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CHAPTER 5

Identification of Novel Small Molecule Inhibitors of ETS Transcription Factors

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ABSTRACT

The **E**-**T**wenty-**S**ix_(ETS) family of transcription factors is a large and diverse protein family which regulates cellular development and differentiation. ETS factors have a substantial role in several disease states, particularly cancer, because many of these transcription factors are downstream of the major signal transduction pathways, which play critical roles in cell proliferation. Over the last two decades, multiple approaches have been developed to inhibit receptors and kinases with the aim of blocking tumor growth. However, most therapies have failed to deliver the anticipated clinical benefits due to drug resistance and toxicity. Consequently, there is an urgent need for new therapies. This study aims to identify potential small molecule inhibitors of ETS transcription factors binding to their consensus DNA binding site. The overall rationale is that this will limit overt toxicity and overcome the severe redundancy problems in cell signaling pathways and associated acquired resistance to therapy. To this end, we have performed proof-of-principal high throughput screens to find unique small molecules that inhibit the activity of ETS transcription factors by blocking their association with their DNA consensus sites. These screens and the characterization of resulting hit compounds suggest that targeting ETS activity could enable the identification of novel inhibitors of cancer cell proliferation, which exhibit relatively less toxicity than currently available treatments and may suffer less from problems of acquired drug resistance.

INTRODUCTION

In the past decade, oncology has witnessed a shift from traditional chemotherapies toward precision treatments that specifically target tumor cells to limit cytotoxicity in healthy tissues. While there have been some improvements in patient outcomes, the promised revolution has thus far failed to materialize. The two principal reasons for this are the acquisition of resistance to treatment and the fact that this class of inhibitors has, to date, been designed to block the activity of a relatively limited range of enzymes and receptors, e.g., kinases. Therefore, one ultimate goal is to design therapies that effectively treat the disease, do not cause off-target toxicity, and are not susceptible to resistance (Zhong, 2021; Manzari, 2021). In this light, there is a critical need to identify novel targets, particularly now that there is broader acceptance of the utility of searching beyond the historical constraints of the previously termed 'druggable genome. The druggable genome essentially encompasses enzymes and proteins expressed at the cell surface, thus making it accessible for treatment. However, such a restricted approach has proven not to be an unqualified success, and attention is now shifting to alternative targets previously considered to be 'non-druggable. Therefore, different novel targets, which have a demonstrable role in tumor development and evolution, need to be identified to develop more targeted treatments for tumors (Manzari, 2021; Hopkins, 2002; Zhong, 2021; Bushweller, 2019)

There are compelling reasons to think the ETS family of transcription factors (TFs) represents an excellent target (Sizemore, 2017). ETS factors were first discovered in 1983 as a fusion protein expressed by the avian retrovirus E26 (E-Twenty-Six). They are essential for normal cell proliferation, growth, and differentiation. Moreover, they sit downstream of the major MAP kinase signaling pathway and link it to other critical signaling networks, including the NOTCH system and the transforming growth factor beta (TGFβ) pathways (Hollenhorst, 2012; Wasylyk, 1998). In this way, they act as a central hub for translating extracellular stimuli into the changes in gene expression required to modulate cellular behavior. Mechanistically, these proteins function by binding to consensus DNA binding sites and thereby either activate or repress gene expression. Crucially, dysregulation of ETS factors often plays a role in tumor development by influencing cellular proliferation, invasion, migration, and evasion of apoptosis (Bazin, 2002; Butler, 2017; Fry & Inoue, 2018; Schober, 2005). Indeed, knock-down studies have demonstrated that loss of ETS activity blocks tumor cell growth (Fry & Inoue, 2018; Hsing, 2020). In addition to targeting tumor cells, there is good reason to assert that targeting ETS factors would also block other essential features of the tumor microenvironment (Li , 2010). For example, ETS

transcription factors are indispensable for angiogenesis (Lelièvre, 2001) , suggesting they could play an essential role in the angiogenic switch, a critical phase of tumorigenesis during which tumors stimulate the local vasculature to grow into the tumor, thereby enabling its growth and dissemination (Gu, 2020). Thus, because ETS factors underpin blood vessel growth, specific inhibitors could potentially accomplish two goals simultaneously: they could inhibit the proliferation of tumor cells and block tumor angiogenesis. (Oettgen, 2010).

Approaches for identifying ETS factor inhibitors

Targeting transcription factors is challenging owing to a lack of suitable targetable structural motifs due to their non-enzymatic mechanism of action (Chen, 2020). In this study, we apply novel methodologies for targeting ETS factors to identify (and characterize) small molecule inhibitors. For several reasons, small molecules are potentially the most effective means of targeting transcription factors compared to peptides or antibodies. Firstly, small molecules can generally penetrate cells more easily due to their size, allowing them to reach the transcription factor within the cell. Secondly, small molecules are unlikely to suffer the same problems of immunogenicity associated with therapeutic peptides and antibodies (Kuriakose, 2016; Jarvi,2021; Wei, 2021).

There are two main strategies for targeting transcription factors using small molecules. One involves selecting molecules that directly bind to the protein, such as DNA-encoded libraries or NMR-based assays (Goodnow, 2017). The second involves high-throughput screens (HTS) using multi-component functional assays in which the process of ETS DNA-binding is targeted. In this study targeted immobilization NMR screening (TINS) and HTS have been used to recover small molecule ETS factor inhibitors. The TINS approach uses small molecular scaffolds, which can sample significantly more chemical space (approximately 200 daltons) than bulkier small molecules (approximately 400 daltons). Thus, their small size enables a broader exploration of suitable chemical interfaces. The second screen was a multi-component high throughput functional screen yielding larger lead-like compounds capable of blocking ETS from associating with its consensus DNA site (Kim, 2015; Bon, 2022).

RESULTS

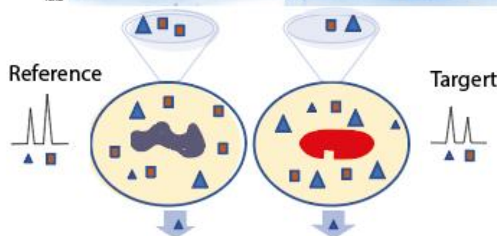
A fragment library screen (Targeted Immobilization NMR Screening (TINS)) to identify novel inhibitors of ETS transcription factors

To discover specific small molecule inhibitors of ETS factors, we aimed to screen for small molecule inhibitors that bind to the ETS DNA binding domain of ETS proteins. To that end, optimized purification protocols were developed to

produce milligram quantities of pure recombinant ETS DNA Binding Domain (DBD), initially of two ETS factors, TEL and FLI1. Figure 1A shows a Coomassie™ stain of FLI1 DBD, which has been double purified via two steps of column chromatography: nickel column chromatography followed by heparin column chromatography.

A

B



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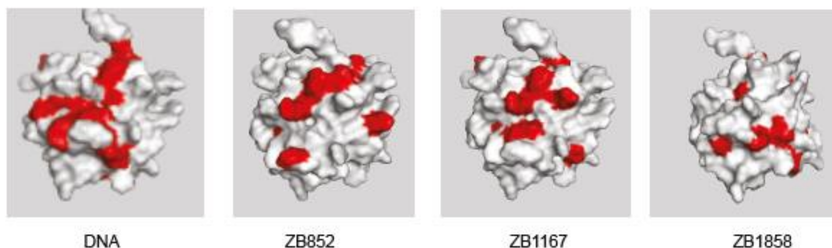

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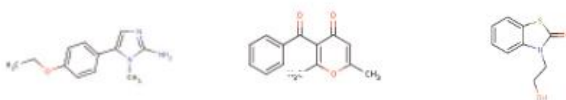

Figure 1. (A) Coomassie™ stain of FLI1 DBD. Two different columns (nickel column chromatography followed by heparin column chromatography) have been used for purifying the FLI1 DBD. Arrows indicated fractions were processed for further purification (B) Applying TINS method for Fragment-based drug discovery. A fragment library screen was done by simultaneously injecting a mix of fragments into a dual-cell sample holder with an immobilized TEL_{ETS} DNA-binding domain as a target and the reference protein. NMR spectroscopy detects peaks corresponding to fragments, as depicted by a reduced peak for the binder (red square) in the cartoon, while non-binders (blue triangle) exhibit unchanged peaks. Screening for a decrease in fragment peak intensity compared to a reference indicates binding. (C) Validation of the candidate fragments from the Targeted Immobilized TEL-DBD NMR Screen. NMR Chemical shift mapping of the TEL-DBD residues upon

fragment binding. The protein-DNA binding interface has been comparing to fragment binding to $TEL_{ETS.}$ (D) The chemical structure of fragment hits selected for follow-up studies.

Subsequently, the purified ETS DNA-binding domain was employed to screen a fragment library consisting of 1364 commercially available fragments using TINS (schematically represented in Figure 1B) in collaboration with the Chemistry department of Leiden University. This proof of principle screen, in which direct binding of molecular fragments to the TEL-DBD proteins of interest is assayed, identified multiple hits.
To validate these hit molecules, as the first step, NMR was deployed to map the site of binding of several 'top' hits to the DNA-binding domain. The structure of a protein-ligand complex was determined for three of the initial fragment hits obtained from the screen (Figure 1C). The chemical structures of the validated fragments are illustrated in Figure 1D. Notably, the pattern of contact between the ETS DNA binding domain (EDBD) and a consensus DNA site overlaps with the points of contact between the EDBD and a sub-set of hit compounds, suggesting that such molecules might disrupt ETS factor binding to DNA.

Fragments disrupt the binding of the ETS DNA binding domain to DNA, with low affinity.

To further validate the hit fragments, initially, we established an *in vitro* assay employing a biotinylated consensus ETS DNA-binding site and *in vitro* translated EDBDs to recapitulate the binding of ETS protein to DNA. This optimized assay has been used to assess the ability of fragments to block TEL binding to its DNA-binding site. Figure 2A illustrates at least two distinct classes of hits. For instance, one fragment (number 1838) displayed an enhancing DNA binding effect, while another fragment (number 852) exhibited the ability to inhibit DNA binding. The requirement for a high concentration (10 mM) due to the small size of the fragments indicates that these fragments exhibit low potency and low affinity for the target. This observation is not unexpected, as the fragment screen was anticipated to be a starting point for exploring bulkier novel analogues with superior affinities for the EDBD (Bon, 2022). Despite the direct interaction of fragments with the target protein, the affinities are far too low to elicit effects in cell-based assays because the fragments are not potent enough in practice. Broadly, there are two ways to address this issue: 1) with medicinal chemistry, which specifically modifies fragment side chains; 2) screen computationally for analogues that share common structural features with the original fragment candidates identified in the initial screen.

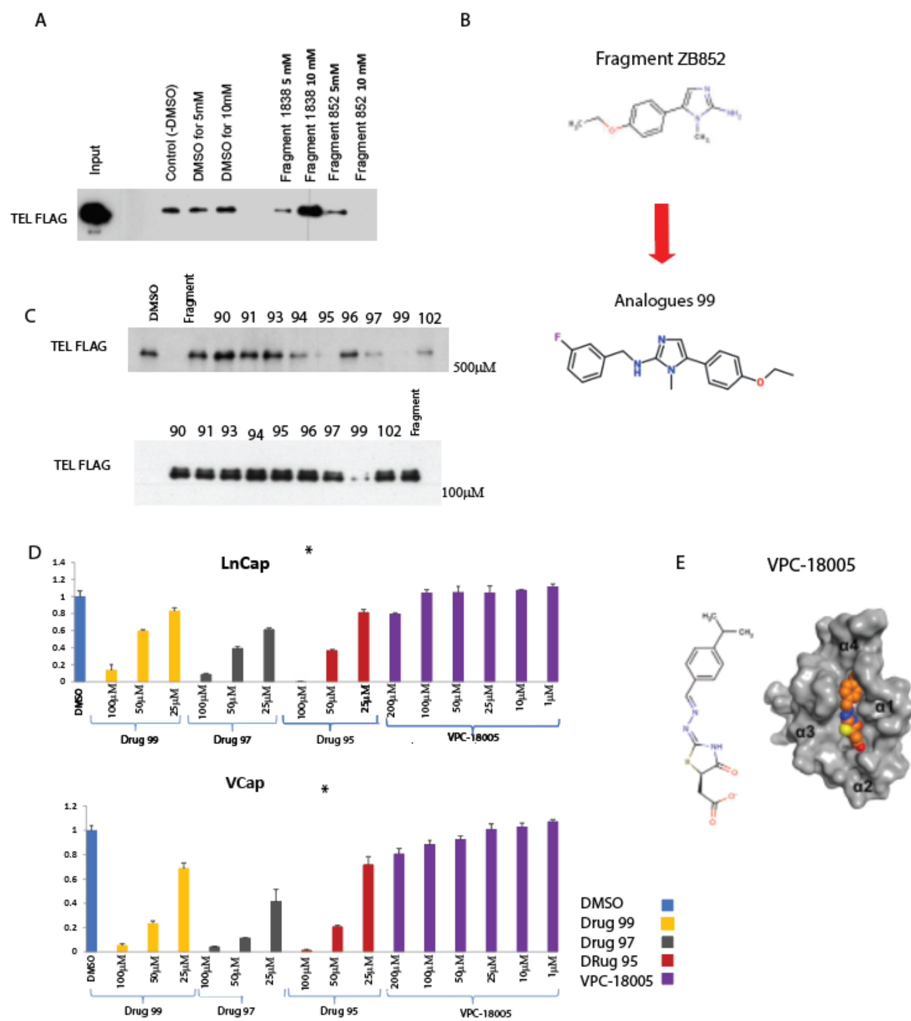

Figure 2. (A) Shown is hit validation using *in vitro* binding assay. A biotinylated consensus ETS DNA-binding site and *in vitro* translated proteins were used to validate the ability of binding to (TEL). DNA-bound proteins were detected by Western blotting using a FLAG mouse monoclonal antibody. Fragments disrupt binding with low affinity. **(B)** A chemical compound database (CHEMDOC) has been used to identify analogues. **(C)** Analogues disrupt TEL binding to a consensus DNA site at lower concentrations in *in vitro* binding assays. **(D)** Cell viability assay have been done using analogues versus VPC-18005 on prostate cancer cell proliferation. The indicated cell lines were incubated in the presence or absence of a range of analogues 95, 97, and 99 concentrations in LnCap (upper panels) and VCap (lower panels). Proliferation was assessed using the Cell Titer-Blue cell reagent. Each data point is the mean of triplicate measurements. Error bars represent the standard deviation of the mean. **(E)** The chemical structure of VPC-18005 and a space-filling representation of the predicted VPC-18005 binding pocket within the ERG-ETS domain. VPC-18005, as an anti-ERG drug, has been developed recently and targets the DNA-binding domain of ERG protein (Butler, 2017).

In general, analogues are identical to the original fragment except for additional chemical side chains (Figure 2B). Since medicinal chemistry is time-consuming and high-risk (modifying the fragment may make it less potent), we initially implemented the second strategy. Consequently, over 100 fragment analogues were selected through computational screening of selected hits. For example, Figure 2B shows a typical analogue of fragment 852. These analogues were chosen for further characterization in biochemical and biological assays.
Figure 2C demonstrates an improved effect of the analogues (500-100 µM), upwards of 100-fold in terms of potency, in a functional *in vitro* binding assay. Subsequently, we investigated whether the proliferation of tumor cells could be influenced when they are exposed to the analogues. This was examined on two prostate cancer cell lines: the TMPRSS2-ERG mutant line (VCaP cells) and the TMPRSS2 wild-type tumor line (LnCaP cells). The analogues efficiently inhibited cell proliferation of both lines, with IC50 values within the range of 25-100 µM (Figure 2D). By contrast, a recently reported putative ETS factor inhibitor discovered by computational chemistry methods, VPC-18005 (Figure 2E), failed to inhibit tumor cell proliferation at comparable drug concentrations (Figure 2D). Collectively, these data suggest that fragment-based screening could potentially identify molecular scaffolds that bind to the EDBD, and subsequent selection of the analogues might generate clinically valuable compounds. Such an approach has been routinely adopted by the industry as a proof-of-principle test of the suitability of targets for larger-scale screens (Kirsch, 2019). In this context, we next performed an ultra-high-throughput screen of a substantial small molecule library.

Ultra-high Throughput Screen (HTS) to identify novel ETS factor inhibitors

The second screen was conducted in collaboration with the European Lead Factory (ELF). This type of screening is only feasible when the assay is rapid, scalable, and automated. For this purpose, HTRF (Homogeneous Time-Resolved Fluorescence) has been applied by using the His-epitope tagged ETS DNA-binding domains (of TEL or FLI1) and a biotinylated oligonucleotide harboring three ETS binding sites (Figure 3A). It should be emphasized that HTRF is the most frequently used generic assay technology, which is widely used in drug target studies involving high-throughput screening (Degorce, 2009). This technology is based on a combination of fluorescence resonance energy transfer technology (FRET) with time-resolved measurement (TR). This energy transfer occurs between two Fluorophore conjugated antibodies (donor and acceptor) when they come close (Bazin, 2002; Mathis, 1999). In the case of the ETS screen, one fluorophore was attached to the antibody, which binds to the His tag of the protein. Another fluorophore was coupled to the antibody, which recognizes the

biotinylated DNA site. When two fluorophores are in close proximity, upon excitation of the donor by a light source, energy is conveyed from the donor to the acceptor. Subsequently, the activated acceptor emits fluorescence at a specified wave length. Compounds that could disrupt the binding of the ETS protein to the DNA site lead to a loss of the fluorescence signal. Figure 3A schematically represents the mode of action of the HTRF biomarker assay.

Using this assay, 450000 small molecules have been screened to identify lead-like compounds capable of blocking ETS from associating with DNA (a so-called functional screen). This screen led to the identification of five compounds. For simplicity, ELF compounds have the designations A-E in the following experiments. The chemical structures of the small molecule inhibitors are depicted in Figure 3B.

As mentioned, a structural comparison of fragments and small molecule inhibitors showed that these molecules contain more drug-like molecular features, whereas the fragment molecules are smaller and less potent.

Biochemical validation of small molecule inhibitors in functional *in vitro* binding assays

Using *in vitro* assays shown earlier for the fragments and analogues (Figure 2A and 2C), we showed that the hits clearly inhibit TEL DNA binding, particularly drugs A and D (Figure 3C and 3D). It should be noted that unlike the fragment assay, in which fragments were used at a concentration of 10 mM, here only 50 µM concentrations were sufficient to observe an inhibitory effect. A critical issue to address is whether the drugs function by specifically blocking TEL binding to DNA or by intercalating into the oligonucleotide (what we would call non-specific inhibition). To address this, we tested the ability of the drugs to block the binding of the unrelated JUN protein to its consensus Activator Protein (AP)-1 transcription factor binding site (Figure 3E). Our evidence suggests that JUN binding is not obviously affected by drugs A, B, C, and D but is inhibited by drug E. These experiments are consistent with the idea that drugs A, B, C, and D might inhibit ETS DNA binding by targeting the ETS protein rather than the DNA. By contrast, drug E inhibited the binding of full-length TEL, FLI1, and JUN binding to the AP-1 consensus site. Therefore, the binding is likely non-specific, and this molecule was subsequently excluded from further studies.

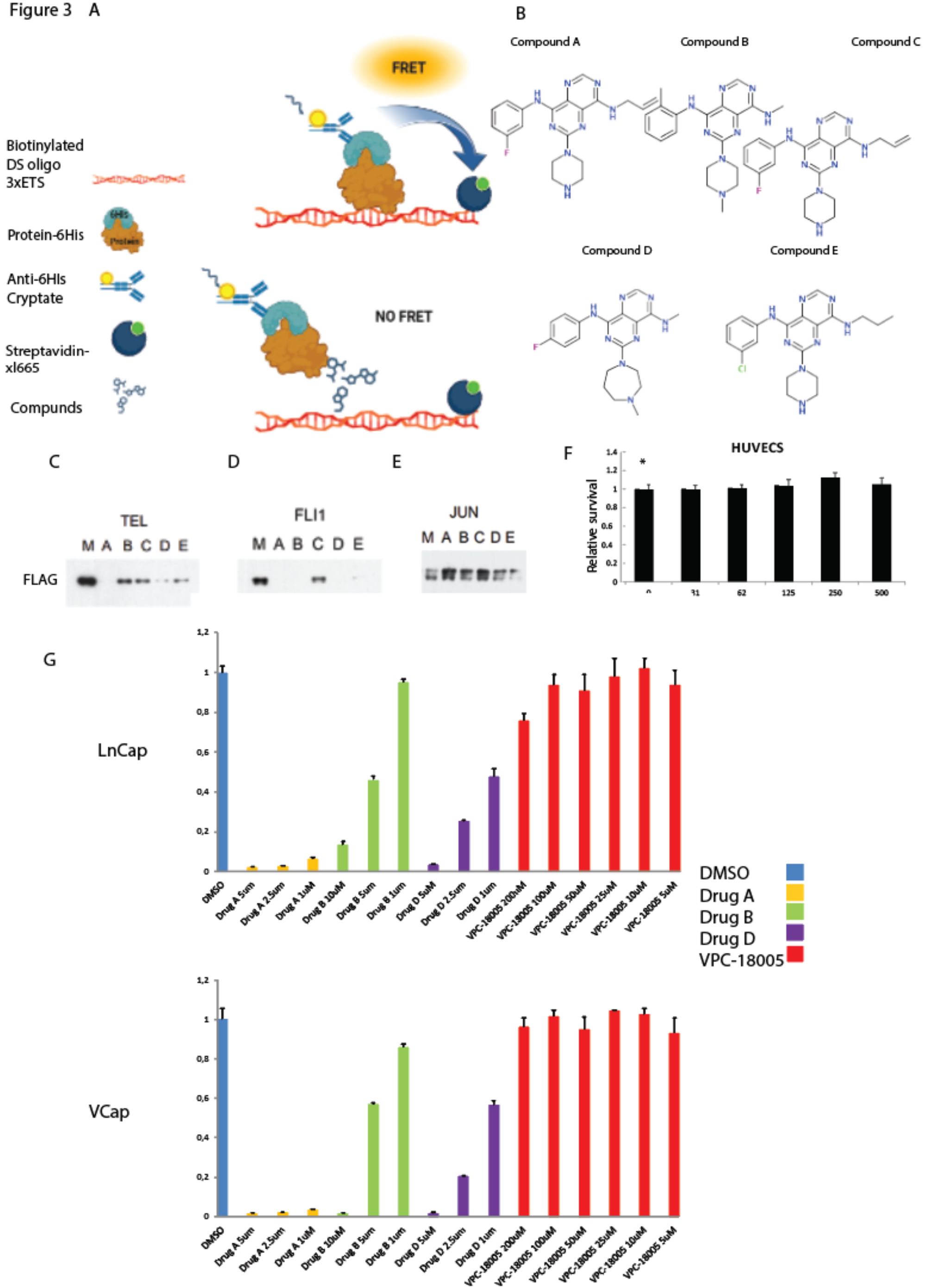

Figure 3. (A) Schematic representation of disruption of Protein-DNA binding through Ultra-high throughput screen.

< Figure 3 (continued). (B) Chemical structures of the small molecule inhibitors (ELF drugs, A, B, C, D, E). **(C)** ELF compounds inhibit ETS protein binding to DNA (*In vitro* DNA-binding assay). Double-stranded oligonucleotides harboring either three consensus ETS binding sites for TEL **(C),** FLI1 **(D)**, or three AP-1 binding sites for JUN **(E)** were incubated with *in vitro* translated TEL, FLI1 and JUN respectively. All constructs were FLAG epitope-tagged to enable a formal comparison. ELF drugs were added to a final concentration of 50 µM. DNA-bound proteins were detected by Western blotting using a FLAG mouse monoclonal antibody. **(F)** The impact of ELF drugs on HUVECs' cell viability is illustrated. Unaffected growth of 'normal' HUVECs was observed upon treatment with the shown concentration of drug A. **(G)** The treatment effects of drugs on the proliferation of prostate cancer cells (VCaP and LnCaP) compared to VPC-18005 are depicted. Cell proliferation reduction due to treatment with ELF Drugs A, B, and D on prostate cancer cells is compared to an in silico developed ETS inhibitor, VPC-18005. ELF compounds inhibit the proliferation of cancer cells harboring ETS fusion proteins. The indicated cell lines were incubated in the presence or absence of a range of concentrations of compounds A, B, and D (both left and right-side panels) compared to VPC-18005. Error bars represent the standard deviation of the mean.

Candidate-hit compounds significantly inhibit tumor cell proliferation

ETS transcription factors play critical roles in normal cell growth and proliferation, and cancer cells often exhibit aberrant ETS protein activity. In this section, initially, we have compared the growth of 'normal' primary cells (human umbilical vein endothelial cells (HUVECs) with the growth of 2 prostate cancer lines: VCaP (which contain a chromosomal translocation resulting in sharply elevated ERG protein levels) and LnCaP cells.

Figure 3F clearly illustrates that the growth of 'normal' HUVECs was relatively unaffected by the presence of the most potent compound, drug A, across all tested concentrations (31 nM – 500 nM). However, the proliferation of the two cancer cell lines was significantly suppressed by drugs A, B, and D, each showing half-maximal inhibitory concentration (IC50) values in the 1-10 µM range (Figure 3G). This evidence strongly suggests that the ELF drugs might exhibit tumor-specific effects, given their lack of impact on the growth of normal cells. In addition, as it has been shown, cell proliferation was not significantly inhibited by treatments with VPC-18005 (Butler, 2017).

Candidate hit compounds inhibit angiogenic sprouting

Considering the data obtained from our functional studies and the established role of ETS transcription factors in sprouting angiogenesis, we sought to elucidate the effect of the ETS inhibitors on angiogenesis. Three different angiogenesis assays were deployed.

- ***In vivo* analyses using the zebrafish embryo model**

The fli1a:gfp transgenic line (Lawson, 2002) produces embryos in which all of the endothelial cells are marked by GFP. Coupled to their optical transparency, this enables direct visualization of angiogenesis. During the first 2 days of development, the reiterated pattern of intersegmental trunk vessels (ISV) are formed by angiogenic sprouts from dorsal aorta endothelial cells that grow to

the dorsal side of the trunk where they interconnect to form the dorsal longitudinal anastomotic vessel (DLAV). Interestingly, incubation of zebrafish embryos with 10µM of ELF compound for 16 hours resulted in clear disruption of angiogenesis, manifested by a reduction in the number of vessels, aberrant vessel trajectories, and the premature stalling of dorsal aorta sprouts (Figure 4A). Effects were most obvious for drugs A, B, and D. Incubation with drug C resulted in less disruption vessels that have been visualized using confocal microscopy.

- **Primary cell angiogenic tube formation assay**

To further characterize the inhibitory effect of the small molecules, they were added to three-dimensional (3D) cultures of primary HUVECs. HUVECs were grown on Matrigel in the presence or absence of resynthesized drugs (10 µM). Sprouting was quantified after 24 hours using in-house computer software. Figure 4B demonstrates that all drugs inhibited sprouting, with drugs A and D showing the most apparent effects. Together, these findings suggest that the ELF drugs (most notably drugs A, B, and D) might exert specific inhibitory effects on angiogenesis by targeting ETS.

- ***Ex vivo* fetal bone metatarsal explant assay**

Mouse fetal metatarsals allow the analysis of blood vessel growth in a dish and recapitulate critical features of angiogenesis observed *in vivo* (Song, 2015). Fetuses at embryonic stages E15.5–18.5 can be used for metatarsal preparation, as they exhibit similar vessel outgrowth.

For this study, metatarsals were isolated from fetuses at embryonic stages E17.5 and incubated in a defined medium containing vascular endothelial growth factor (VEGF) in the presence or absence of 1 µM ELF compounds. The assay has been performed according to the protocol provided in the Methods section. Vessel formation was monitored by phase-contrast light microscopy from day 2. Compounds A, B, and D each inhibited ectopic angiogenesis of metatarsals isolated from fetal mice. Immunofluorescence staining with PECAM-1 (CD31 antibody) was performed to visualize metatarsal vessel outgrowth. Confocal images of microvessel are shown (Figure 4C). A quantitative analysis of vessel branching was determined based on the number of pixels in the vessel area. Taken together, these data demonstrate the potential anti-angiogenic inhibitory effect of the hit compounds.

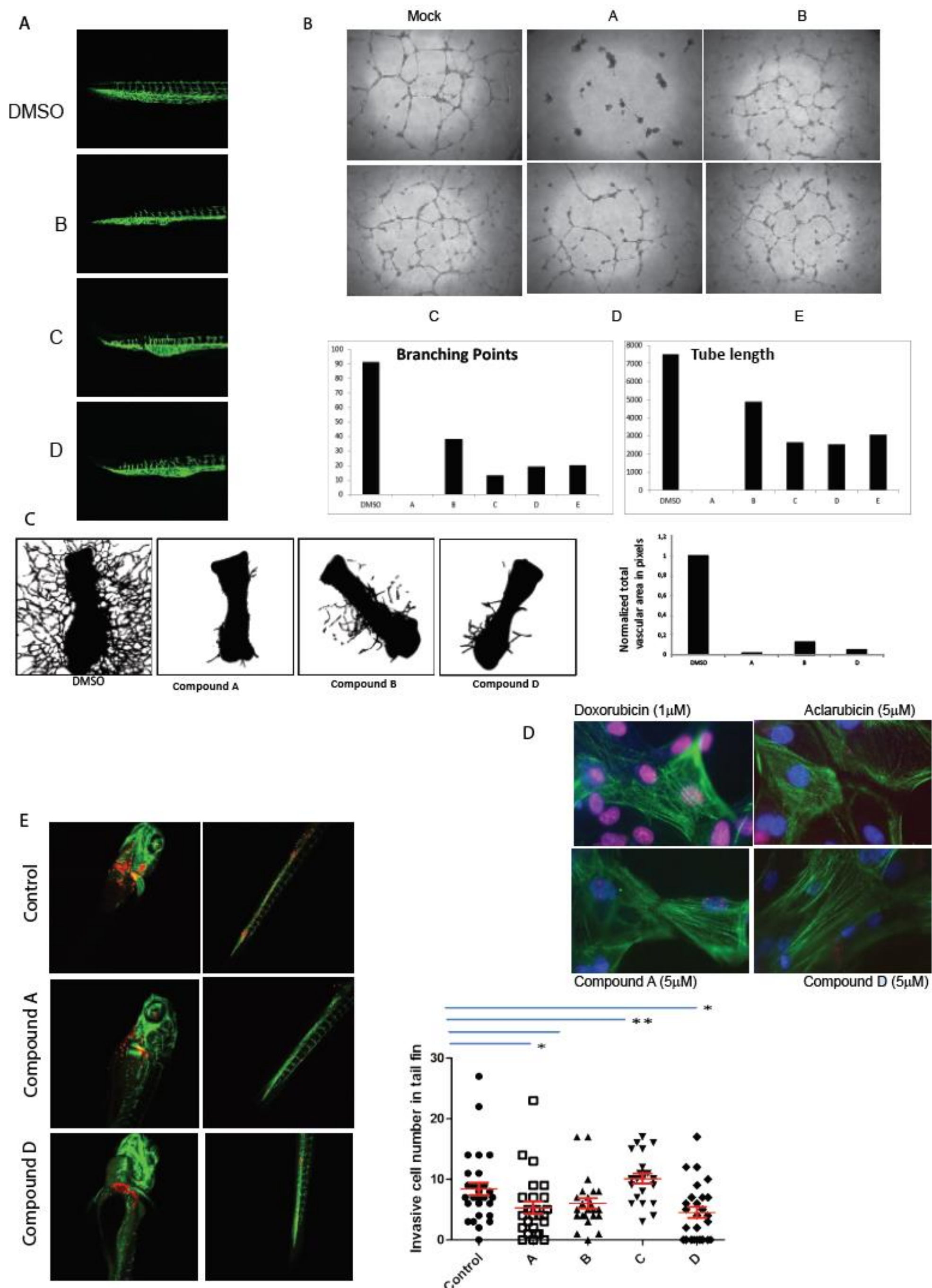

Figure 4. (A) The *fli1*a:gfp transgenic zebrafish embryos were treated with 10 µM of ELF compounds for 16 hours. Blood vessels were imaged by confocal microscopy.

< **Figure 4 (continued). (B)** Effect of ELF compound on human primary endothelial cell tube formation. HUVECs were grown on Matrigel in the presence or absence of ELF drugs (10 µM). Sprouting was quantified after 24 hours using in-house computer software.
(C) Mouse metatarsal assay. Metatarsals isolated from fetal mice were incubated ex-vivo in a defined VEGF medium in the presence or absence of 1 µM ELF compounds. Blood vessels were visualized using a CD31 antibody. Ten metatarsals were scored per condition. The scale bar represents 500 µm. **(D)** Evaluating DNA Damage in hPSC-derived cardiomyocytes and HUVECs. Assessing cardiotoxicity in hPSC-derived cardiomyocytes that were cultured overnight in the indicated conditions. Shown are confocal images of cells stained for Troponin I (TNNI3; green) and phospho-histone H2A.X (red- to visualize DNA damage). All nuclei were stained with DAPI (blue). **(E)** ELF compounds inhibit breast cancer cell invasion in xenotransplanted zebrafish embryos. Fluorescently labeled breast cancer cells were microinjected into the duct of Cuvier of 3-day-old zebrafish embryos. Tumor cell invasion was measured 3 days later.

Evaluation of hit compound cardiotoxicity

Toxicity is a fundamental problem associated with chemotherapy treatments, in particular treatments that damage DNA. In the case of doxorubicin, which is a widely used chemotherapy reagent, the main problem is cardiotoxicity, which leads to damage to cardiac tissue and heart failure. Therefore, cardiotoxicity is one of the most important considerations when developing novel therapies that improve current treatments. Doxorubicin acts by inhibiting topoisomerase II, leading to double-stand DNA breaks, which activate the DNA-damage response machinery, ultimately resulting in apoptosis. While this is an efficient means of killing cancer cells, doxorubicin and similar chemotherapy reagents also target 'normal' cells, leading to serious side effects of toxicity.
As the primary activity of ETS inhibitors takes place within the nucleus, genotoxic testing was conducted by assessing γ-H2AX levels in human iPSC-derived cardiomyocytes (hiPSC-CM). Phosphorylation of histone H2AX is a standard measure of DNA-damage response activation, which can be visualized using a specific antibody (Kuo, 2008). Cardiotoxicity was evaluated in both the presence and absence of the specified drugs, utilizing an antibody targeting phosphorylated histone H2AX. The ETS inhibitors exhibited relatively minimal levels of damage when compared to the conventional chemotherapy drug, doxorubicin (Figure 4D).

In vivo inhibition of migration and invasion of tumor cells in zebrafish

The importance of ETS factors in cellular migration and invasion has often been observed within aggressive tumors. Therefore, an effective ETS inhibitor might suppress cellular migration (Buchwalter, 2005; Schober, 2005). We employed zebrafish xenotransplantation to investigate cell extravasation and intravasation. Approximately 400 human breast cancer MDA-MB-231 cells were injected into

the duct of Cuvier (Doc) 48h after fertilization. Injected zebrafish embryos were treated with compounds in their water. The embryos also remained viable when cultured in the presence of up to 25 µM of the compounds for five days. EGFP-labeled vasculature of Tg (*fli1:gfp*) and mCherry-labeled tumor cells enabled visualization of cancer cell invasion and dissemination in the living zebrafish. The Figure 4E shows, that the dissemination of the breast cancer cell lines toward the head and tail was significantly reduced in the presence of compounds A, B and D (10 uM).
Taken together, these data suggest that, in addition to directly inhibiting proliferation of tumour cells, selected hit compounds exert an inhibitory effect on angiogenic sprouting.

DISCUSSION AND FUTURE PERSPECTIVES

While the past two decades witnessed considerable efforts to target receptors and kinases to impede tumor growth, the clinical success of such therapies has often been hampered by drug resistance and toxicity issues. In response to this pressing challenge, our study has tested an innovative approach to inhibiting cancer proliferation by focusing on inhibiting ETS transcription factors. Although targeting transcription factors was historically disregarded due to structural complexities and intrinsically disordered regions, recent progress in drug discovery and modern chemistry has reignited interest in targeting such proteins. Here, we explored the possibility of targeting the ETS family, knowing that such an approach holds the promise of effectively targeting tumor growth and angiogenesis. This introduces a multidimensional strategy for cancer treatment. Our main approach aims to mitigate overt toxicity and address prevalent redundancy issues in cell signaling pathways, ultimately combating acquired resistance to therapy. As compared to other signaling pathways, TFs are the most downstream component, so there are fewer parallel pathways to bypass. Therefore, in principle, targeting ETS should be less prone to therapy resistance.
Here, we presented the discovery of novel small-molecule inhibitors targeting the ETS DNA binding domain of transcription factors (TEL and FLI1). Initially, two types of screens have been done for hit identification. Firstly, fragment-based screening selects molecules that directly bind to the DBD. Secondly, HTS of lead-like small molecules, which are multi-component functional assays, have been used to discover compounds capable of blocking ETS DBD binding to DNA. We have initiated a hit-to-lead characterization of these molecules through a variety of biochemical and cell-based experiments to select the most promising candidates.
As tumor proliferation and migration are the primary drivers of tumorigenesis, assessing the suppression of these two events is a satisfactory starting point for

testing the inhibitors (Erkizan, 2009; Butler, 2017). We showed that the inhibitors reduced the proliferation of cancer cell lines and also blocked angiogenesis and vessel growth in different assays. Furthermore, a zebrafish xenotransplantation study suggests that the compounds may block cancer cell invasiveness (see Figure 4E). The results of our *in vitro* biochemistry assays also verified the mode of action of these compounds, which includes the inhibition of ETS protein DNA binding. These data provided proof-of-principle evidence that targeting ETS factors might represent a novel approach to inhibiting tumor growth. Follow-up studies will be required to validate this idea by using mouse models and organoids.

Concerning toxicity, testing DNA damage in cultured cardiomyocytes showed significantly lower damage levels in the cells that had been treated with the ETS inhibitors compared to doxorubicin. There was an increase of γ-H2AX caused by compound A (Figure 4D), but this was much lower than the effect of doxorubicin at comparable drug concentrations. This suggests that the novel compounds cause less damage than the conventional chemotherapy treatment. Regarding toxicity, it is noteworthy that zebrafish embryos tolerated low µM quantities of the compound. However, it will also be essential to conduct toxicology and pharmacokinetic studies in mammalian model systems. Minimizing on-target toxicity is indeed also a significant challenge in drug development, particularly as ETS transcription factors have an important role in normal cell proliferation and differentiation. One might expect on-target toxicity of these compounds when given systemically. In order to balance therapeutic efficacy, we could use specific ETS inhibitors selectively acting on specific ETS factors associated with. In addition, combination therapies might help in optimizing drug dosage and minimizing toxicity.

There are clearly potential specificity issues because of the number of ETS factors that have a common core binding motif. There are potential differences in their mode of action and the specific sequence of the DNA binding sites that could be explored, but due to this overall conservation, we may end up with inhibitors which inhibit more than one ETS factor. One way to limit specificity problems is by using different ETS sites and also purifying and screening full-length ETS proteins. This has proven to be very challenging technically, but once overcome, it opens up the possibility of selecting even more targeted compounds. Furthermore, the purification of full-length ETS protein will offer us the possibility of obtaining a deeper understanding of these factors, their function, and their involvement in the pathology of disease. With a complementary effort to characterize the nature of ETS-controlled gene networks, we can more dissect the effects of the novel compounds at the molecular level through transcriptome analyses and proteome analyses.

MATHERIALS AND METHODS

Cell culture, biochemistry

The prostate cells, LnCaP, and VCaP were cultured in Iscove's Modified Dulbecco's Medium (IMDM) from Gibco, supplemented with 15% Fetal Bovine Serum (FBS). The primary HUVECs (Lonza) were cultured in EGM-2 (Lonza) medium supplemented with the Bullet kit (Lonza) and 10% fetal bovine serum (Gibco).. The culture dishes were coated for 30 minutes with 0.2% gelatin at 37℃. All cell lines were maintained in a 5% CO_2, 37 °C humidified incubator, tested monthly for mycoplasma contamination and checked for authenticity by short tandem repeat (STR) profiling. Cardiomyocytes generously provided by Dr. Richard Davis (Anatomy & Embryology, LUMC).

Proliferation assays

The cells were seeded in triplicate in appropriate concentrations; 30,000 cells/mL LnCaP and VCaP/60,000 cells/ml into 96-well white plates with a clear flat bottom in 100µl of regular medium. The next day, the medium was supplemented with the different concentrations of compounds. The number of viable cells was determined using a Cell Titer-Blue reagent (Promega, Madison) at 0, 3, and 5 days after treatment. The analysis was done by removing the excess medium and adding 85µl of regular media with 15µl of the Cell Titer-Blue Reactions were left at 37°C for 2 hours, and absorbance readings were taken at 544 nm/590 nm (RFU) using the Victor X3 multi-label plate reader from Perkin Elmer.

Protein Expression and purification

Phage-resistant BL21 E. coli were transformed with pETHIS-Fli1DBD constructs, followed by selection of colonies on LB + kanamycin. Subsequently, the selected colonies were grown in 200 ml LB media + kanamycin at 30°C and induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD600. Protein expression was carried out for four hours. After induction, cells were centrifuged at 5,000 rpm at 4°C, and the resulting pellets were snap-frozen in liquid nitrogen and stored at -80°C.

Frozen cells pellets were lysed in 3ml of N.1 buffer (Imidazole 16.6 mM, NaCl 500 mM, Dithiothreitol (DTT) 2mM, 5% glycerol. pH 7.4) and sonicated at 60% amplitude, with a 10-second on and 15-second off cycle for 10 minutes, until the lysate cleared. The sonicated lysates were centrifuged at 17,000 rpm at 4°C for 10 minutes, and the resulting pellets and supernatant were subjected to 12% SDS

gel. Protein release into the supernatant was confirmed by staining with Instant Blue™ Protein Stain. The FLI-1 binding domain was purified by passing the sample diluted in N.1 buffer through a 1 ml nickel column, eluting fractions with N.2 buffer (Imidazole 332 mM, NaCl 500 mM, Dithiothreitol (DTT) 2 mM, 5% glycerol, pH 7.4). Subsequently, the cleanest fractions were diluted in H.1 Buffer (Hepes 20 mM, EDTA 0.5 mM, DTT 2 mM, 5% glycerol, pH 7.4) and run through a 1 ml heparin column, eluting fractions with H.2 buffer (NaCl 1 M, Hepes 20 mM, EDTA 0.5 mM, DTT 2 mM, 5% glycerol, pH 7.4). The clean fractions were then pooled and snap-frozen in liquid nitrogen for storage at -80°C. All fractions were assessed by running on a 12% SDS gel and staining with Instant Blue™ Protein Stain.

***In vitro* DNA binding assay**

In vitro translated protein was made using the TNT-coupled reticulocyte *in vitro* translation system (transcription and translation-Promega). Subsequently, 50 pmol of biotinylated double-stranded oligonucleotides harboring either three consensus ETS binding sites (for TEL and FLI1) or three AP-1 binding sites (for JUN) were coupled to MyOne streptavidin C1 beads (Invitrogen). Double-stranded oligonucleotides were incubated with *in vitro* translated TEL, FLI1, or JUN. It is important that all constructs were FLAG epitope tagged to enable a formal comparison with monoclonal antibodies. ELF drugs were also added to the reaction with the final concentration of 50 µM. Reactions were incubated at 4 °C with vigorous shaking for 30 min in the presence of 1 µg of poly (dI/dC), 4 mm spermidine. Beads were successively washed three times with the Binding buffer (50 mm KCl, 10 mm HEPES (pH 7.6), 5 mm MgCl2, 10 mm Tris (pH 8), 0.05 mm EDTA (pH 8), 0.05 mm, 0.1% Triton X-100, 20% glycerol). Associated proteins were eluted in Laemmli buffer, and protein-DNA interactions were determined by western blotting, which has been described previously (Forghany, 2018) using a FLAG mouse monoclonal antibody (Sigma).

HUVEC tube formation/sprouting assay

96-well plates were coated with 60 µl of Matrigel/well 30 min prior to seeding 25000 HUVECs/well. Cells were grown on Matrigel in the presence or absence of ELF drugs (10 µM). EGM-2 medium was supplemented with 50 ng/ml recombinant human VEGF 165 (R & D Systems) EGM2 medium(Endothelial Cell Growth Medium-2 with Bullet Kit/Lonza) was supplemented with 50 ng/ml recombinant human VEGF 165 (R&D Systems). Images were taken at multiple time points. After 24 hours, sprouting was analyzed with Stacks (in-house software; Department of Cell and Chemical Biology, Leiden University Medical Center). The analysis of sprouting was done after 24 using in-house computer software developed by the Department of Chemical and Cell Biology at LUMC

(van IJzendoorn,2017). Each individual space bounded by connected tubes constitutes a loop. Branches are the intersections made by connected tubes, and total length is the combined length of all tubes (not all the data shown here).

Zebrafish assay

The toxicity of ELF drugs has been explored using the fli1a:gfp transgenic line (Lawson, 2002). Zebrafish care, maintenance, and handling in the Laboratory have been done according to the international guidelines (Avdesh , 2012). All animal experiments were approved by the local institutional committee for animal welfare (Dier Ethische Commissie "DEC" of the Leiden University Medical Center).

For the experiments described here embryos were kept in egg water (60 µg/mL sea salts; ~60 embryos/dish) at incubate at 28 c. Chorions were removed mechanically using two forceps before 24hp. As a popular solvent, Dimethyl sulfoxide (DMSO) has been used to deliver compounds. Drug administration has been done with titration and final concentrations of 5, 10, 20, and 50 µM. Embryos were anesthetized using tricaine methane sulfonate (Sigma-Aldrich) with the final 40 mg/ml concentration in egg water, approximately 10 minutes before imaging. Imaging of vessels was carried out by using a Leica SP2 confocal microscope (Leica Microsystems) using a ×10 or ×20 objective. Vessel characteristics of 20 embryos per condition were scored.

Metatarsal assay

This protocol comprises multiple steps, including dissecting, seeding the metatarsals, staining, imaging, and quantifying. It is important to isolate the embryos from the uterus for the metatarsal assay and keep them in phosphate buffered saline (PBS) with calcium and magnesium on ice until the metatarsals are isolated. The metatarsal dissection procedure was done according to nature protocol (Song, 2015) from a mouse embryo at day 17 of gestation. In the whole experimental procedure, it is essential to prevent the isolated metatarsals from drying, which compromises the sprouting step. We used DPBS (Dulbecco's PBS) with calcium and magnesium to isolate the metatarsals. The medium for culturing the metatarsals was MEM Alpha Medium (Thermo Scientific) and GlutaMAX (Gibco™), which contained FBS and penicillin-streptomycin. This medium was then supplemented with 50 ng/ml recombinant human VEGF 165. The metatarsal should be placed in the middle of a 0.1 % gelatin-coated plate. The plates were kept in the back of the incubator, especially for the first 2–3 days, without any movement to avoid floating the bones. From day 2, the culture medium needs to be changed. Then, approximately every other day until the experiment ends, the medium should be refreshed and supplemented with 10µm small molecule inhibitors. In addition, fibroblast migration from

metatarsals begins to grow underneath on day 2. The vessel sprouting was visible with phase-contract microscopy on day 7, and the treated metatarsal indicates significant differences compared to the control.
To visualize vessel outgrowth from metatarsals immunofluorescence, staining was done after removing the culture medium on day 5. Metatarsals were washed with DPBS (Dulbecco's PBS; Gibco, with calcium and magnesium), and metatarsals were fixed in Zink Macrodex Formalin (PFA; Sigma-Aldrich) for 15 min. Staining on the metatarsals was performed using the CD31 antibody (BD Pharmingen™) as previously described (Forghany, 2018). For a detailed analysis of vessel formation, images were processed in Photoshop. After thresholding, vessel configurations were converted into black-and-white binary images. Vascular area (Black) in pixels was quantified using in-house computer software developed by the Department of Cell and Chemical Biology at LUMC. In all the treatments, the total vascular area or tube length in pixels has been normalized against the pixel of the metatarsal bones (Song, 2015). All animal experiments were approved by the local animal ethics committee.

Cardiomyocytes

The hiPSC-CM (stem cell-derived cardiomyocytes) were grown on 5mm coverslips in 100µl Matrigel™(BD Matrigel™ Basement Membrane Matrix) and 1ml culture medium. The culture medium for the hiPSC-CM was DMEM 10% FBS with Supplement 100x) stock from Gibco, 450 µM α-MTG, 0.05 mg/ mL L-Ascorbic acid 2-phosphate from Sigma-Aldrich, 2 mM GlutaMAX supplement 100x stock from Gibco, 0.5 % Penicillin/ streptomycin (Thermo Scientific). Matrigel™ Matrix Growth Factor Reduced (BD) was diluted with 0.5 mg Matrigel in a 6 mL cold culture medium.
Cells were treated with ETS inhibitors, Aclarubicin, and doxorubicin. Following 16 hours of treatment, the cells were fixed with 4% paraformaldehyde-phosphate buffered saline (PBS) for 15 min and permeabilized in 0.2% Triton X-100-PBS for 5 min. Subsequently, the cells were washed with PBS 0.5% Tween and blocked with a solution containing PBS 0.5% Tween and 5% bovine serum albumin for 30 min. Primary antibodies Troponin I and anti phospho-histone H2A.X (Ser139) (Cell signaling) were then added to the blocking buffer and incubated with the cells for 1 hour, followed by washing and incubation with secondary antibodies in the blocking buffer for 30 min. After another round of washing, the cells were mounted with a DAPI mounting solution (10 µg/ml) and sealed with nail varnish. All immunostaining procedures were performed at room temperature and were visualized using a Leica SP8 confocal microscope.

Antibodies and drugs

The following primary antibodies were used: FLAG mouse M2 monoclonal (Sigma-Aldrich), anti-HA.11 mouse monoclonal (Covance), anti-HA rabbit polyclonal (Abcam), Secondary antibodies; anti-Rabbit Alexa Fluor® 488 and anti-Mouse Alexa Fluor® 594 (Thermo Fisher scientific), anti-JUN rabbit (Cell Signaling Technology), anti-JUN mouse (Santa Cruz Biotechnology), anti-FLAG rabbit (Sigma), anti-γ-tubulin (Sigma), Rat Anti-Mouse CD31 (BD Pharmingen), anti-TroponinI (Santa Cruz), anti-phospho-histone H2A.X (Ser139)(Cell signaling), Aclarubicin (Sigma Aldrich), doxorubicin (Sigma Aldrich), Triton (Merck), Puromycin (Sigma-Aldrich).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9. The unpaired Student's t-test was used for most analyses, and $P < 0.05$ was considered statistically significant. All measurements in this study were taken from distinct samples.

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