Allosteric Activation of PP2A Inhibits Experimental Abdominal Aortic Aneurysm

Xianming Zhou¹, ²,§, Chao Zhang¹, §, Fei Xie¹, Wei Wei³, Rui Li¹, Qian Xu², Yu Wang², Philip A. Klenotic⁴, Goutham Narla⁵, Nianguo Dong¹, #, and Zhiyong Lin², #

¹Department of Cardiovascular Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
²Cardiology Division, Emory University School of Medicine, Atlanta, GA, USA
³Department of Pediatrics, St John Hospital and Medical Center, Detroit, MI, USA
⁴Department of Pharmacology, Case Western Reserve University, Cleveland, OH, USA
⁵Division of Genetic Medicine, Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA

§These authors contributed equally to this work.

#Address correspondence to:

Zhiyong Lin, 101 Woodruff Circle, Room 3004, Atlanta, GA 30322, USA. Phone: 404-712-0974, Email: zhiyong.lin@emory.edu

Or Nianguo Dong, 1277 Jiefang Avenue, Wuhan, China. Phone: 86-27-85351610, Email: dongnianguo@hust.edu.cn
Conflict of Interest: The Icahn School of Medicine at Mount Sinai, on behalf of G. Narla, has filed patents covering composition of matter on the small molecules disclosed herein for the treatment of human cancer and other diseases (International Application Numbers: PCT/US15/19770, PCT/US15/19764; and US Patent: US 9,540,358 B2). RAPPTA Therapeutics LLC has licensed this intellectual property for the clinical and commercial development of this series of small molecule PP2A activators. G. Narla, has an ownership interest in RAPPTA Therapeutics LLC.

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Abstract:
Although extremely important, the molecular mechanisms that govern aortic aneurysm (AA) formation and progression are still poorly understood. This deficit represents a critical roadblock toward the development of effective pharmaceutical therapies for the treatment of AA. While dysregulation of Protein Phosphatase 2A (PP2A) is thought to play a role in cardiovascular disease, its role in aortic aneurysm is unknown. The objective of this study is to test the hypothesis that PP2A regulates abdominal aortic aneurysm (AAA) progression in a murine model. In an angiotensin II-induced AAA murine model, the PP2A inhibitor, LB-100, markedly accelerated AAA progression as demonstrated by increased abdominal aortic dilation and mortality. AAA progression was associated with elevated inflammation and extracellular matrix fragmentation, concomitant with increases in both metalloproteinase activity and reactive oxygen species production. Conversely, administration of a novel class of small molecule activators of PP2A (SMAPs) resulted in an antithetical effect. SMAPs effectively reduced AAA incidence along with the corresponding pathologies that were increased with LB-100 treatment. Mechanistically, modulation of PP2A activities in vivo functioned in part via alteration of the ERK1/2 and NFκB signaling pathways, known regulators of AAA progression. These studies, for the first time, demonstrate a role of PP2A in AAA etiology and demonstrate that PP2A activation may represent a novel strategy for the treatment of abdominal aortic aneurysms.

Key words: abdominal aortic aneurysm, protein phosphatase 2A
**Introduction:**

Over the last two decades, studies conducted in both human samples and in rodent models have spurred numerous efforts to identify potential therapeutic targets to limit abdominal aortic aneurysm (AAA) progression. These targets include key signaling cytokines, inflammatory molecules, and matrix metalloproteinases (MMPs). Despite these advances, no effective medical therapy has been found to decrease the rates of growth or rupture of asymptomatic AAAs [1, 2]. Available and experimental therapies, including ACE inhibitors, antagonists of the angiotensin receptors, anti-inflammatory and antihypertensive agents, and antibiotic treatment, all have limited efficacy in halting or reversing the progression of AAA. As it stands today, timely surgery and endovascular aneurysm repair (EVAR) are the only definitive treatment options available.

In light of this critical unmet need to develop effective new therapeutic strategies for the treatment of AAA, the discovery and validation of novel molecular driver pathways and needed. Toward that end, we focused our efforts on the role of protein phosphatases in AAA biology. Reversible protein phosphorylation plays a ubiquitous and central regulatory role in a wide array of biological processes. The aberrant balance between kinase/phosphatase activity contributes to an increasing array of human disease states, including cardiovascular diseases [3, 4]. Protein kinases have been a major focus of drug development efforts, with hundreds of inhibitors either in the pipeline or already in the clinic. Protein phosphatases, however, have been largely ignored for drug development because of their reputed lack of substrate specificity as well as the toxicity associated with natural product active site inhibitors [5]. Interestingly,
as summarized in a recent report [6], this paradigm could be changing. Efforts to unpack the therapeutic utility of protein phosphatases are progressing with unprecedented potential via an allostERIC drug discovery approach.

In the present study, we focused on the major serine/threonine protein phosphatase 2A (PP2A) family of phosphatases, that regulates multiple key molecular pathways, including MAPK, NFκB, and Akt, all known regulators of AAA development and progression [7-10]. PP2A, a potent family of de-phosphorylating enzymes within mammalian cells, is a critical serine/threonine phosphatase that has been implicated in the regulation of many signaling pathways, including apoptosis and cell cycle progression as well as the negative regulation of many oncogenic and cell survival pathways [11-13]. PP2A’s ability to broadly regulate diverse signaling pathways is due to its dynamic heterotrimeric composition that allows over 60 different PP2A holoenzymes to form, each with its own substrate specificity [14, 15]. All three subunits of PP2A are required for its full enzymatic activity. Most PP2A exists in cells and tissues as ABC heterotrimerics. The Scaffolding subunit (A Subunit) positions the complex together; the C subunit contains the catalytic site, and the B subunit (Regulatory Subunit), regulates the target specificity of PP2A.

The restoration of PP2A activity has been shown to be therapeutically effective in model systems. Several compounds have been shown to activate PP2A indirectly, mainly through inhibiting endogenous PP2A inhibitors or targeting PP2A regulators. However, these indirect methods of PP2A activation have many additional downstream effectors, limiting their use as a tool to study PP2A activity specifically. As reported in previous publications by the Narla group, in order to improve the tolerability and
translational potential of a PP2A activating compound, a medicinal chemistry-based approach using a tricyclic chemical scaffold as a starting point was undertaken to develop a novel series of SMAPs (small molecule activators of PP2A) [16]. SMAPs (DT-1154 and the latest generation DT-061) have been demonstrated to reactivate PP2A and inhibit tumor progression in a variety of murine tumor models [17].

In the current study, we leveraged the availability of a unique PP2A inhibitor (LB-100) as well as SMAPs (DT-1154), to perform pharmacological gain- and loss-of-function studies in a preclinical murine AAA model. Results obtained from these studies strongly support PP2A activation as an AAA antagonist as well as validate PP2A as a novel molecular target for future AAA therapy development.
**Methods:**

*Study approval.* For human samples used in this study, aortic tissues were obtained from patients undergoing open thoracoabdominal aortic aneurysm surgery by protocols approved by the Ethics Committee at the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Both aneurysmal tissues and adjacent non-aneurysmal tissues were collected from the same patients. Written informed consent was received from patients prior to inclusion in the study. For animal experiments involving PP2A activator (SMAPs), studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University (IACUC protocol # 2013-0128) and Emory University (PROTO201800048) and performed at both institutions. The PP2A inhibition (LB100) studies were approved by the IACUC at Tongji Medical College, Huazhong University of Science and Technology (IACUC #2439), experiments were carried out at Union Hospital, Tongji Medical College.

*Mice and AngII infusion model:* Apoe-null mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or Beijing HFK Bioscience CO. LTD. Mice were housed under standard light-dark conditions (12h:12h) and allowed ad libitum access to standard rodent chow and water. To induce AAA formation in mice, male mice at age 10-12 weeks were infused with AngII as previously described. Alzet model 1004 osmotic minipumps (DURECT Corp., Cupertino, CA) implanted subcutaneously were used to deliver AngII (1µg/kg/min, Sigma) or vehicle (normal saline) for 28 days, depending on the experimental design described in the results section. Apoe-null mice
purchased from the vendors were randomized into control and treatment groups (vehicle vs LB-100 or DT-1154), followed by concurrent AngII infusion and treatment (LB-100 or DT-1154). The PP2A inhibitor LB-100 (Selleck Chemicals, 1.0mg/kg, dissolved in normal saline) was administered through ip injection every other day, the treatment was initiated one week prior to AngII infusion and continued until the completion of AngII infusion. SMAPs (DT-1154, 100mg/kg/d, or DT-061, 15mg/kg BID) were administered via oral gavage 3 days prior to AngII infusion and continued until the completion of experiments. Working solution for SMAPs were prepared in a N,N-Dimethylacetamide (DMA)/Kolliphor HS-15 (Solutol)/diH2O solution. Mice were anesthetized with ketamine (1.5mg/kg)/xylazine (0.3mg/kg), followed by mini pump implantation. To relieve pain from surgery, post-operative analgesia was administered using intra-peritoneal injection of buprenorphine (0.05-0.10mg/kg IM q8-12h) on the day of surgery and on post-operative days 1-3. At the completion of AngII infusion, transcardial perfusion in mice was accomplished via butterfly catheters to perfuse normal saline followed by 4% paraformaldehyde at 100 mmHg for 8 minutes. The aorta was exposed under a dissecting microscope (Leica, Model DM 500), peri-adventitial tissues were carefully removed from the aortic wall, and a caliper was used to measure the external diameter of suprarenal abdominal aorta. To compute for AAA incidence, an aneurysm was defined by a 50% or greater increase in the external diameter of the suprarenal aorta in comparison to aortas from saline-infused mice.

ROS generation assay: A subset of study mice were perfused only with ice-cold PBS (pH 7.4) without any fixatives at 100 mmHg for 5 min at 4°C. The abdominal aorta was
dissected and embedded in OCT (Tissue-Tek; Miles Inc., Naperville, IL) and sectioned using a cryostat (Leica, Wetzlar, Germany). DHE (dihydroethidine hydrochloride, 5μM) was topically applied to the freshly cut frozen aortic sections (10μm) and incubated at 37°C for 30 min. The presence of ROS is shown as red. DHE fluorescence images were acquired with a Leica microscope (Model: DM2000LED) using 510 nm excitation/ 580 nm emission filters at the same photomultiplier tube voltage, gain and offset. The analyzer was blinded to the identity of sections. Images were converted to gray scale and the integrated density per image area of interest was measured using NIH ImageJ.

**PP2A phosphatase activity assay** — Aortic PP2A activity was measured using threonine phosphopeptide as the substrate with the PP2A immunoprecipitation phosphatase assay kit (Millipore). Tissues were lysed using a lysis buffer (20mM imidazole-HCl, 2mM EDTA, 2mM EGTA, 1mM benzamidine, pH 7.0 with 10μg/ml aprotinin and 1mM PMSF). Tissue extracts were sonicated for 10 s and centrifuged at 2000g for 5 min. 200μg of total protein extracts were incubated with anti-PP2A-C subunit antibody (4μg) 18h at 4°C with gentle rocking. Then 40μL of Protein A agarose slurry were added and rocked 2h at 4°C. Beads were washed 3 times with 700μL TBS, and once with 500μL Ser/Thr assay buffer. The beads were then incubated with 60μL diluted phosphopeptide and 20μL Ser/Thr assay buffer at 30°C for 10min in a shaking incubator. The beads were centrifuged briefly, and the samples were analyzed in a colorimetric assay using malachite green at an absorbance of 650 nm.

**Tissue lysis and western blot** — Snap-frozen aorta tissues were extracted using a Total
Protein Extraction Kit (Pierce) with protease and phosphatase inhibitor from Millipore. Tissues were homogenized using a pellet pestle motor (Kimble-Kontes, NJ) according to the protocol in the protein extraction kit. Protein concentrations were determined by BCA kit (Pierce). Equal amounts of the resultant protein were run on 8-12% SDS-PAGE gel under denaturing conditions, transferred to nitrocellulose membrane, and subjected to western blot analyses using the indicated antibodies. ERK1/2, phospho-ERK1/2, phospho-p65 are from Cell Signaling Technologies (Danvers, MA). PP2A-A subunit mAb detects endogenous levels of both α and β isoforms of PP2A-A subunit. PP2A-B Subunit Antibody detects endogenous levels of the PR55 PP2A B subunit (α isoform). The antibody may also recognize the β, γ, and δ isoforms of the PR55 PP2A B subunit. PP2A-C subunit antibody detects endogenous levels of PP2A catalytic subunit protein (both α and β isoforms). Depending on the availabilities of the antibodies in the lab at the time of running the experiments, membranes were re-probed with antibodies against either β-Actin or GAPDH (both from Santa Cruz) to confirm equal loading. Detailed antibody information is provided in supplemental table I. Target proteins were detected by ECL kit (Pierce) and bands intensity analyzed by ImageJ.

Cell culture — Macrophage cells RAW264.7 were obtained from American Type Culture Collection and cultured in DMEM according to the instructions provided by the supplier. SMAP compounds were diluted with DMSO to a stock concentration of 80μM and stored at room temperature. RAW264.7 cells plated in a 6-well plate were pretreated with DT-061 (5μM in DMSO) for 45 minutes, followed by TNFα (20ng/ml) for 15 minutes
or 12 hours for protein or RNA isolation respectively. Cellular proteins were extracted with RIPA buffer supplemented with protease inhibitor tablet (Roche).

**RNA extraction and Quantitative RT-PCR analysis** — Cells were treated as indicated and then harvested for total RNA using Qiagen RNeasy mini kit. Abdominal aorta tissues from saline or AngII treated mice were snap-frozen in liquid nitrogen. Total RNA was isolated from cultured cells or tissues with Trizol (Invitrogen Corp., Carlsbad, CA) as described by the manufacturer. Total RNA (1µg) was DNase-treated and reverse-transcribed using Bio-Rad iScript Reverse Transcription Supermix kit. The resulting cDNA was diluted to 100µL and used in subsequent real time PCR reactions. Gene expression was assessed by SYBR green on QuantStudio 6 (Applied Biosystems). Gene expression was standardized to GAPDH or β-actin using the ΔΔCt method. Depending on the availability of primers in the lab, GAPDH or β-actin were used interchangeably in our studies as they did not alter our data in the current study based on our experience. To ascertain our samples are free of contamination, as a standard practice for RT-PCR experiments in the lab, no template and no RT controls were always included.

**MMP activity measurement in situ** — In situ MMP zymography was performed on freshly cut OCT-embedded murine aorta sections. Fluorescein-conjugated gelatin substrate DQ gelatin (Invitrogen) was prepared according to the manufacturer’s instructions. The substrates were then applied to sections and allowed to incubate at 37°C for 24 hours, after which green fluorescence was examined using a fluorescent microscope. Negative controls were performed on parallel sections in the presence of 5
mM EDTA. Pictures were taken and fluorescence intensity calculated with ImageJ. The results are expressed as percentage of fluorescence area over each cross-section.

**Histology and Immunohistochemistry** — Mice were euthanized and tissues were harvested, rinsed in PBS, fixed in 4% paraformaldehyde for 48 hours, embedded in paraffin, and serial 5 μm sections were cut. Morphology was evaluated by hematoxylin and eosin (H&E) staining. Aorta wall elastin integrity was assessed by Verhoeff-van Giesen staining using the Accustain Elastic Stain kit (Sigma). Elastin fragmentation was graded as follows according to Libby et al [18]: grade 1, intact, well-organized elastic laminae; grade 2, elastic laminae with some interruptions and breaks; grade 3, severe elastin fragmentation or loss; and grade 4, severe elastin degradation with visible ruptured sites. Detailed antibody information is provided in supplemental table II. For all histological analysis, the individuals who did the work were blinded to the groupings.

**Statistics** — Data are expressed as mean ± SEM. Statistics was performed using GraphPad Prism 8. In general, when comparing two unpaired groups of samples, if the data from either group did not pass normality test, Mann-Whitney test was used. If the data passed normality test, two-tailed unpaired Student t test was used. To compare the incidence of AAA formation between two groups, chi-square test and two-tailed Fisher’s exact probability test were used. For comparison of the effects of two variables, two-way ANOVA followed by Bonferroni post-hoc correction was used. Post-hoc testing was only undertaken if the ANOVA analysis reached significance. Also refer to figure legends for
specific statistical methods used in each figure. A value of $P \leq 0.05$ was considered significant.
Results:

Inhibition of PP2A promotes abdominal aortic dilation and vascular inflammation

The regulation of protein phosphorylation involves the net balance of protein kinase vs protein phosphatase activity. Protein phosphatase 2A family of holoenzymes possess major serine/threonine phosphatase activity in mammalian cells [19]. Considerable experimental evidence indicates that several major signaling pathways involved in AAA progression, including MEK/ERK1/2, P38, JNK1/2 and NFκB [8-10, 20, 21], are downstream targets of PP2A. Armed with this understanding, we hypothesized that the loss of PP2A function promotes abdominal aortic dilation. To test this, we used the well characterized active catalytic site PP2A inhibitor, LB-100. The ability of LB-100 to affect AAA development was tested in an established mouse AAA model that involves the infusion of Angiotensin II into Apoe-null mice. This model successfully recapitulates many of the characteristics of human AAA, including luminal dilation, ECM fragmentation, the inflammatory response and reactive oxygen species (ROS) generation within the arterial wall [22]. Upon the completion of AngII infusion, we evaluated the AAA phenotype in Apoe-null mice. In line with the inhibition of PP2A activity (Suppl. Figure 1), in the aorta during AngII infusion, as demonstrated in Figure 1A and Supplemental Figure 2A, treatment of Apoe-null mice undergoing AngII infusion with LB-100 (4 weeks) markedly aggravated aortic dilation as manifested by increased maximal suprarenal abdominal aortic diameter and AA incidence (Figure 1A&B, Suppl Figure 2A&B). The maximum abdominal aortic diameters of aneurysms in the LB-100 treated group were increased compared to the vehicle-treated control group (LB-100:
1.91mm; versus vehicle: 1.61mm, \( P = 0.019 \) (Figure 1A). Consistent with these observations, LB-100 treatment dramatically increased elastin degradation resulting in compromised aortic vessel wall integrity, enhanced aortic aneurysm severity (Figure 1C and Suppl. Figure 1B&C) and increased mortality (Figure 1D).

It is commonly believed that inflammation plays an important role in the development and progression of most AAAs. We, therefore, examined the impact of PP2A inhibition on vascular inflammation. Concordant with the enhanced severity of aortic aneurysm, LB-100 profoundly amplified vascular inflammation following AngII infusion as demonstrated by heightened AngII–elicited inflammatory cell (macrophage and T cells) infiltration in the adventitia (Figure 1E). The expression of several key inflammatory factors (MCP-1, MMP2, and VCAM-1) was also significantly augmented (Figure 1F, Suppl. Figure 1D). Furthermore, LB-100 substantially elevated MMP activities and reactive oxygen species generation (Figure 1G&H), two key mediators of AAA pathogenesis.

**PP2A activation strongly attenuates AAA progression**

The above results support our hypothesis that loss of PP2A activity augments AngII-induced abdominal aortic dilation. To further substantiate PP2A as a critical regulator of AAA, we next sought to determine the effect of PP2A activation in the same AngII infusion model. Restoration of PP2A activity has been shown to be of significant therapeutic value, however pharmaceutically tractable approaches to directly activate PP2A remain elusive. In order to improve the tolerability and translational potential of PP2A activating compounds, the Narla lab developed a new chemical series of SMAPs
These molecules are unprecedented in their ability to simultaneously down-regulate multiple critical signaling pathways. A series of in vitro phosphatase assays proved the specific interactions between SMAPs and PP2A [16]. Additional studies demonstrated potent anti-tumor properties of these novel SMAPs by targeting multiple pathways involved in tumor progression including mitogen-activated protein kinase (MAPK) and Akt signaling [16]. In light of the potent inhibitory effects of SMAPs on these kinases (specifically MAPK), we postulated that PP2A activation by SMAPs might mitigate AAA progression.

We administered DT-1154 (a validated PP2A activator compound) at 100 mg/kg/day, a dose that is well tolerated and effective in murine cancer models [16, 23], three days prior to AngII treatment and continued treatment throughout the ensuing 4-week AngII infusion. Importantly, DT-1154 was well tolerated at the treatment dose tested in this AAA model and did not result in altered mouse behavior, weight loss or mortality. As shown in Suppl. Figure 3, SMAPs administration in mice increased PP2A activity (a newer generation of SMAPs DT-061 was used because DT-1154 has been discontinued). Following AngII infusion, we assessed the effects of SMAPs on AAA progression.

In contrast to what was found with PP2A inhibition, treatment of Apoe-null mice undergoing AngII infusion with SMAPs (DT-1154) administration markedly reduced aortic dilation (Figure 2A and Supplemental Figure 4A&B) as well as AAA incidence (Figure 2B). The maximum abdominal aortic diameters of aneurysms in the DT1154-treated group were reduced compared to the vehicle-treated control group (vehicle: 2.02mm, versus DT-1154: 1.57mm) (Figure 2A). To confirm the beneficial effects of DT-
1154 treatment on pathological manifestation of murine AAA, we performed studies on key pathological processes involved in aneurysm formation, specifically loss of vessel wall integrity, oxidative stress, MMP elaboration, and vascular inflammation. Our studies demonstrated that PP2A activation markedly prevented elastin breakdown and deterioration of the extracellular matrix in the aorta (Figure 2C, Suppl. Figure 4B&C), concordant with the reduced mortality (Figure 2D). Similarly, DT-1154 exerted strong ameliorative effects on the AngII-induced vascular inflammatory response. DT-1154 treatment significantly blocked macrophage and T cell infiltration (Figure 2E). Consistent with these observations, vascular tissue expression of several key inflammation markers (MCP-1, VCAM-1 and MMP2) were markedly reduced in DT1154-treated aortas (Figure 2F and Supplemental Figure 4D). Additionally, contrary to what was observed for PP2A inhibition by LB-100, DT-1154 profoundly decreased AngII-induced MMP activity (Figure 2G) and ROS formation in abdominal aortic tissues (Figure 2H).

Taken together, these data clearly demonstrate that SMAP-dependent activation of PP2A significantly inhibits experimentally induced abdominal aortic aneurysm formation and reduces the severity of abdominal aneurysm, supporting a protective role of PP2A against AAA formation.

**Manipulation of PP2A modulates ERK1/2 and NFκB pathways**

To gain additional insight into how alterations in PP2A function affect aortic dilation and aneurysm progression, we assessed several well documented signaling pathways critical in the activation of extracellular remodeling enzymes and vascular inflammation in AAA. As shown in Figure 3A, PP2A inhibition by LB-100 strongly
upregulated phosphorylated p65 and ERK1/2 within suprarenal abdominal aortic samples of mice, findings in line with its strong enhancement of vascular inflammation, MMP activation and ROS generation. Conversely, an antithetical effect was obtained with PP2A activation by DT-1154. In DT-1154 treated animals, substantially attenuated AngII-induced ERK1/2 and NFκB activation was seen (Figure 3B).

Accumulating studies have demonstrated both vascular cells and bone marrow derived cells are involved in the pathogenesis and progression of AAA. While assessing the signaling response in multiple cell types in the context of SMAPs administration is beyond the scope of current study, we investigated the effect of SMAPs in macrophages given their central importance in driving inflammation and producing enzymes responsible for extracellular matrix degradation. As shown in Figure 4, SMAPs treatment (DT-061) strongly inhibited TNFα-induced ERK1/2 and NFκB activation in macrophage cells (RAW264.7). Consistent with this, a marked inhibition of several key inflammatory genes (MCP-1, IL-1β, COX-2 and MMP9) was also seen in SMAPs-treated cells.

**Decreased PP2A activity in aortic aneurysm**

Through gain and loss of function studies, we have demonstrated the protective role of PP2A activation in a murine model of aortic aneurysm formation. The next logical step was to determine if PP2A activity is altered in human aortic aneurysms. PP2A activity was assayed in human thoracoabdominal aortic aneurysm samples and corresponding non-aneurysmal controls. Our studies revealed a marked reduction of PP2A activity in aortic aneurysmal samples (P=0.02) when compared to control tissue.
(Figure 5A), findings in corroboration with the hypothesis that the decrease of PP2A activity may predispose aneurysm formation. Similarly, in AngII-induced murine AAA, a marked reduction of PP2A activity was seen (Figure 5B). These findings prompted us to wonder whether PP2A subunits or holoenzyme complex might be altered in AAA. Given that there are 17 potential PP2A subunits and there are approximately 96 unique holoenzyme configurations, we recognize it is difficult to address which holoenzyme complex is involved. To gain initial insights into which specific PP2A subunits might be affected in AAA, we assessed the protein expression of several PP2A subunits in human AA tissues. As shown in Figure 5C&D, a significant reduction of the scaffolding PP2A-A subunit was seen, while no difference was observed for the regulatory PP2A-B and catalytic PP2A-C subunits. These data strongly suggest that PP2A is relevant in human aneurysm formation and, coupled with our results obtained from an experimental AAA model, suggest that activation of PP2A may serve as a novel therapeutic target for the clinical treatment of aortic aneurysms.
Discussion

Protein kinases have been a major research focus for the development of viable therapeutics for cardiovascular disease treatment. In stark contrast, modulation of phosphatases for this same purpose has remained largely unexplored. The goal of this study was to determine the utility of PP2A activation as a potential AAA therapy. Gain- and loss-of function studies were performed via pharmacological activation and inhibition of PP2A in a murine model of AAA. Results described here show that inhibition of PP2A activity significantly worsened the observed AAA pathophysiology and disease progression in mice. This, to our knowledge, is the first direct evidence implicating PP2A in abdominal aortic aneurysm development and progression while also offering novel insights into AAA pathobiology.

Conversely, our data demonstrate that PP2A activation, using a first-in-class direct small molecule PP2A activator series (SMAPs), markedly attenuated the progression of AngII-induced AAA in Apoe-deficient mice. Consistent with these observations, SMAPs administration potently inhibited vascular inflammation, MMP activity, ROS production and vessel wall deterioration, all key pathologic features of AAA. Importantly, alteration of PP2A function may be relevant in human aneurysm formation and progression. We observed a substantial reduction of PP2A activity in human aortic aneurysmal tissues, findings that are in line with what was observed in our animal studies. These findings, together with our studies in an experimental aortic aneurysm model, suggest that PP2A could serve as a novel therapeutic target for the treatment of human aortic aneurysmal disease. We recognize that a natural extension
of the current study is to determine whether SMAPs can stabilize existing aneurysms and these efforts will be the focus of future studies. Nevertheless, our proof-of-principle experimental findings here point to therapeutic reactivation of PP2A as a novel strategy for the treatment of AAA.

In our current study, we have not thoroughly explored the mechanisms by which SMAPs negatively regulate AAA progression. We showed that SMAPs inhibited vascular inflammation after AngII infusion into Apoe-null mice. Our data supported that the inhibition of two important pathways (ERK1/2 and NFκB) that promote inflammation and have been demonstrated to be required for AAA progression, likely contributed to the observed mitigation effect by SMAPs. Of note, both ERK1/2 and NFκB pathways have been demonstrated to be required for MMP and ROS generation in AAA. It would be necessary to conduct additional studies to comprehensively determine whether SMAPs also affected other major pathways that are known important regulators of inflammation and AAA, including but not limited to p38, mTOR, STAT, c-Jun N-terminal kinases. Similarly, our studies have not comprehensively explored the effects of SMAPs in different cell types that are known to be important in the inflammatory responses leading to AAA pathology. While our current data support that administration of SMAPs in macrophages recapitulated the inhibition effect on ERK1/2 and NFκB activation seen in AngII-infused aortas, we do not know the impact of SMAPs on other cell types such as endothelial cells, smooth muscle cells, fibroblasts and other immune cell types. Lastly, whether the alteration of PP2A activity is manifested as changes in transcription regulation in our experimental conditions merits further exploration. For instance, a prior study showed that PP2A inhibition in lung epithelial cells leads to accumulation of a
hyperphosphorlated inactive form of tristetraprolin (an anti-inflammatory molecule and a direct target of PP2A) that caused the augmentation of mRNA of proinflammatory molecules. Importantly, PP2A activation by FTY720 reverses this effect [24]. Whether SMAPs affect tristetraprolin function will be explored in future investigations.

In addition to the anti-inflammatory effects, SMAPs treatment also mitigated elastin degradation (Figure 2C, Suppl Figure 4C), suggesting an action for SMAPs in limiting pathological vascular remodeling. Of note, a role for PP2A and its inhibition in regulating vascular remodeling have been implicated in the literature. PP2A B56γ in proliferating vascular smooth muscle cells (VSMCs) has been demonstrated to serve as a critical mediator for phosphodiesterase PDE1A in the regulation of β-catenin / T-cell factor (TCF) signaling - an important pathway in regulating vascular remodeling. PP2A inhibition by Okadaic acid (OA) or silencing of B56γ in VSMCs abrogated PDE1-mediated β-catenin / T-cell factor (TCF) signaling [25]. A recent study showed that B55α plays an important role specifically in the remodeling vasculature. B55α/PP2A was found to limit endothelial cell apoptosis during vascular remodeling [26]. These observations, coupled with SMAPs’ negative regulation of pathological vascular remodeling in AAA, strongly support the notion that PP2A activation might be protective against disease development and progression where aberrant vascular remodeling plays an important contribution.

Another limitation of our current animal studies is related to the systemic nature of administration of PP2A inhibitors and activators. The precise cellular and molecular mechanisms underlying the observed effects undoubtedly require further investigation. It is conceivable that SMAPs and LB-100 could introduce “off-target” effects in our
experimental animal model. We recognize that pharmacological gain- and loss-of-function approaches could be undermined by “off-target” effects. However, based on previous studies in multiple cancer (e.g., lung, prostate and breast) models, all data supported SMAPs’ target specificity and favorable toxicity profiles[16, 23, 27]. In the first study describing the use of SMAPs, SV40 small T antigen was utilized as a form of target validation to determine whether the biological effect of SMAPs was through PP2A activation. Expression of the small T antigen in lung cancer H358 cells conferred resistance to SMAP treatment in the xenograft model, indicating that SMAP-mediated growth inhibition was dependent on functional PP2A holoenzymes [16]. In another study, DT-1310 (a biologically inactive analog that lacks a N-H sulfonamide hydrogen bond donor function) is structurally similar to SMAP but is biologically inactive, was used. Treatment with DT-1310 neither induced an increase in annexin V positivity nor inhibited PP2A-regulated signaling pathways, such as AKT and MAPK signaling in human advanced lung adenocarcinoma (LUAD) cell line H1975 [28]. Recently, the 3.6 Å cryo-em structure provided insight into the unique trimeric pocket recognized by DT-061 and the molecular interactions of DT-061 with all three PP2A subunits. Specifically, these studies showed that DT-061 shifts the equilibrium of PP2A heterogeneity to favor B56α ultimately [29] suggesting that B56α could play a major role in AAA.

Nevertheless, to gain in-depth insights into the requirement of specific PP2A subunit for SMAPs’ effect, as well as the importance of each specific PP2A subunit in AAA, studies involving genetic manipulation of PP2A subunits via knock in or knockout approaches in vivo and in vitro are warranted. Along this line, previous studies offered several important clues. In our future investigations, we will manipulate PP2A Aα and
B56α in our model systems to dissect their roles in AAA and their importance in mediating SMAPs’ anti-AAA effects. We will then extend our studies to other PP2A subunits.

Our findings may have broader implications in cardiovascular disease other than AA. For example, previous studies indicate that PP2A is critical in the maintenance of endothelial barrier function [30-32]. Given the central importance of the endothelial barrier in maintaining vascular homeostasis, one can postulate that enhancing PP2A activity by SMAPs will offer benefits in a variety of vascular disease settings (such as atherothrombosis, coronary artery disease, acute coronary syndrome and stroke) where endothelial barrier function is disrupted. Recent studies also showed that cardiomyocyte restrictive deletion of PP2A in adult mice causes cardiac hypertrophy [33]. We believe that this present study sets the groundwork to extend the use of specific SMAPs herein described to other cardiovascular disease model systems, providing the field with the opportunity to gain new mechanistic insights into the structural and pathophysiological roles of phosphatases in cardiovascular biology.
Clinical perspectives

The number of patients diagnosed with abdominal aortic aneurysm (AAA) is increasing worldwide as the population ages, yet the current available and experimental therapies have demonstrated limited efficacy for AAA treatment.

We provide the first direct evidence implicating protein phosphatase 2A (PP2A) in AAA development and progression and offers novel insights into the pathobiology of AAA. Our studies revealed a marked reduction of PP2A activity in aortic aneurysmal samples when compared to control tissue, suggesting PP2A is relevant in human aneurysm. Importantly, PP2A activation via an orally bioavailable first-in-class small molecule activators of PP2A (SMAPs), strongly mitigates the progression of AAA.

Our studies highlight a potential new target for pharmacologic therapy and management strategies of AAA.

Data availability statement

All supporting data for this manuscript is included in the Figures and the accompanying supplemental files.
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Figure legends

**Figure 1.** PP2A inhibition by LB-100 augments AngII-induced AAA in mice. (A) Maximal abdominal aortic diameter; two-way ANOVA followed by Bonferroni post-hoc correction was used. (B) Incidence of AAA including rupture, n=30; Chi-square test. (C) Elastin integrity grading in Vehicle versus LB-100 in Apoe-null mice following 4 weeks of AngII (1μg/kg/min) infusion. Data are presented as mean ± SEM (Vehicle, n=10; LB100, n=8); Elastin score: 1-4, higher score indicates more severe elastin breakdown. (D) Kaplan-Meier survival curve (n=30 each group); (E) Representative images of immunohistochemical staining for macrophages (MAC3, left panel), T cells (CD3, right panel); (F) Representative images of immunohistochemical staining for MCP1, (G) in situ MMP activity, and (H) ROS production in Apoe-deficient aortae following 28 days of AngII infusion; quantification shown in the right panels. n = 5–7. Scale bars: 100 μm. Statistical analysis was performed using Student’s t test except MAC3 and MCP1 (Mann Whitney).

**Figure 2.** PP2A activation by SMAPs inhibits AngII-induced AAA in mice. (A) Maximal abdominal aortic diameter in Apoe-null aortae following 4 weeks of Ang II (1μg/kg/min) infusion (Vehicle, n =33; SMAPs, n =35). Mann Whitney test. (B) Incidence of AAA including rupture (Vehicle, n=47; DT-1154, n=43); Chi-square test. (C) Elastin integrity grading in Vehicle versus DT-1154 in Apoe-null mice following 4 weeks of AngII (1μg/kg/min) infusion. Data are presented as mean ± SEM (n=11); Elastin score: 1-4, higher score indicates more severe elastin breakdown. Mann Whitney test. (D) Kaplan-
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Figure 3. LB-100 enhances while DT-1154 mitigates AngII-induced ERK1/2 and NFκB activation following 4 weeks of AngII infusion. (A) Western blot of phospho-ERK1/2 and NFκB in Apoe-null aortae treated with LB-100 following saline or AngII treatment (4 weeks). n = 5 for vehicle group; n = 6 for LB-100 group. (B) Western blot assessment of NFκB and ERK1/2 activation in abdominal aortae in Apoe-null mice with and without SMAPs (DT-1154) administration. Quantification of p-P65 and p-ERK1/2. V: Vehicle; D: DT-1154; AngII + Vehicle, n=7; AngII + DT-1154, n=6. Statistical analysis was performed using Student’s t test.

Figure 4. SMAPs inhibit ERK1/2 and NFκB activation and inflammation in macrophage cells. (A) Western blot of p-ERK1/2 in macrophage cells (RAW264.7) following TNFα (20ng/ml, 15 minutes) treatment in the presence of absence of SMAPs (DT-061). T-ERK1/2 = Total ERK1/2; (B) Western blot of NFκB (p-p65) under the same conditions as in (A); (C) qRT-PCR assessment of Mcp1, Mmp9, Cox2 and Vcam1, n=4 per group. Macrophages were treated with TNFα for 12 hours in the presence of absence of...
SMAPs (DT-061). Two-way ANOVA followed by Bonferroni post-hoc correction was used.

**Figure 5.** Decrease of PP2A activity in aortic aneurysms. (A) PP2A activity assay in control human non-aneurysm and thoracoabdominal aortic aneurysmal samples, n=8; (B) PP2A activity in murine control and abdominal aortic aneurysm from AngII-infused Apoe-null mice, n=5-6. (C) Western blot of PP2A subunits in the same set of samples as in (A); (D) Quantitation of PP2A subunits. n=6. Mann Whitney test.
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