinity determination of DNA binding to TEL_{ETS} by SPR was achieved by immobilizing the protein on the chip surface to an immobilization level of around 9000 RU. Following the immobilization, increasing concentrations of the same oligonucleotide used in the gel retardation assay (with three recognition sites) were injected in a single cycle manner. The binding response at each concentration was then plotted against the DNA concentration and K_D was determined by a 1:1 binding model using GraphPad Prism software (Figure 4). The fit yielded a K_D of 1.9 μM. The affinity appears to be tighter than that estimated from the NMR titration. This difference in the K_D is likely due to the presence of three -GGAA- recognition sites on the oligonucleotide used in the SPR assay compared to one in the oligonucleotide used in the NMR titration.

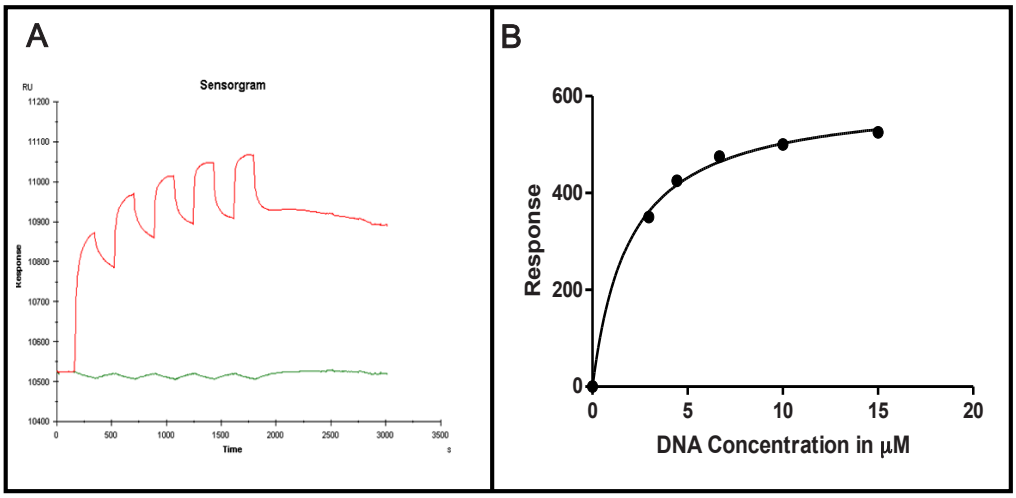

Figure 4. The determination of the DNA binding affinity of TEL_{ETS} using SPR. [A] The sensorgram obtained by single cycle injections of increasing concentrations of DNA (1 µM, 5 µM, 7.5 µM, 10 µM, 15 µM) onto immobilized TEL_{ETS} . The green line corresponds to the background response. [B] Shows the graph obtained by a fit to 1:1 binding model as analyzed by GraphPad prism software and exhibited a KD of 1.9 µM.

Validation of fragment hits by HSQC

The validation of fragment binding to TEL_{ETS} was carried out by protein observed NMR using HSQC experiment (described earlier). The main goal at this stage was to validate a limited number of fragments that would be easier to follow-up by medicinal chemistry (Fragments for which commercial analogs were available and those that can be easier to undergo synthetic chemistry). Three fragments as shown in Figure 5 were selected from a set of hits for this purpose. The resulting CSPs observed upon addition of the fragments were then mapped onto the available TEL_{ETS} solution structure (PDB 2DAO). Interestingly, a similar region of TEL_{ETS} is affected upon addition of all three fragments. As seen in Figure 6, CSPs for these fragments are localized mainly on the protein surface which is common to DNA binding (Figure 3A). The key residues that show significant chemical shift perturbations are N44, R56, K63, R66, A67, R69, I76, F87, T101 and S113 for fragments **1** and **2.** For fragment **3**,

the key residues that gave significant chemical shift perturbations were V15, G51, S66, I77, E80, F87, T101, L112 and S113.

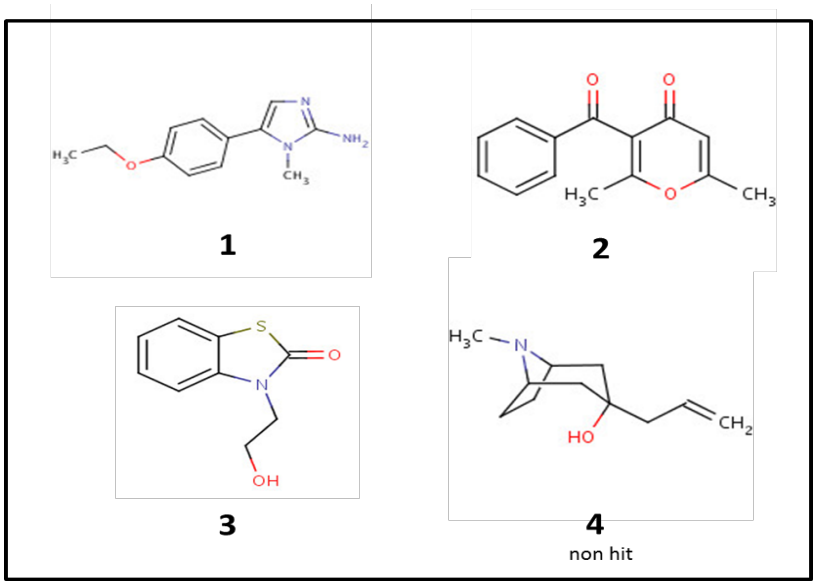

Figure 5. The chemical structures of fragment hits that were employed for follow-up studies after the initial TINS screen on TEL_{ETS}. Fragment 4 is not a hit and was used as a control for gel retardation assay.[16]

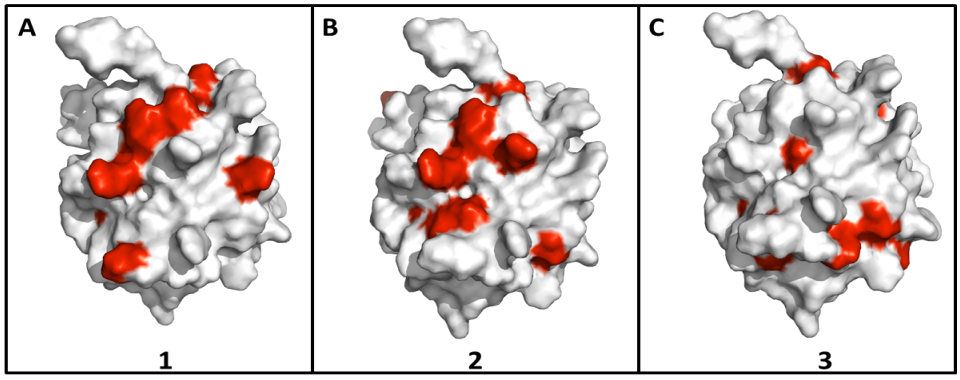

Figure 6. Chemical shift mapping of the protein residues involved in fragment binding to TEL_{ETS}. The chemical shift perturbations (in red) caused by the presence of the indicated fragment hit as determined by [^{1}H,^{15}N] HSQC NMR are mapped onto the available PDB structure of TEL_{ETS} (2DAO). The most significant chemical shift perturbations ($\Delta\delta$ > two times standard deviation + $\Delta\delta_{avg}$) are shown.[17,18] The figure was created in PyMOL.[19]

Disruption of the DNA binding to TEL_{ETS} ***by fragments in gel retardation assay***

In order to investigate the effect of fragments on oligonucleotide binding, we carried out gel retardation assay. First, TEL_{ETS} and oligonucleotide were incubated to form a stable complex at a DNA to protein ratio of 1:200. Subsequently, fragments **2** and **3** were added and the reaction mixture was further incubated for 2 hrs at room temperature. The reaction mixture for fragment **1** showed signs of precipitation and as a result was not considered for further characterization. As a control, a fragment that did not appear as a hit in the TINS screen was also used. As seen in Figure 7, in the presence of 10 mM of the fragment, DNA binding to TEL_{ETS} is significantly reduced. In the presence of control fragment, the DNA binding is not affected indicating the specificity of the fragments that bind to the TEL-ETS domain.

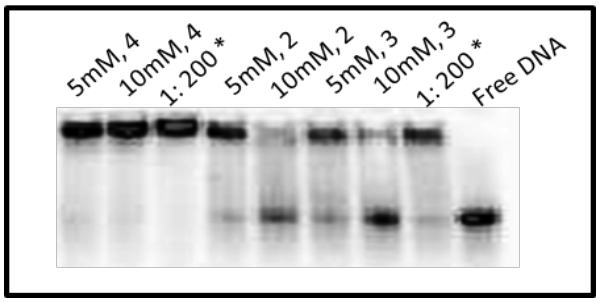

Figure 7. Disruption of DNA binding observed in the presence of validated fragment hits 2 and 3. The final concentration of the respective fragment in the reaction mixture is indicated. Fragment 4 was not a hit in the TINS screen and was used as a control. The asterisk (*) indicates a reaction without added fragment with equal percentage of DMSO. The disruption of oligonucleotide binding is clearly seen at fragment concentrations of 10 mM and comparatively less at a lower fragment concentration (5 mM).

Discussion and Conclusions

Here we report a study that focuses on the interaction of small molecules with TEL_{ETS}. To date there are no known inhibitors that disrupt the TEL-DNA binding. To identify potential fragment hits, we have applied a fragment based screen by NMR using a library of 1364 molecules.[14,16] The advantage of screening molecular

fragments rather than drug-sized molecules is that a dramatically larger portion of chemical structure space is explored with a smaller number of compounds.

In the study presented here, the interaction of the DNA oligonucleotide with TEL_{ETS} was analyzed using protein observed NMR, gel retardation assay and SPR. The binding affinity for the DNA was determined to be 1.9 μM**.** To find small molecule inhibitors that would target the DNA binding site, it would be first necessary to obtain a structural basis for the binding of TEL_{ETS} -DNA complex. We have used protein-observed HSQC NMR to obtain low resolution information to define the DNA binding interface of TEL_{ETS}. The CSP data obtained from the [^{1}H,^{15}N] HSQC experiment clearly demonstrate that DNA binding to TEL_{ETS} is mediated by a highly positively charged protein interface. Of a number of fragments hits discovered from the TINS NMR screen, three fragments were further selected from medicinal chemistry point of view (fragments that can be easily chemically modified). These were further characterized and validated for binding using protein-observed NMR experiments as described earlier. Interestingly, the ligand induced chemical shift changes clearly illustrate that all three fragments bind at the DNA binding interface. The commonality of the binding site between the fragments and the DNA is also reflected in the gel retardation assay. The presence of fragments **2** and **3** at high concentrations in the assay caused significant disruption of TEL_{ETS} -DNA binding. The use of high concentrations was necessary and indicates that the fragments weakly interact with TEL_{ETS}. This is not surprising as these are starting points and not elaborated compounds.

Inhibitors that would specifically disrupt DNA binding should lead to loss of the downstream signaling cascade and influence the transcription repressor function of TEL. This would allow transcription of other proteins that might play a critical role in

the regulation of angiogenesis in tumors tissues.[12] The functional inhibition of the TEL_{ETS}-DNA complex exhibited by the validated fragments **2** and **3** in gel retardation assay suggests they might be starting points to develop tool compounds to enable further target validation studies. However, the selectivity of these fragment hits against the ETS- domain of other proteins and in the presence of CtBP, remains to be tested.

Experimental Section

Protein expression and purification:

C-terminal hexahistidine tagged- TEL_{ETS} (1-118 as per numbering in the PDB-2DAO; 334-452, the numbering as per UniProt ID P41212) was cloned into pET28a and cultures in LB medium containing kanamycin (50 µg/mL) at 37°C in *E.coli* strain BL21 (DE3) (Stratagene). Protein expression was induced at an OD600 of 0.6 with 0.5 mM IPTG and growth was continued for 4 hours at 30°C. The cell pellets were collected by centrifugation at 5000 rpm for 30 minutes. The cell pellets were resuspended in 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol and stored at -80°C. Cells were lysed by addition of 50µl lysozyme (50 mg/mL) followed by incubation for 1 hour at 4°C and passing through French press at 1500 psig twice. The recovered lysate was centrifuged at 35000 rpm at 4°C for 45 minutes using a Beckman Ti35 rotor. The supernatant was applied to a 5 mL HisTrap HP Ni^{2+} affinity column (GE healthcare) equilibrated in the manufacturer's suggested binding buffer supplemented with protease inhibitor PMSF. The column was washed with 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 200 mM imidazole, 1 mM β-mercaptoethanol and protein was eluted with 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole and 1 mM β-mercaptoethanol. The eluted fractions were further purified on a Superdex G75 equilibrated with buffer

of 25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mM β-mercaptoethanol.

TINS NMR screening

C-terminal hexahistidine tagged- TEL_{ETS} (334-452) was cloned into pET28a and expressed in *E.coli* strain BL21(DE3) (Stratagene). The protein was first purified on Ni-Hitrap FF (GE lifescience) followed by purification on Superdex G75 with buffer of 25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mM β-mercaptoethanol. TEL_{ETS} (334-452) and Akt PH domain (aa 1-123) were immobilized via amine-coupling to 500 µL Actigel-ALD resin (Sterogene) in 25 mM Hepes pH 7.5, 100 mM NaCl and 2 mM $MgCl_2$ at 4°C using the coupling reagent provided by the manufacturer. The immobilization efficiency was above 90% and the final concentrations of the immobilized targets were typically in the range of 100 µM. TINS NMR experiments were performed on a 500 MHz Bruker NMR spectrometer using spatially selective Hadamard pulse sequences and analyzed as described previously.[14-16,20,21]

Enzyme Mobility Shift Assay

The DNA binding to TEL_{ETS} was detected using a gel retardation assay. The indicated amount of protein was diluted in a buffer consisting of 10 mM HEPES, pH 7.8, 2 mM $MgCl_2$, 0.1 mM EDTA, 100 µg/mL bovine serum albumin, 15% glycerol, (0-0.8) µg/mL poly(dI-dC) (Boerhringer Mannheim) and 2 mM dithiothreitol in a total volume of 15 µL. 20 fmol of the ^{32}P-labeled DNA oligonucleotide in 5 µL was added, incubated on ice for 30 minutes and applied to a non-denaturing 8% Tris-glycine acrylamide gel containing 2% glycerol. Electrophoresis was performed at 80V for 20 minutes and subsequently 120V for 40 minutes at 4°C in 25 mM Tris-HCl, pH 8.5 and

200 mM glycine. The gel was dried and radioactivity was detected using a phosphorimager (Biorad).

The three validated fragments determined as hits to TEL_{ETS} domain from TINS NMR screening were subjected to gel shift assay analysis. The indicated concentrations of the fragment were added to the reaction mixture and incubated for 2 hours at room temperature. The amount of DMSO in the controls was matched to the equivalent amount in the presence of a fragment.

NMR backbone sequential assignment

Uniform ^{13}C, ^{15}N labeling of TEL-ETS domain was achieved by expression of C-terminally hexahistidine tagged TEL_{ETS} (aa 334-452) in *E.coli* BL21 (DE3) cell grown in M9 minimal medium supplemented with $^{15}NH_4Cl$ and ^{13}C-D-Glucose (CIL) as a sole nitrogen and carbon source. The protein was purified as described above. The protein was concentrated down to 0.7 mM in 25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mM β-mercaptoethanol. In order to perform sequential assignment of backbone amide, the following experiments were acquired at 296K on a 600MHz BRUKER DMX NMR spectrometer equipped with a TXI cryo-probe; [^{1}H, ^{15}N]-HSQC, HNCO, HNCaCb, CbCaCONH, HNCaCO, HNCA, and HNCOCA . The acquired data was processed using nmrpipe and visualized on Sparky.[22,23] The assignment process was guided by the predicted chemical shifts calculated by SHIFTX in automatic assignment program MARS using PDB-2DAO as an input structure.[24,25]

Chemical shift perturbation and generation of binding site

^{15}N labeled C-terminal hexahistidine tagged TEL_{ETS} (aa 334-452) in *E.coli* BL21

(DE3) cell grown in M9 minimal medium supplemented with $^{15}NH_4Cl$ as a sole nitrogen source, and purified as described above. The [^1H,^{15}N]-HSQC were acquired at 296K on a 600MHz Bruker DMX NMR spectrometer equipped with a TXI cryo-probe. The NMR sample was prepared in 25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mM β-mercaptoethanol. The typical NMR sample contained 0.130 mM of the protein, fragments at various concentrations (see text) and 5% d_6-DMSO. The pH of the samples was adjusted carefully within +/- 0.05 units after addition of the compound. During the experiment, total of 128 indirect increments with 16 scans per increments were acquired. The data was processed using Topspin 1.2/2.1 (Bruker) and visualized on Sparky.[23] Chemical shift perturbations in [^1H, ^{15}N]-HSQC were calculated based on the ($\Delta\delta$ > two times standard deviation + $\Delta\delta_{avg}$) for change in ^1H and ^{15}N ppm value between in the presence and absence of a compound. The potential binding sites of a compound were mapped onto the surface of the TEL-ETS domain (PDB-2DAO) structure using chemical shift perturbation data and viewed in PyMOL.[19]

Surface Plasmon Resonance studies

DNA binding to TEL_{ETS} domain was tested using surface plasmon resonance on a T200 biacore instrument (GE healthcare). Initially, pH scouting was performed to determine the optimum pH for protein immobilization on the CM5 chip surface. Following immobilization of the protein to required response units (6000 RU), increasing concentrations of DNA were titrated in a single cycle kinetic mode. The buffer conditions used was 25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mM β-mercaptoethanol + 3% DMSO. The analysis was performed using a Biacore evaluation software and GraphPad Prism software.

Contributions

The clones for the production of the protein were provided by David A. Baker (Leiden Univeristy Medical Centrum). Ruta K. Nachane was responsible for protein and compound preparations for TINS NMR screening. Johan G. Hollander helped in the NMR set-up of the TINS screen and Eiso AB performed the computational analysis of the fragment screen.

SUPPLEMENTRY INFORMATION

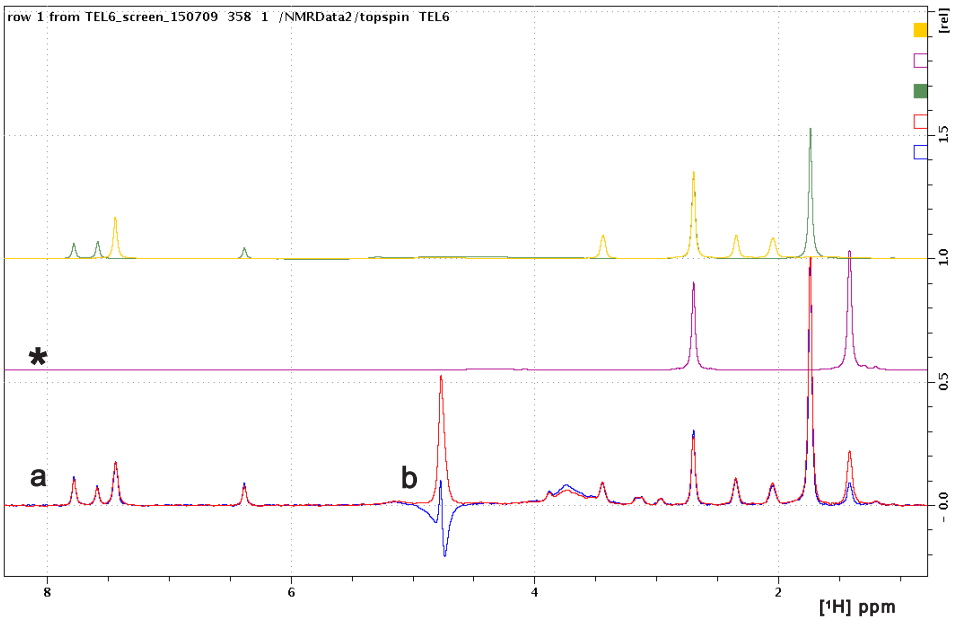

Figure S1. The screening of fragment library using TINS for binding to immobilized TEL_{ETS}.[14,15] The figure shows detection of ligand binding to the protein in a fragment mix. The overlaid ^{1}H resonances spectrum at the bottom represented by **a** shows the binding of a fragment to TEL_{ETS} as seen by the reduction in the peak height of the blue spectrum as compared to the red one which is in the presence of reference protein, PH domain of AKT. The asterisk (*) indicates the spectrum (in purple) of fragment that preferentially binds TEL_{ETS} in solution. The residual signal from water is indicated by **b**. All other resonances belong to the other fragments in the mix.

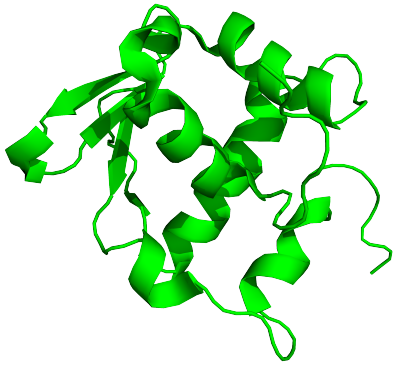

Figure S2. The NMR structure of TEL_{ETS} domain (PDB-2DAO)

REFERENCES

1. Verger A, Duterque-Coquillaud M. (2002) When Ets transcription factors meet their partner. Bioessays 4: 362-70.
2. Mavrothalassitis G, Ghysdael J. (2000) Proteins of the ETS family with transcriptional repressor activity. 19 (55): 6524-6532.
3. Sharrocks, AD. (2001) The ETS-domain transcription factor family. Nat Rev Mol Cell Biol 2; 827-837.
4. Lopez RG, Carron C, Oury C, Gardellin P, Bernard O, et al. (1999) TEL is a sequence- specific transcriptional repressor. J. Biol. Chem. 274: 30132–30138.
5. Karim FD, Urness LD, Thummel CS, Klemsz MJ, McKercher SR, et al. (1990) The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. Genes Dev 4: 1451-1453.
6. Green SM, Coyne HJ, 3rd, McIntosh LP, Graves BJ. (2010) DNA binding by the ETS protein TEL (ETV6) is regulated by autoinhibition and self-association. J Biol Chem 285: 18496–18504.
7. Graves, B.J, Petersen J.M. (1998) Advances in Cancer Research (Woude G.V., Klein G., editors. eds) pp 1-55, Academic Press, San Diego.
8. Golub TR, Goga A, Barker GF, Afar DE, McLaughlin J, et al. (1996) Oligomerization of the ABL tyrosine kinase by the ETS protein TEL in human leukemia. Mol. Cell. Biol. 16: 4107-4116.
9. Lacronique V, Boureux A, Valle VD, Poirel H, Tran Quang, C, et al. (1997) A TEL-JAK2 Fusion Protein with Constitutive Kinase Activity in Human Leukemia. Science 278: 1309-1312.
10. Jousset C, Carron C, Boureux A, Tran Quang C, Oury, C, et al. (1997) A domain of TEL conserved in a subset of ETS proteins defines a specific oligomerization interface essential to the mitogenic properties of the TEL-PDGFR beta oncoprotein EMBO J. 16: 69-82.
11. Carroll M, Tomasson MH, Barker GF, Golub TR, Gilliland DG. (1996) The TEL/platelet-derived growth factor b receptor (PDGFβR) fusion in chronic myelomonocytic leukemia is a transforming protein that self-associates and activates PDGFβR kinase-dependent signaling pathways. Proc. Natl. Acad. Sci. 93: 14845-14850.
12. Roukens MG, Alloul-Ramdhani M, Baan B, Kobayashi K, Peterson-Maduro J, et al. (2010) Control of endothelial sprouting by a Tel–CtBP complex. Nat Cell Biol 12: 933–942.
13. Hajduk PJ, Greer J. (2007) A decade of fragment-based drug design: strategic advances and lessons learned. Nat Rev Drug Discov 6(3): 211-219.
14. Vanwetswinkel S, Heetebrij RJ, van Duynhoven J, Hollander JG, Filippov DV, et al. (2005) Chem Biol 12: 207-216.
15. Marquardsen T, Hofmann M, Hollander JG, Loch CMP, Kiihne SR, et al. (2006) Development of a dual cell, flow-injection sample holder and NMR probe for comparative ligand-binding studies. J. Mag. Reson 182: 55-65.
16. Siegal G, AB E, Schultz J. (2007) Integration of fragment screening and library design. Drug Discov Today 12: 1032-1039.
17. Sklenar V, Piotto M, Leppik R, Saudektim V. (1993) Gradient-Tailored Water Suppression for ^{1}H - ^{15}N HSQC Experiments Optimized to Retain Full Sensitivity. J Magn Reson 102 (A): 241 -245.

18. Bodenhausen G, Ruben, DJ. (1980) Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy. Chem Phys Lett 69 (1): 185-189.
19. The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.
20. Kobayashi M, Retra K, Figaroa F, Hollander JG, Ab E, et al. (2010) Target immobilization as a strategy for NMR-based fragment screening: comparison of TINS, STD, and SPR for fragment hit identification. J Biomol Screen 15 (8): 978-989.
21. Murali N, Miller WM, John BK,Avizonis DA, Smallcombe SH. (2006) Spectral unraveling by space-selective Hadamard spectroscopy. J Magn Reson 179: 182-189.
22. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, et al. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J Biomol NMR 6(3): 277-93.
23. Goddard TD, Kneller DG. SPARKY 3, University of California, San Francisco.
24. Neal S, Nip AM, Zhang H, Wishart DS. (2003) Rapid and accurate calculation of protein ^{1}H, ^{13}C and ^{15}N chemical shifts. J. Biomol. NMR 26: 215-240.
25. Jung, YS and Zweckstetter, M. (2004) Mars-robust automatic backbone assignment of proteins. J Biomol NMR 30(1): 11-23.