Investigation of the Biological Function of Fibroblast Activation Protein and its Potential as a Therapeutic Target in Cancer and Diabetes

A thesis

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Andrew L. Coppage

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Advisor: William Bachovchin

Thesis Chair: Larry Feig

Committee Member: Andrew Bohm

Committee Member: Brian Schaffhausen

Abstract

Fibroblast activation protein is an extracellular post-proline cleaving serine protease belonging to the S9 family of prolyl oligopeptidases. Other members of this family include DPPIV, DPP8, DPP9 and PREP, all of which share the relatively rare ability to cleave peptides after proline residues. This unique specificity suggests possible roles for these proteases in regulation of bioactive peptides as exemplified by DPPIV, which regulates glucose homeostasis through proteolysis of the incretin hormones. DPPIV is now a validated target for the treatment of diabetes, but interest is increasingly being directed at the closely related FAP based on the metabolic syndrome-resistant phenotype of the FAP knockout mouse. This observation implicates FAP as a potential therapeutic target in diabetes. Evidence also exists for FAP as a target in cancer based on observations that FAP is strongly and selectively upregulated on cancer-associated fibroblasts and the demonstrated efficacy of pharmacological inhibitors of FAP in multiple models of cancer, although most of this work has been done with the dipeptide boro-prolines that cross-react with other S9 family members.

Work to assess FAP as a drug target in either pathology has thus far been limited by a lack of FAP-specific inhibitors or an FAP-specific substrate to accurately measure inhibition. Fortunately a number of FAP-specific inhibitors and an FAP-specific substrate are now available. Here, we leverage these new tools to show that specific pharmacological inhibition of FAP, DPPIV, FAP+DPPIV, DPP8/9 and PREP are all unable to replicate either the anti-cancer or immune-stimulating effects of the dipeptide boro-prolines. In fact, PREP-specific inhibition appears to accelerate tumor growth. We

also show that a FAP-specific inhibitor, although unable to recapitulate many of the aspects of the FAP knockout mouse phenotype, does appear to enhance glucose tolerance, and moreover this effect is additive to that obtained with DPPIV inhibition.

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ACE2; angiotensin-converting enzyme-related carboxypeptidase

AFC; 7-amino-4-trifluoromethylcoumarin

AMC; 7-amino-4-methylcoumarin

APCE; antiplasmin cleaving enzyme

APJ; apelin receptor

BCA; Bicinchoninic Acid Assay

B.I.D.; Twice-daily dosing

BMSCs; bone marrow stromal cells

Bp; base pair

CAFs; cancer associated fibroblasts

CAR; chimeric antigen receptor

CCL11; eotaxin

CCL17; C-C motif chemokine 17

CXCL-1; C-X-C motif chemokine 1

DASH; DPPIV structure and activity homologs

DIO; diet-induced obese

DMSO; dimethyl sulfoxide

DPP10; dipeptidyl peptidase 10

DPP2; dipeptidyl peptidase 2

DPP6; dipeptidyl peptidase 6

DPP8; dipeptidyl peptidase 8

DPP9; dipeptidyl peptidase 9

DPPIV; dipeptidyl peptidase 4

ECM; extracellular matrix

EGR1; early growth response 1

EMT; epithelial to mesenchymal transition

FAK; focal adhesion kinase

FAP; fibroblast activation protein

FGF-21; fibroblast growth factor 21

FGFRs; fibroblast growth factor receptors

GBM; glioblastoma

G-CSF; granulocyte colony stimulating factor

GIP; gastric inhibitory polypeptide

GLP-1; glucagon-like peptide 1

GLUT 1; glucose transporter 1

HbA1c; hemoglobin A1c

HEK mFAP; HEK293 cells which overexpress mouse FAP

HFD; high-fat diet

HSCs; hepatic stellate cells

IL-1β; interleukin 1β

IL-6; interleukin 6

IPGTT; intraperitoneal glucose tolerance test

kb; kilobase

kDa; kilodaltons

KO; knockout

LC/MS; liquid chromatography–mass spectrometry

MES; 2-(*N*-morpholino)ethanesulfonic acid

MIP-1β; macrophage inflammatory protein

MMP; matrix metalloprotease

mpk; milligrams/kilogram

NSCLC; non-small cell lung cancer

ObRs; leptin receptors

P.O.; oral gavage

PPCE; post-proline cleaving enzyme

PREP; prolyl oligopeptidase

Pyr1-apelin-13; pyroglutamate apelin-13

RCF; relative centrifugal force

SBEs; SMAD binding elements

SGLT2; sodium/glucose cotransporter, member 2

SMAD; Mothers Against Decapentaplegic

SPRY2; sprouty homolog 2

T2D; type II diabetes

Tb4; thymosin b4

TCA; trichloroacetic acid

TGFβ; transforming growth factor beta

TNFα; tumor necrosis factor alpha

WT; wild type

Xaa-boroPro; dipeptide boro-proline

α2AP; alpha-2-antiplasmin

αSMA; α smooth muscle actin

Introduction

1.1 Fundamentals of FAP Biology

1.1.1 The Phylogeny of FAP

Fibroblast activation protein (FAP) is a serine protease of the S9 family under the umbrella of the SC clan of proteases [125]. Within this group, FAP belongs to the S9b subfamily and more specifically the dipeptidyl peptidase IV-like gene family. This family encompasses four enzymatically active members, dipeptidyl peptidases IV, 8, 9 (DPPs IV, 8, 9) and FAP, as well as two catalytically inactive members, DPP6 and DPP10. This family is also referred to as the DPPIV structure and activity homologs (DASH) group of enzymes. FAP is most closely related to DPPIV, a protein with which it shares 52% amino acid identity. The DPPIV and FAP gene can be found sequentially on the largest arm of chromosome two (2q24.2 and 2q24.3) with each gene consisting of 26 exons spanning approximately 80 kilobases (kb) [117]. The high degree of similarity and location of these genes suggest they are a result of a duplication event; however both genes are conserved throughout vertebrate evolution indicating this duplication event did not occur in our immediate evolutionary past [107]. The mouse and human forms of FAP appear to be true homologs, with similar expression patterns and enzymatic activities [174].

1.1.2 The Post-Proline Cleaving Enzymes

From a functional standpoint, FAP is a post-proline cleaving enzyme (PPCE), which means it has the ability to cleave peptide bonds immediately following proline residues. It shares this activity with other members of the DPPIV-like gene family as well as other select proteases [60]. The ability to cleave after proline is somewhat rare among proteases

due to the structure of proline, which contains a secondary rather than primary amine and has a cyclic structure that can adopt either the cis or trans conformation within a peptide chain. These differences render proline residues particularly resistant to cleavage from most other proteases, making post-proline cleaving enzymes useful for the regulation of proline containing bioactive peptides [56].

In addition to the specialized post-proline cleaving function, all enzymatically active members of the DPPIV gene family, including FAP, possess dipeptidyl peptidase activity (Fig. 1). This activity allows these enzymes to cleave dipeptides from the N-terminus of peptides and proteins [62, 226]. This ability is also shared by the protease dipeptidyl peptidase 2 (DPP2, QPP, DPP7), which is a member of the S28 protease family [224, 264]. Although these enzymes prefer a penultimate proline residue for this exopeptidase activity, they are also capable of hydrolyzing peptides with alanine or serine immediately N-terminal to the cleavage site (P1 position). Preferences for amino acids two residues N-terminal of the cleavage site (P2 position) are less stringent, as are positions C-terminal to the scissile peptide bond. Given these relatively lax requirements, substrate specificities of the post-proline cleaving dipeptidyl peptidases overlap considerably. However, the activity of these enzymes is not likely to be completely redundant given their varied tissue expression patterns and cellular localizations.

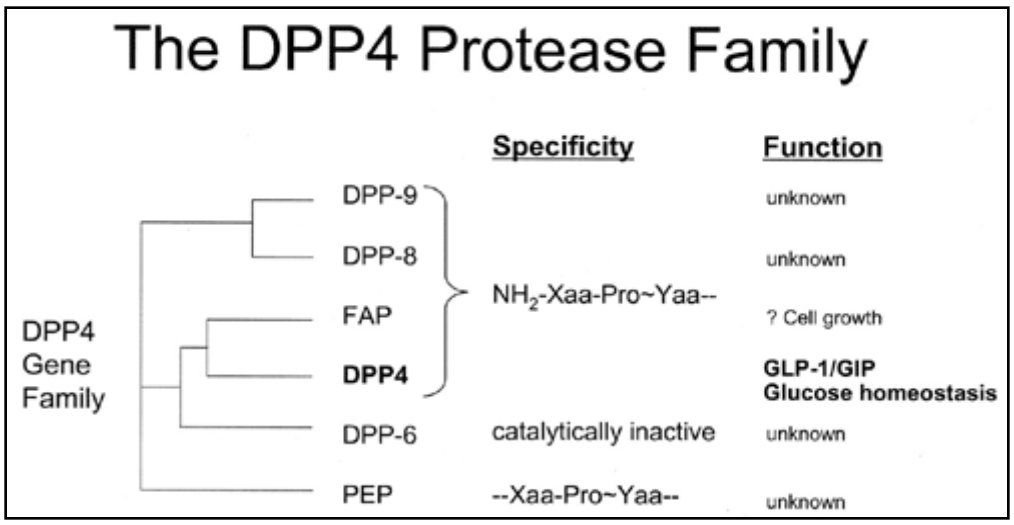


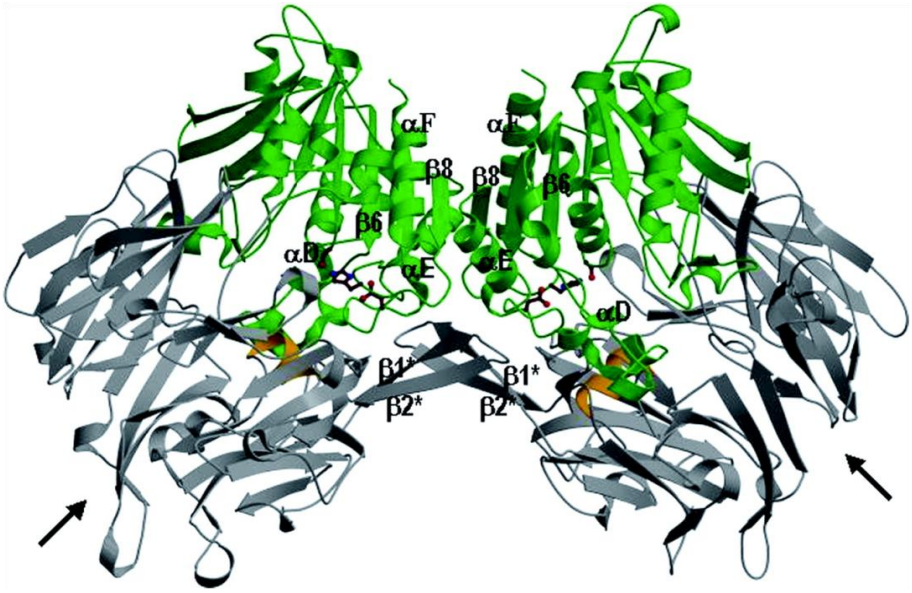
Figure 1. Family of DPPIV-related proteases and their substrate specificities. For the majority of enzymes, the biological roles and identity of endogenous substrates remains poorly understood. Figure and caption from: Drucker, Daniel J. Dipeptidyl Peptidase-4 Inhibition and the Treatment of Type 2 Diabetes: Preclinical Biology and Mechanisms of Action. Diabetes Care. 2007;30(6):1335-1343.

FAP is unique among the DPPIV gene family because, in addition to dipeptidyl peptidase activity, it also possesses post-prolyl endopeptidase activity, which is the ability to cleave after proline within peptide chains [195]. It shares this endopeptidase activity with prolyl oligopeptidase (PREP, POP, PEP), although PREP has far more promiscuous substrate preferences than FAP, which requires glycine in P2 and proline in P1 for endopeptidase cleavage [43]. Additionally, PREP is exclusively an intracellular enzyme while FAP is extracellular, suggesting that these proteases are unlikely to be acting on the same substrates *in vivo* [220, 239].

1.1.3 Fibroblast Activation Protein

1.1.3.1 The Biochemistry of FAP

As a monomer FAP is ~85 kilodaltons (kDa) in size, although the protein is most often found in its catalytically active, dimeric form [137]. FAP is a type II transmembrane protease possessing a six amino acid N-terminal intracellular region and 20 amino acid transmembrane domain, while the extracellular region is composed of two large domains, an α/β hydrolase domain and an eight bladed propeller domain (Fig. 2) [169]. FAP appears to be extensively N-glycosylated as baculovirus-expressed human FAP has sugar residues at nine of ten available sites on each homodimer [237]. Eight of these sites are present on the beta propeller domain, while the other two are on the hydrolase domain. Despite this extensive glycosylation, the contribution of these carbohydrates to the function or production of mature FAP remains unknown.



Kathleen Aertgeerts et al. J. Biol. Chem. 2005;280:19441-19444

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Figure 2. Ribbon diagram representing the overall structure of the FAPα dimer. The β-propeller domain and α/β-hydrolase domain are shown in gray and green, respectively. The dimer is viewed perpendicular to its 2-fold dyad axis. Active site residues Ser624, Asp702, and His734 are represented in ball-and-stick representations. The α-helix containing the Glu motif is indicated in yellow. Arrows indicate the direction of the

central pore. The figure was made using the programs MOLSCRIPT (22) and Raster3D (23). Figure and caption from Aertgeerts et al., 2005 [237].

The active site of FAP is present at the interface of the two major extracellular domains and is accessible in two ways, through a 24 angstrom side opening created by the hydrolase and propeller domains, or through a smaller pore of 14 angstroms in the propeller domain [237]. The catalytic triad of FAP consists of three residues, Ser624, Asp702 and His734 [137, 168]. The residues surrounding the catalytic serine are G-W-S-Y-G, which are consistent with the G-X-S-X-G classical consensus sequence of active site serines in other proteases [261]. The catalytic triad of FAP cooperates in the hydrolysis of peptide bonds with the histidine activating the serine nucleophile for attack on the scissile peptide bond, while the glutamic acid functions to stabilize the tetrahedral intermediate formed during catalysis [259, 261]. Although the three residues of the catalytic triad are classical, their orientation within the primary structure is not. FAP is therefore classified as a non-classical serine protease, a label it shares with DPPIV [136].

Like, DPPIV, FAP is primarily recognized as a membrane-anchored protein. Although there are reported isoforms of FAP with intracellular localization, their existence is largely unconfirmed [134]. However, also like DPPIV, substantial evidence does exist for a soluble extracellular form of FAP present in circulation. This form of FAP is reported to have near identical substrate specificity to the membrane bound form and was first labeled antiplasmin cleaving enzyme (APCE) before being identified as a soluble form of FAP [12, 262]. While the source of soluble FAP is not known, it has been suggested that an

unidentified sheddase enzyme is responsible for cleaving FAP free of the cell membrane [209].

In its soluble form, FAP is not known to interact with any other proteins, however as a membrane bound enzyme, FAP forms heterodimers with DPPIV [206]. These heterodimers localize to the invadopodia of endothelial cells together with matrix metalloproteases (MMPs). FAP also exists in close proximity to urokinase plasminogen activator receptor on the surface of invasive melanoma cells, an association which is dependent on β1 integrins [172]. In a separate study, α3/β1 integrin is identified as existing in an invadopodia complex with FAP only in the presence of collagen, suggesting a role for this complex in the function and formation of invadopodia [182].

1.1.3.2 FAP Substrates and Substrate Preferences

Like other enzymes in the DPPIV gene family, FAP is capable of cleaving after a proline bond two amino acids from the N-terminus of peptides [22, 107]. Investigation of FAP dipeptide substrate specificity using NH_2-Xaa-Pro linked to the fluorescent group 7-amino-4-trifluoromethylcoumarin (AFC) revealed that FAP is capable of hydrolyzing these substrates with any amino acid in the P2 position, although there was a clear preference for Ile, Pro or Arg residues [226].

The biological significance of FAP dipeptidyl peptidase activity remains unknown as FAP has not been shown to cleave any dipeptidyl peptidase substrate *in vivo*. However, a recent study has shown that FAP shares a number of dipeptidyl peptidase substrates with DPPIV, including glucagon-like peptide 1 (GLP-1), gastric inhibitory polypeptide (GIP)

and the neuropeptides substance P, neuropeptide Y, beta natriuretic peptide and peptide YY [179]. Although FAP cleaves these peptides, all of these substrates are also hydrolyzed by DPPIV with considerably greater efficiency. In fact, DPPIV is a superior dipeptidyl peptidase, as evidenced by its ability to cleave the substrate Gly-Pro-AFC with approximately 100 fold greater catalytic efficiency than FAP [237].

This difference in hydrolysis efficiency of dipeptidyl peptidase substrates can be ascribed to a small variation in the otherwise nearly identical active sites of DPPIV and FAP. In both DPPIV and FAP there is a glutamic acid residue in a negatively charged pocket of the active site that interacts with the N-terminus of substrates. In the DPPIV active site Asp^{633} pushes this residue, Glu^{206}, further into the active site where both negatively charged residues can form hydrogen bonds with the N-terminus of a substrate [259]. In comparison, FAP lacks an analogous aspartate residue and instead has an alanine (Ala^{657}) at this position [237]. This substitution likely explains the difference in dipeptidyl peptidase activity between FAP and DPPIV as the FAP/A657D mutant has greatly increased dipeptidyl peptidase activity [237]. The substitution of alanine for aspartic acid in the active site of FAP also contributes to the ability of FAP to cleave endopeptidase substrates as the FAP/A657D mutant results in a nearly 350 fold loss of catalytic efficiency towards the endopeptidase substrate Z-Gly-Pro-7-amino-4-methylcoumarin (Z-Gly-Pro-AMC) [237]. Later work demonstrated that A657 along with five conserved residues in the substrate pocket of FAP contribute to transition state stabilization of substrates within the active site [10].

The ability to cleave after proline within a peptide chain, rather than only in the penultimate position, is what separates FAP from other proteins of the DPPIV family. In contrast to the substrate specificity of its dipeptidyl peptidase activity, the endopeptidase activity of FAP is highly restricted. FAP can only hydrolyze endopeptidase substrates with Pro in P1 and Gly in P2 [226]. Substrates meeting these requirements appear to be preferred over dipeptidyl peptidase substrates, evidenced by the fact that FAP cleaves the substrate Z-Gly-Pro-AMC with 10 fold higher catalytic efficiency compared to Gly-Pro-AMC [237]. FAP activity towards endopeptidase substrates is also affected by amino acids in positions P4 through P4', although the requirements are not as stringent as in the P1 and P2 positions [39, 195, 262]. The P5-P7 positions can also contribute to substrate binding and Arg in these positions is shown to be favorable [262].

Given the preference of FAP for endopeptidase substrates, its unique activity towards GP containing endopeptidase substrates in the extracellular space, and its inferiority to DPPIV as a dipeptidyl peptidase, it has been posited that FAP is first and foremost an endopeptidase. Indeed, the only *in vivo* substrate of FAP yet discovered is the endopeptidase substrate alpha-2-antiplasmin (α2AP) [77]. FAP cleaves a 12 amino acid sequence off the N-terminus of the nearly 70kDa α2AP protein and this truncated form has been detected in the circulation. This proteolytic event also appears to be functionally significant, as the FAP truncated form of α2AP is more readily cross-linked to fibrin, where it inhibits clot resolution [181]. However, the FAP cleavage site in α2AP is not conserved in other species, such as mouse, suggesting cleavage of this substrate may not be the only biological role for FAP enzymatic activity.

Two other endopeptidase substrates of FAP have been described. The first, sprouty homolog 2 (SPRY2), is an intracellular protein that is unlikely to be a relevant substrate of FAP, but together with α2AP, highlights the ability of FAP to cleave large globular proteins [39]. This is in contrast to DPPIV and DPP8/9, which preferentially cleave peptides shorter than 10kDa [154]. PREP, the only other PPCE to share endopeptidase activity with FAP, also strongly prefers peptides less than 30 amino acids in length, likely due to exclusion of larger substrates by the beta propeller domain [204]. Why FAP, which also has a beta propeller domain and a similar structure to DPPIV, can hydrolyze larger substrates when related enzymes cannot remains unknown.

The final endopeptidase substrate described for FAP is collagen. Early reports indicated FAP was capable of cleaving type I collagen, but it was later revealed that FAP is actually unable to cleave collagen in its native form without prior denaturation, hydrolysis or the aid of other proteolytic systems such as the MMPs [69, 106, 255]. Collagen I derived gelatin is cleaved after internal Gly-Pro residues with consensus sequences of PPGP and (D/E)-(R/K)-G-(E/D)-(T/S)-G-P. FAP also is capable of cleaving type III collagen, but not collagen IV [69]. Though FAP is not able to cleave intact collagen, the expression of FAP in areas of active extracellular matrix (ECM) remodeling suggest pre-digested or unwound collagen may be an important substrate of this protease.

1.1.4 Expression of FAP

1.1.4.1 FAP in Adult Tissues

FAP was named in 1993, although the protein was discovered earlier as the antigen bound by the F19 antibody [26]. This antibody was found to selectively recognize human sarcoma cell lines and cultured fibroblasts, yet failed to bind cells in most adult tissues [26, 27]. Further work has confirmed that FAP is nearly, but not completely, absent from most healthy adult tissues [33]. FAP expression was originally observed in the human pancreas using the F19 antibody and the subset of cells labeled by this antibody were later confirmed to be alpha cells [26, 42].

The F19 antibody also recognizes a small number of fibroblasts and there does appear to be a subset of stromal cells that express FAP in healthy adult tissues. A recent study in transgenic mice expressing luciferase under the control of the FAP promoter showed the highest levels of FAP promoter activity in bone, adipose, pancreas and muscle tissues [49]. Detectable FAP activity in the bones of mice likely reflects the expression of FAP on bone marrow stromal cells (BMSCs) and later work in humans have identified BMSCs as the only FAP-expressing cells in human bone marrow [101, 138]. Activity profiling of FAP in mouse tissues using an FAP-specific fluorescent substrate also revealed low, albeit detectable levels of FAP in a number of tissues, with particularly high activity detected in uterus, pancreas and submandibular gland [210].

FAP is also present in the circulation as a soluble enzyme [12]. In humans the concentration of FAP in plasma is approximately 100-500ng/ml, however there is substantial interspecies variation as mouse plasma has approximately 15 fold more FAP activity than

human plasma [38, 198, 210]. In fact, mouse FAP activity is substantially higher in plasma than in any healthy tissue when corrected based on total protein concentration [210].

The relative paucity of FAP in healthy adult tissues provides few clues as to the biological function of this enzyme. However, substantial upregulation of FAP is observed in a number of pathological situations, particularly those involving tissue remodeling. FAP expression during these processes may provide insight into the biological role of this protease.

1.1.4.2 FAP During Development

FAP expression has been reported during development in both frogs (*Xenopus laevis*) and mice (*Mus musculus*). In frogs, FAP expression is induced during loss of the tadpole tail in response to thyroid hormone. This process involves extensive tissue remodeling characterized by cell death and resorption [253]. In mice, FAP transcript is present during embryogenesis, particularly days E14 through E17 [175]. Use of a transgenic FAP knockout (KO) mouse expressing β-galactosidase under the control of the FAP promoter revealed embryonic FAP expression in areas of tissue remodeling including somites and perichondrial mesenchyme [88]. Despite significant expression of FAP during development, the FAP KO mouse develops normally [245].

1.1.4.3 FAP in Wound Healing

Though absent from most healthy adult tissues, FAP expression has been reported to increase greatly in certain remodeling tissues. A non-pathological situation in which FAP expression has been reported is during the healing of wounds [26, 117]. FAP upregulation is

observed in rats following both superficial and deep scald burns [90]. In this model, expression peaked seven days after burning and was detectable for at least 21 days. In a mouse model of incised wound healing, a similar temporal pattern of FAP expression is observed with FAP transcript levels peaking six days after wounding [85]. Though the FAP KO mouse has no reported phenotype related to wound healing, the upregulation of FAP following skin injuries suggests a role for FAP in the healing process. In humans, this role may be reflected in the cleavage of α2AP, which leads to greater incorporation of this protein into fibrin clots resulting in slowed clot resolution [181].

1.1.4.4 FAP in Pulmonary Fibrosis

Further evidence for a role for FAP in tissue remodeling comes from the plethora of diseases in which upregulation of this protease is observed. One such disease is pulmonary fibrosis, a scarring of the lung tissue. FAP is not expressed in the healthy lung, but it is substantially upregulated in the human disease as well as in both mouse and canine models [11, 109, 158]. FAP localizes to the fibrotic foci of the scarred lung and its expression positively correlates with the degree of fibrosis [158].

1.1.4.5 FAP in Liver Fibrosis

Another pathology where FAP correlates with disease severity is liver fibrosis [148]. Liver fibrosis is characterized by excess deposition of ECM by hepatic stellate cells (HSCs), and it is these cells that are recognized to be key drivers of the fibrotic process. Interestingly, FAP upregulation occurs on HSCs which may be significant given the role of HSCs in extracellular matrix remodeling [108]. Plasma levels of FAP are also increased

in patients with liver cirrhosis and examination of plasma FAP activity may prove useful in stratifying disease severity [157, 210].

1.1.4.6 FAP in Atherosclerosis

FAP expression has also been documented in atheromas, specifically in aortic plaques. In this disease, FAP is reported to be expressed by the smooth muscle cells [105]. Surprisingly, patients with acute coronary syndrome actually have lower levels of circulating FAP, suggesting that FAP is likely not shed or secreted from these plaques in substantial amounts [38]. FAP expression in atheromas appears to be higher in thin cap plaques, which are more prone to rupture. This has led to the development of a radiolabeled FAP inhibitor as a potential imaging agent, however FAP levels in atherosclerotic plaques may not be high enough for this agent to be clinically useful [80].

1.1.4.7 FAP in Arthritis

The pathology of arthritis entails inflammation of both bone and cartilage. This reaction can result in significant tissue remodeling, leading to degeneration of the joint [152]. Not surprisingly, FAP expression is strongly upregulated in patients with arthritis. Synovial fibroblasts, part of the synovial membrane which separates the outer joint capsule from the joint cavity, are the cells responsible for the upregulation of FAP in this disease [104]. Patients with rheumatoid arthritis, an auto-immune form of arthritis, have increased levels of FAP expression relative to patients with osteoarthritis [104]. The role of FAP in rheumatoid arthritis appears to be destructive as knockout of FAP prevents cartilage degradation in a mouse model of the disease [47, 145, 164].

1.1.4.8 FAP in Cancer

By far the most well studied aspect of FAP is its expression and involvement in cancer [100]. Since its discovery as a marker of fibroblasts in culture and select sarcomas cells, FAP expression has been investigated in a myriad of cancers and related diseases. FAP is expressed in greater than 90% of epithelial carcinomas, although expression was found to be restricted to stromal cells, rather than the tumor cells [27]. Epithelial tumors of the stomach, ovary, bladder, lung, colon and breast all express FAP, while benign colorectal adenomas, benign ovarian tumors and fibroadenomas show little to no FAP expression [33, 107, 228]. Expression of FAP is consistently low in normal tissues and elevated in the desmoplastic stroma of epithelial cancers making it an excellent marker for the reactive mesenchyme [89, 168]. FAP expression is also reported on endothelial cells and surrounding pericytes during angiogenesis, where it can be observed in heterodimers with DPPIV [75, 86, 206].

Although FAP is most well known as a marker of stromal fibroblasts in epithelial cancers, its expression has also been observed in cancer cells. FAP is expressed in cells cultured from select osteogenic sarcomas, fibrosarcomas, Ewing's sarcomas as well as astrocytomas and melanomas [26]. However, care must be taken when interpreting these results because culturing fibroblasts has been shown induce FAP in cells originally devoid of FAP *in situ*. A similar upregulation of FAP in tumor cells under culture conditions cannot be ruled out and this may explain a conflicting report of Ewing's sarcoma tumors lacking FAP expression [128]. Studies utilizing immunohistochemistry on tumor sections also provide additional evidence for the expression of FAP on select

colorectal cancer and gastric cancer cells [84, 140]. FAP immunostaining is also detected on melanoma and ovarian cancer cells, although in the latter instance FAP was inexplicably localized to the cytoplasm [87, 110]. Additionally, FAP expression is observed on the cancer cells of invasive cervical carcinomas [89]. These examples demonstrate that FAP can, in select cancers and situations, be expressed by tumor cells, but this appears to be the exception rather than the rule.

Expression of FAP in epithelial tumors has also been linked to progression and patient prognosis. FAP expression positively correlates with liver metastases in gastric cancer and lymph node metastases in both colorectal and thyroid cancers [140,167, 228]. Staging of gastric cancers and breast ductal carcinomas can also be aided by examining the degree of FAP expression [73, 83]. Consistent with the data on metastases and staging are a bevy of studies highlighting FAP expression as a negative prognostic factor in solid tumor cancers. FAP is reported to be a negative prognostic factor in digestive, ovarian, breast, colorectal, pancreatic and lung cancers [40, 41, 87, 92, 102, 127, 200, 235]. However, two studies of breast ductal carcinoma and head and neck cancer found FAP expression to correlate with better overall survival [233]. Excluding these two studies FAP appears, at the very least, to be a marker of cancer progression and perhaps an active participant in advancing malignant disease.

1.1.5 Regulation of FAP Expression

Despite extensive evidence linking the presence of membrane bound FAP to physiological processes and pathological conditions involving inflammation and ECM

remodeling, the molecular mechanisms which regulate FAP expression remain poorly understood. Examination of the FAP gene revealed a 2kb upstream promoter region in cells expressing FAP and a core 245bp region that was mainly responsible for this promoter activity [131]. Utilizing mouse cells expressing FAP, this study found that early growth response 1 (EGR1) bound to the core FAP promoter. EGR1 is a zinc finger transcription factor known to be involved in the transcription of genes including fibronectin, PTEN and transforming growth factor beta (TGFβ) [254]. Knockdown of EGR1 or mutation of the EGR1 binding site within the FAP promoter reduced, but did not eliminate transcription of FAP, indicating EGR1 may not be the only important regulatory element.

Interestingly, FAP expression also appears to be driven by TGFβ, a gene whose own transcription is regulated by EGR1. TGFβ signaling is critical for regulation of mothers against decapentaplegic (SMAD) sensitive genes and FAP was found to be one such SMAD sensitive gene. Both SMAD 3 and 4 bind to SMAD binding elements (SBEs) within the FAP promoter of FAP-expressing invasive melanoma cells and promote its transcription. These invasive melanoma cells were found to express high levels of TGFβ, which acts in an autocrine manner to regulate transcription of SMAD sensitive genes [256]. EGR1 and TGFβ may participate in a positive feedback loop whereby EGR1 drives FAP and TGFβ expression, which leads to further increases in FAP transcription [251]. While exogenous TGFβ was capable of increasing FAP expression in invasive melanoma cells, it was not sufficient to cause expression of FAP in non-invasive melanoma cells that did not constitutively express FAP. In fact, addition of exogenous TGFβ also fails to induce

binding of SMAD 3 and 4 to the SBEs of the FAP promoter in the non-invasive cells, despite functional TGFβ signaling. This suggests other transcriptional elements or perhaps epigenetic regulation may provide a master on/off switch for the FAP gene. Only after this switch has been turned on, can TGFβ regulate transcription of FAP. It is likely that TGFβ has a significant function in regulation of FAP expression *in vivo*, given that TGFβ is known to play a key role in the same pathologies in which FAP expression is observed. Specifically, TGFβ is known to cause epithelial to mesenchymal transition (EMT), which is a hallmark feature of both fibrosis and cancer invasion and metastasis.

Other studies have examined different conditions or factors which may indirectly or directly regulate FAP expression. Overexpression of the transcription factor TWIST1, which is upregulated in human glioblastoma (GBM), leads to increased FAP expression in human GBM cell lines [258]. Like TGFβ, TWIST1 is also involved in EMT. FAP expression is also upregulated in fibroblasts, melanocytes and melanoma cells in culture by exposure to ultraviolet light and TGFβ signaling was among the pathways implicated in this response [260]. Finally, FAP is upregulated in ovarian tumor cells by exposure to type I collagen, an effect that is abrogated by addition of a β1 integrin antibody [74]. This result, combined with data demonstrating the induction of FAP in fibroblasts placed on silicon nano-wire arrays, but not on flat silicon plates, suggests that FAP expression is regulated depending on extracellular matrix composition and structure [212]. The expression pattern of FAP in areas of tissue remodeling, together with transcriptional drivers known to be central regulators of fibrosis and EMT, suggest a role for FAP in these biological processes.

1.2 Reported role for FAP in Cancer

1.2.1 Cancer Disease Burden

Cancer represents a tremendous continuing challenge to public health systems and is the leading cause of death worldwide. Almost 40% of men and women will be diagnosed with cancer during their lifetime and treating these patients cost the United States healthcare system alone 125 billion in 2010 [201].

The majority of cancers are of epithelial origin with 80-90% of cases reported to be of this type [225]. Among the most common cancers are carcinomas of the breast, lung, colon and prostate. Less common are the sarcomas, which are cancers of mesenchymal origin.

Though treatment options vary by cancer type, the typical first line of treatment for solid tumors is surgery, chemotherapy, radiation or some combination of these three options [20]. All of these treatments can cause profound collateral damage to other tissues in the body. Furthermore, none of these therapies have yet provided a reliable solution as evident by the nearly 600,000 people in the United States project to die from cancer in 2015 [20]. Though advancements have been made and survival rates have begun to improve, novel treatments, particularly for solid tumors, are urgently needed. A greater understanding of the microenvironment of the solid tumor, where FAP+ fibroblasts are found, may aid in discovery of important new therapies.

1.2.2 The FAP+ Cancer Associated Fibroblast

Solid tumors are complex networks of diverse cell types. Cells of the tissue in which the tumor arose comingle with malignant cells as well as resident and infiltrating immune cells. During tumor growth, stromal cells, both within and at the barrier of the tumor, take on a reactive phenotype [214, 223]. Conversion to this phenotype is initiated by tumor cells through secretion of paracrine factors, direct cell-cell contacts and alterations to the ECM [4, 234]. These signals, combined with host inflammatory and immune responses, lead to generation of a tumor stroma [143]. This reactive stroma is characterized by the presence of activated fibroblasts including myofibroblasts that express α smooth muscle actin (αSMA) and pathological remodeling of ECM [116]. In addition to αSMA, FAP is well characterized as a marker of cancer-associated fibroblasts (CAFs) and a reactive stroma [27]. Long ago, the similarities between the tumor microenvironment and a healing wound were recognized. Since then, much work has been done characterizing how inflammatory and anti-inflammatory signals within the tumor microenvironment can affect tumor growth [3, 35, 114]. Mounting evidence now suggests that the tumor stroma, and in particular FAP+ stromal cells, are critical in promoting tumor growth through their suppression of immune cell accumulation and function [19, 21].

Early evidence for the importance of FAP+ CAFs was provided by a study utilizing an FAP DNA vaccine designed to direct a CD8+ T-cell response towards FAP expressing stromal cells. The authors found that this vaccine rendered mouse models of breast and colon cancer more sensitive to therapeutic agents [249]. Additional work in this area utilized a mouse model whereby the diphtheria toxin receptor was expressed in cells with active FAP gene transcription. In this system, addition of the diphtheria toxin causes death of

cells expressing FAP, which in tumor models includes FAP+ stromal fibroblasts. The authors found that transplantation of cells from the LL2 line into these transgenic mice resulted in tumors that continued to grow, despite the expression of chicken ovalbumin on the cancer cells that induced strong systemic CD8+ T cell responses [242]. This observation suggested that the tumor microenvironment may promote tumor growth by holding back anti-cancer immunity. Addition of the diphtheria toxin to tumor bearing mice resulted in depletion of FAP+ fibroblasts within the tumor and this was accompanied by a substantial immune-dependent reduction in tumor size, proving ablation of the FAP+ cells helped to unleash the immune system on the tumor [242].

Later work determined that FAP+ cells are responsible for local secretion of the chemokine C-X-C Motif Ligand 12 (CXCL12) and that an antagonist of the CXCL12 receptor, CXCR4, restored the ability of the immune system to cause tumor regression [247]. Therefore, the coating of tumors cells with FAP+ fibroblast derived CXCL12 may represent a key mechanism by which CAFs shield tumors from the immune system. In addition to downregulating immune responses, CAFs are also thought to shield tumors from the immune system through physical exclusion of immune cells and by production of ECM. Existence of these "cancer cell nests" has been observed in a number of tumors [25, 149]. Consistent with this role for FAP+ CAFs is data demonstrating that adoptive transfer of FAP-targeted chimeric antigen receptor (CAR) T cells results in reduced growth of lung and pancreatic tumors through immune-independent mechanisms [257]. This suggests multiple roles for the FAP+ stromal fibroblast in promotion of tumor growth and suppression of anti-tumor immune responses.

1.2.3 The FAP Protein in Cancer

There is ample evidence that FAP+ stromal cells are intimately involved in tumor growth, however the specific role of FAP as a protein is much less clear. Perturbations to the ECM are a salient feature of all the pathologies in which FAP is induced. Much of the literature has thus focused on the role of FAP collagenase activity in cancer. FAP has the ability to cleave type I and type III collagen, but only after denaturation or cleavage by matrix metalloproteases [69, 106]. Melanoma cells expressing FAP were found to degrade gelatin matrices, while FAP negative melanoma cells do not, highlighting the potential importance of this activity [1]. However, the significance of this gelatinase activity in the context of the tumor microenvironment remains a matter of debate.

One key study examined FAP+ fibroblasts in a cell culture model and found that they create ECM with parallel collagen I and fibronectin fibril organization that is conducive to invasion by pancreatic cancer cells [93]. Invasion by these cells is reduced when FAP is inhibited during ECM production, however the small molecule used in this study inhibits both FAP and the related prolyl oligopeptidase. Despite this drawback, this study remains one of the few to examine the role of FAP in its proper biological niche, on fibroblasts.

In contrast, nearly all other efforts to determine the biological role of FAP in cancer have utilized systems whereby the cancer cells express FAP. Exogenous FAP was found to promote invasion, migration and proliferation of the ovarian cancer cell line HO-8910PM and overexpression of FAP in SKOV-3 ovarian cancer cells results in xenografts with enhanced growth [189, 251]. FAP has also been shown to govern the ability of another

ovarian cancer cell line, SB247, to both invade and contract type I collagen gels [74]. *In vivo,* knockdown of FAP reduced the metastatic potential of the same SB247 cells. Contrary to these tumor promoting functions, FAP has also been reported as a tumor suppressor in melanoma cells [94].

Regardless of the findings of these various studies, the role of FAP proteolysis is likely confounded by its non-enzymatic functions. This is at least partially the case with respect to the findings with HO-8910PM cells, as FAP was shown to promote invasion, migration and proliferation of these cells through non-enzymatic mechanisms involving the GTPase Rac1 [98]. FAP also clearly has non-enzymatic roles in ECM degradation, likely through modulation of cell signaling pathways such as those regulated by focal adhesion kinase (FAK) [92]. In another study, over-expression of FAP in MDA-MB-231 breast cancer cells led to accelerated tumor growth and enhanced expression of other ECM remodeling proteins such as MMP9 regardless of whether the FAP was wild type (WT) or catalytically inactive [99]. This work highlights the ability of FAP to affect other protease systems, further complicating studies attempting to parse out the enzymatic and non-enzymatic effects of FAP when using techniques such as overexpression, genetic deletion and knockdown.

1.2.4 FAP Enzymatic Activity in Cancer

Two techniques have been used for the study of FAP enzymatic activity in isolation. The first involves mutation of the active site of FAP. Genetic mutation of the active site serine of FAP to alanine renders the protease catalytically dead, but mature protein is still

produced. This allows enzymatic effects to be separated from non-enzymatic effects. One key study on the role of FAP enzymatic activity in cancer utilized HEK293 cells expressing WT (HEK mFAP) or catalytically inactive mouse FAP and these cells were compared in a mouse xenograft model. In this work, tumors expressing WT FAP grew significantly faster than cells expressing catalytically inactive FAP [2]. While this result is interesting, the HEK mFAP model places FAP in an unusual biological niche on the tumor cells, rather than on the surrounding stroma. Although some tumor cells have been demonstrated to express FAP, the vast majority of clinical samples suggest FAP expression is typically restricted to the stromal compartment.

Only one study has attempted to address the role of FAP enzymatic activity in the stroma of tumors. This work was done in the CT-26 model, a syngeneic mouse model of colon cancer in which FAP+ stromal cells are induced by the host in response to tumor formation. In this work, two inhibitors, Glu-boroPro (PT-630) and LAF237 (vildagliptin) were compared to vehicle control for their effect on tumor growth [248](Fig. 3). Glu-boroPro is a nM range inhibitor of all the PPCEs, but is poorly cell permeable, while LAF237 is an excellent DPPIV inhibitor that also somewhat cross-reacts with dipeptidyl peptidases 8 and 9. In this experiment, Glu-boroPro significantly reduced tumor growth in the CT-26 model, while LAF237 did not. As DPPIV and FAP are thought to be the only extracellular PPCEs, Glu-boroPro was assumed to be inhibiting only DPPIV and FAP, while LAF237 was assumed to be inhibiting DPPIV. Therefore, FAP inhibition was designated as being responsible for suppression of tumor growth by Glu-boroPro. This finding was supported by a lack of tumor growth inhibition by Glu-boroPro in FAP KO

mice. However, the non-specific nature of Glu-boroPro still renders suspect the conclusion that FAP inhibition is responsible for its anti-cancer effects.

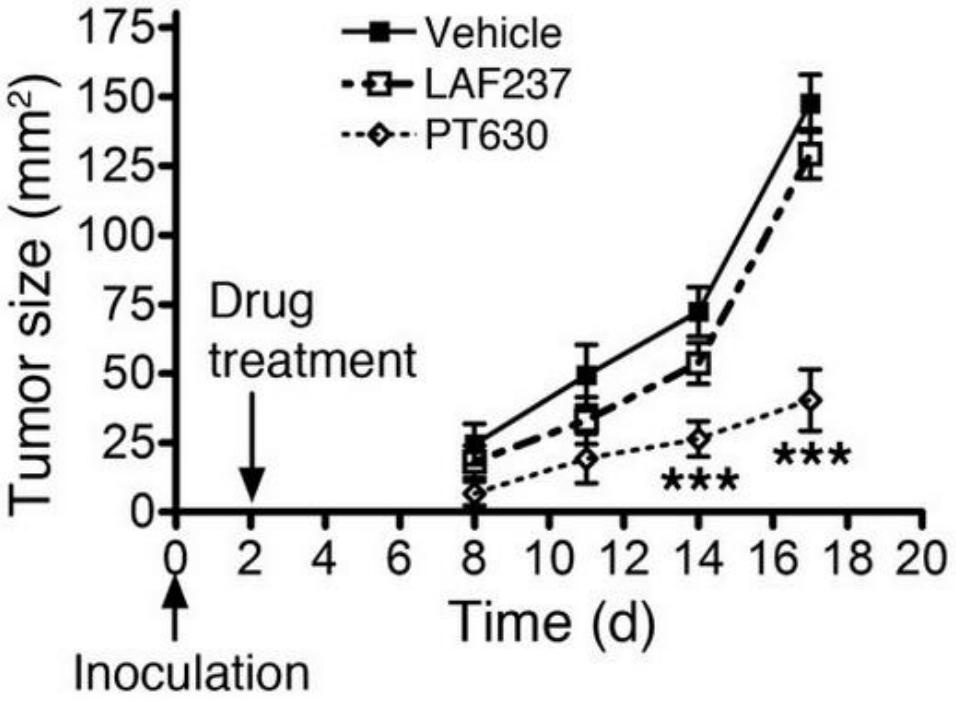


Figure 3. Effect of inhibition of FAP and/or DPPIV on tumor growth. PT630 treatment inhibited CT26 tumor growth. Mice were treated by oral gavage with vehicle control, LAF237, or PT630 twice daily, starting on day 2, after tumor cell inoculation. Data represent mean ± SEM (n = 11 animals per group in 2 independent experiments). ***P< 0.0001 versus vehicle control. Figure and legend adapted from Santos et al., 2009 [248].

1.2.5 The Dipeptide Boro-Prolines

1.2.5.1 Anti-Cancer Mechanisms of the Dipeptide Boro-Prolines

Glu-boroPro is a dipeptide boro-proline (Xaa-boroPro), which were among the first reported inhibitors of FAP. The dipeptide boro-prolines are potent, pan inhibitors of all the post-proline cleaving enzymes PPCEs. These enzymes include FAP, PREP and the

dipeptidyl peptidases 2, IV, 8 and 9 [45, 55, 144]. These compounds, particularly Xaa-boroPros with a β-branched residue in the P2 position, possess potent anti-tumor activity.

Despite inhibiting all the PPCEs, FAP has been posited as the relevant anti-cancer target for the dipeptide boro-prolines [208]. Just two of these inhibitors, Val-boroPro (PT-100) and T-butyl-Gly-boroPro (ARI-4175) have been studied extensively [192, 193, 208, 263]. Though these compounds exhibit no direct cytostatic or cytotoxic effects on any tumor cell line *in vitro*, they both demonstrate potent anti-tumor effects *in vivo* in multiple mouse models of cancer [193, 208, 244]. The compounds were found to be capable of inducing complete regression of even relatively large, established tumors, especially when combined with an anti-tumor vaccine [192, 263]. The tumor responses were significantly attenuated in immunodeficient mice and immunocompetent mice that had rejected their tumors were found to be protected from re-challenge with the same tumor despite discontinuation of treatment, demonstrating the involvement of an adaptive immune response [263].

Val-boroPro was taken into human clinical trials, but unfortunately failed to achieve its endpoints in a pivotal phase 3 trial in non-small cell lung cancer (NSCLC) and was abandoned [197]. Why the striking preclinical efficacy did not translate to the clinic is unclear. However, a lack of knowledge of the molecular mechanisms underlying Val-boroPro's biological activities, together with the then limited understanding of how to investigate immune-based therapy in the clinic, were likely to have been contributing factors. Another contributing factor was that the trial participants were unable to tolerate doses of Val-boroPro projected to be needed for efficacy. Mechanistic understanding

could lead to the identification of Val-boroPro analogs with reduced toxicity, and guide their clinical translation by successfully capturing their remarkable efficacy and ability to elicit immunological memory that protects against tumor recurrence.

Although Val-boroPro's target or targets remain unknown, many of the downstream effects have been identified. Val-boroPro induces a plethora of cytokines and chemokines in mice. For example, Val-boroPro induces the expression of interleukin 1β (IL-1 β), interleukin 6 (IL-6), and granulocyte-colony stimulating factor (G-CSF) in both tumors and tumor draining lymph nodes, and the appearance of G-CSF, eotaxin (CCL11), macrophage inflammatory protein 1β (MIP-1β)/CCL4, C-C motif chemokine 17 (TARC/CCL17) and C-X-C motif chemokine 1 (CXCL-1) in the serum [208]. Many of these cytokines and chemokines have an Xaa-Pro on their N-termini, suggesting that the extracellular PPCEs, DPPIV or FAP, may play a role in post-transcriptional regulation of their activities through proteolysis. Val-boroPro might function, at least in part, by blocking the enzymatic activity of DPPIV or FAP. Val-boroPro and ARI-4175 also appear to modulate the trafficking of various populations of immune cells such as neutrophils, dendritic cells, macrophages and myeloid suppressor cells, an activity likely mediated via the chemokines they induce [263].

Efforts to identify the target or targets though which the dipeptide boro-prolines mediate their biological activities have not surprisingly focused on the post-proline cleaving enzymes, as both compounds are known to potently inhibit all six of these enzymes. Two of these, DPPIV and FAP, are extracellular enzymes, whereas the other four, DPPs- 2, 8,

9, and PREP are intracellular [205]. Except for DPPIV, the biological functions of these enzymes remain to be fully characterized; therefore, none can yet be eliminated as targets contributing to either the anti-tumor activity or toxicity of Val-boroPro or the dipeptide boro-prolines. In fact, many of these enzymes are reported to play a role in both immune system function and tumor biology.

1.2.5.2 Dipeptide Boro-Proline Targets: DPP8 and DPP9

The leading candidate for the target or targets that are responsible for the induction of cytokine expression by Val-boroPro, as opposed to posttranslational modification, are the intracellular PPCEs, dipeptidyl peptidases 8 and 9. The active site of these enzymes is so similar that no inhibitors specific for either enzyme has yet been discovered [238]. DPP8 and DPP9 are ubiquitously expressed and can be found at high levels in inflammatory lymphocytes and macrophages, where the significant upregulation of DPP9 during monocyte differentiation suggests it might have a functional role [58, 63, 216]. This study also reported that inhibition of DPP8/9 reduces macrophage differentiation and enhances apoptosis in cell culture [63]. The most compelling evidence for DPP8/9 being the Val-boroPro target responsible for cytokine induction is that certain DPP8/9 selective inhibitors mimic Val-boroPro in causing caspase-1 activation and IL-1β release from THP-1 macrophages in culture [163]. In addition, the DPP8/9 inhibitory activity of vildagliptin has been reported to synergize with the compound parthenolide to kill AML cells in culture suggesting that inhibition of DPP8/9 might also sensitize tumor cells to killing [146]. A similar effect has also been observed with t-butyl-gly-boroPro, which has the ability to sensitize tumor cells to T-cell killing in culture [193]. Thus, there is reason to

suspect DPP8/9 as either the target for Val-boroPro's anti-tumor activity, or at least, the key initiator of the cytokine response.

1.2.5.3 Dipeptide Boro-Proline Target: PREP

Another post-proline cleaving enzyme recently implicated in tumor biology is PREP. Like DPP8 and DPP9, PREP appears to be ubiquitously expressed, but unlike DPP8/9, PREP expression is particularly high in the brain. Despite its apparent roles in brain physiology, other functions for PREP have also been proposed [71, 203]. PREP is reported to be upregulated in a number of different tumor types, where it has a putative role in generation of the angiogenic peptide Ac-SDKP from Thymosin b4 (Tb4) [66, 190, 191]. Inhibition of recombinant PREP added to human umbilical vein endothelial cells prevents generation of Ac-SDKP in a cell culture and correspondingly reduces angiogenesis in a matrigel plug assay, suggesting PREP may contribute to the tumor-associated angiogenesis [202]. PREP is also implicated in the generation of Ac-PGP from collagen, which serves as a neutrophil chemoattractant [183]. Despite these reports, the *in vitro* relevance of these substrates is suspect given the cytosolic localization of PREP and the extracellular localization of these peptides. Though there are sparse reports of PREP in the extracellular space, it is unclear how PREP would be released from intact cells to act on these substrates [151].

Mechanisms aside, PREP has been implicated in tumor biology based on the ability of two different inhibitors to reduce tumor growth [243](Fig. 4). The first, M83, is an inhibitor of both FAP and PREP, while the second J94, is PREP-specific. Both of these compounds were shown to reduce growth of HCT116 colon cancer xenografts in mice,

suggesting that PREP enzymatic activity promotes tumor growth. Therefore PREP inhibition could potentially represent a key facet of the dipeptide boro-proline mechanism.

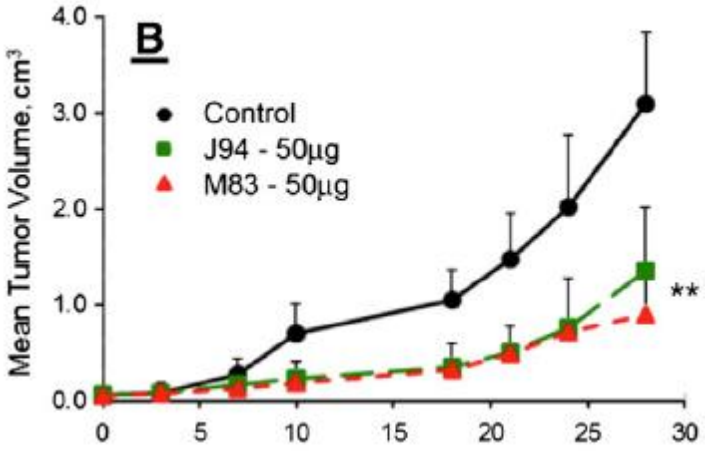


Figure 4. Growth of human xenografts in Foxn1nu mice. Treatment was initiated as I.P. drug injections when tumors reached~0.060cm3 in size. Tumor volumes were measured over the course of 28 days of treatment. Human colon cancer HCT116 xenograft growth. Mice were treated with 50 µl of saline (●) or M83(▲), or J94 (■), 50µg in 50µl of saline per day. **Both the M83 and J94 treatment groups are statistically different than the saline-treated group for days 10 to 28 (P<.001). Figure and legend from Jackson et. al. 2015 [243].

1.2.6 FAP Inhibitors

Though the vast majority of work on pharmacological inhibition of FAP relies on the dipeptide boro-prolines Val-boroPro (Val-boroPro) or Glu-boroPro, the non-specific nature of these compounds and the fact that they inhibit other proteases known to have roles in immunity and cancer makes it nearly impossible to separate out the contribution of FAP inhibition. These non-specific inhibitors of FAP were utilized out of necessity until more specific inhibitors were developed.

The first in a new generation of FAP-specific inhibitors was Ac-Gly-boroPro, which was developed based on the endopeptidase substrate specificity of FAP. Other work utilizing the FAP cleavage site of α2AP as a guide for inhibitor design also resulted in a number of potent FAP inhibitors [226, 262]. FAP inhibitors based on a structure activity relationship study of substituted 4-carboxymethylpyroglutamic acid diamides have also been described [240]. The primary advance of these sets of inhibitors was to eliminate the free N-terminus of the dipeptide boro-proline inhibitors. This modification results in specificity for FAP over other members of the DPPIV gene family which prefer dipeptide, exopeptidase substrates. However, this development did not provide specificity for FAP over PREP. A similar problem plagues the use of the substrate Z-Gly-Pro-AMC, which is cleaved by both PREP and FAP.

Fortunately a number of FAP specific inhibitors and an FAP-specific substrate are now available. Our group has recently synthesized and characterized an FAP-specific inhibitor, 3099, that is 350 fold selective for FAP over PREP, based on the use of D-alanine in the P2 position of this inhibitor [135]. Utilizing this P2 D-Ala, our group has also synthesized the first FAP-specific fluorescent substrate and its specificity has been confirmed using the FAP KO mouse [210]. Additionally, another group has also described a series of FAP-specific inhibitors utilizing a nitrile group to interact with the catalytic serine of FAP [6, 227]. The best inhibitor of this group has low nM affinity for FAP and is reported to provide long lasting inhibition of FAP *in vivo* [91]. Together, these FAP-specific inhibitors and substrate will allow for investigation of the role of FAP in cancer.

1.2.7 Aims of the Thesis: Part I

-Evaluate the role of FAP enzymatic activity in tumor growth

-Investigate the individual contribution of inhibition FAP and the other PPCEs to the mechanism of the dipeptide boro-prolines

1.3 Reported Role for FAP in Diabetes and Obesity

1.3.1 Disease Burden of Type II Diabetes and Obesity

Diabetes, particularly type II diabetes (T2D), is recognized as one of the pre-eminent health threats in the United States and this epidemic is linked to a rise in obesity rates [186]. Increases in obesity and diabetes are driven by a number of factors including a sedentary lifestyle and diets high in calories, trans-fats and simple carbohydrates [187]. Once primarily a disease of developed countries and adults, T2D now affects both developed and developing countries throughout the world afflicting and a startling number of individuals early in life [120]. Diabetes has become so widespread and prevalent that it accounted for approximately 12% of global health care costs in 2010 [119].

T2D begins as a state of mild insulin resistance that progressively worsens, ultimately resulting in peripheral tissues that are resistant to insulin coupled with an inability of the pancreas to produce sufficient amounts of insulin [29, 177, 194]. The ensuing state of high blood sugar is responsible for a number of complications including peripheral neuropathy, kidney dysfunction and impaired wound healing, the latter which can lead to

a need for limb amputation[28, 51, 171]. Individuals with T2D are also at a greatly increased risk of developing cardiovascular disease [159].

With the T2D disease burden expected to increase globally, there is urgent need for new therapies to both treat and modify the course of this disease. Current treatments for type II diabetes include older drugs such as metformin, which remains the standard of care for early stage disease [162]. Other drugs such as the sulphonylureas and insulins can reduce blood glucose levels, but run the risk of causing hypoglycemia [218, 221]. Newer therapies such as the sodium/glucose cotransporter, member 2 (SGLT2) inhibitors and GLP-1 receptor agonists provide patients with better glycemic control and have a low incidence of hypoglycemic events, yet other side effects and issues with patient compliance still represent significant drawbacks [31, 65, 141, 142]. Perhaps the greatest testament to the need for new therapies is the fact that over 60% of T2D patients fail to achieve their hemoglobin A1c goals (HbA1c) [241].

1.3.2 DPPIV Inhibitors: The "Gliptins"

One therapy for T2D that is in widespread use is a class of drugs called the gliptins. These drugs are inhibitors of the protease DPPIV and the top selling drug in this class, Januvia, recently saw sales peak at over 6 billion dollars a year [118]. The gliptins work by inhibiting DPPIV, which plays a key role in degradation of the incretin peptide hormones GLP-1 and GIP [122]. These hormones have penultimate alanine residues which are subject to hydrolysis by the dipeptidyl peptidase activity of DPPIV [48, 139]. In response to a meal, GLP-1 and GIP are released from the L and K cells of the intestine, respectively [121, 153].

They then travel through the portal circulation and activate their cognate receptors in the pancreas, resulting in glucose-dependent insulin secretion from beta cells. However, degradation by DPPIV renders these incretins incapable of engaging their receptors and thus limits post-prandial insulin release [122]. Blockade of DPPIV increases the level of intact incretin hormones, resulting in increased signaling through the GLP-1 and GIP receptors and potentiated post-prandial insulin release [57]. In turn, increased insulin reduces blood glucose levels following a meal [68].

1.3.3 The FAP Knockout Mouse

Although FAP is the protease most closely related to DPPIV, a similar role for FAP in glycemic control may at first seem unlikely. Rightfully so, efforts to determine the biological role of FAP have thus far focused on fibrotic diseases and cancer, where the expression and activity of FAP provide a clear rationale for potential involvement of this protease in these pathological processes. However, recent work has implicated FAP as a player in glucose and lipid metabolism, which represents a true departure from the traditional view of this protease.

Driving this view is work using FAP knockout mice. These mice have been available for a number of years and initial studies revealed that these mice are "fertile, show no overt developmental defects and have no general change in cancer susceptibility [245]." Similarly, no phenotypic differences in growth, organ structure or blood counts were observed. This suggests that FAP may not play a significant biological role in healthy animals, but this does not exclude its involvement during pathological processes.

One such pathological situation in which FAP appears to play a role is in the development of diabetes and obesity. While FAP KO mice do not appear phenotypically different from WT mice on a chow diet, FAP KO mice are reported to be protected from the metabolic abnormalities associated with high-fat diet feeding [180]. In comparison to WT mice fed a high-fat diet (HFD), FAP KO mice fed a HFD have reduced adiposity, fasting serum cholesterol and fasting serum insulin while displaying improved glucose tolerance (Fig. 5). In addition, FAP KO mice fed a HFD gain weight at a significantly reduced rate relative to WT controls.

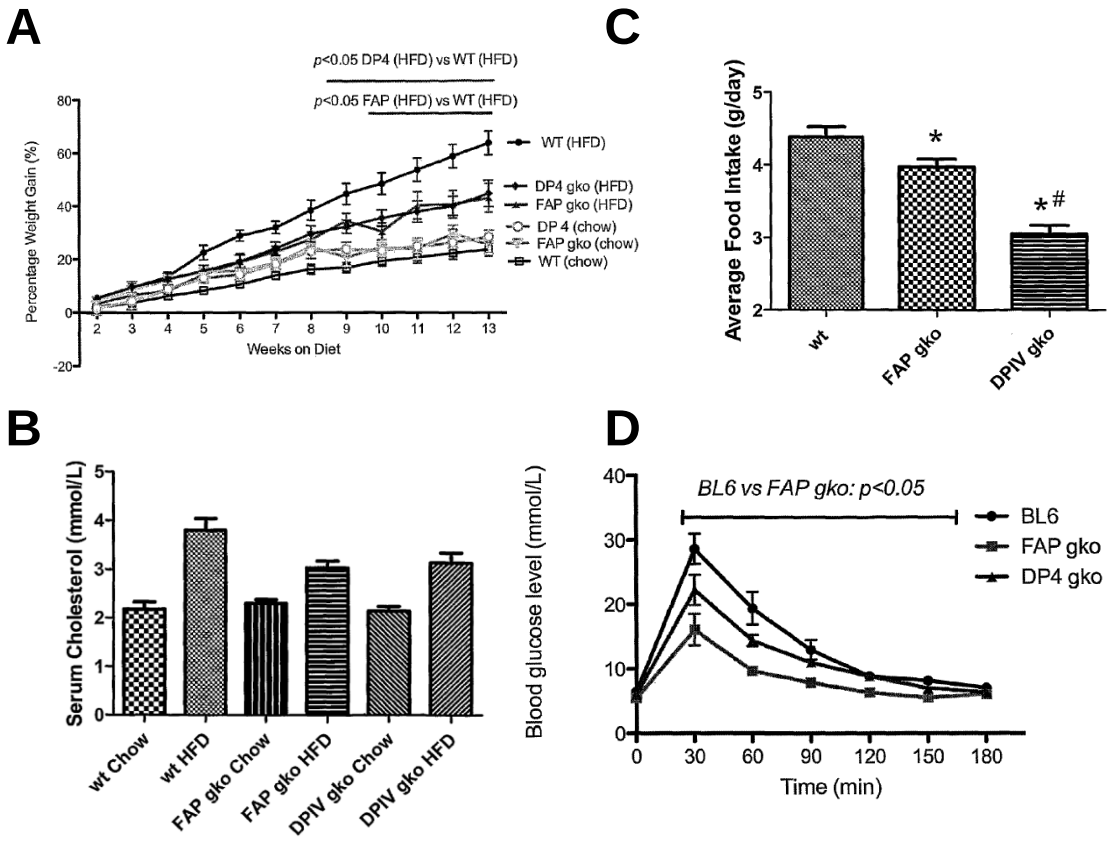


Figure 5. Metabolic phenotype of FAP KO mice. (A) FAP KO mice (FAP gko) are protected from high-fat diet induced weight gain. **(B)** FAP KO mice are protected from hyperlipidemia induced by a HFD. **(C)** FAP KO mice eat only slightly less than WT mice

when on a HFD. **(D)** FAP KO mice on a HFD have improved glucose tolerance relative to WT mice. Figures adapted from Gorrell et. al., Novel Metabolic Disease Therapy. PCT IAU201 0/000066 [180].

The phenotype of the FAP KO mouse is reminiscent of the DPPIV KO mouse which shows the same resistance to the metabolic abnormalities induced by a HFD. However, while DPPIV KO mice eat considerably less than WT mice, FAP KO mice eat only slightly less (Fig. 5C). Reduced food consumption of the DPPIV KO mouse is likely due to increased GLP-1 signaling, which slows gastric emptying and therefore food consumption. However, the fact that the FAP KO mouse eats considerably more than the DPPIV KO mouse suggests that different pathways may be responsible for the similar phenotypes. If a loss of FAP enzymatic activity is responsible for the KO mouse phenotype, then FAP could represent a drugable target for diabetes and obesity.

1.3.4 FAP and the Pancreas

While the knockout mouse data is by the far the most convincing, other work has also implicated FAP in the regulation of metabolism. In mice, depletion of cells in which the FAP promoter is active using diphtheria toxin results in cachexia and anemia, but also a pancreatic endocrine defect [49]. Although the exact nature of the defect is not specified, it most likely affects glycemic control given the role of the endocrine pancreas in secretion of both insulin and glucagon.

However, it remains uncertain whether this defect is due to the loss of FAP expressing cells or the loss of the FAP protein within the pancreas. In addition to this study, FAP

expression has also been documented in the pancreas, both at the transcriptional and protein level [42]. Specifically, FAP is observed within the islets of the human pancreas, presumably on alpha cells. The role of FAP on these cells and in the pancreas remains unexplored.

1.3.5 Confirmed and Potential Metabolic Substrates of FAP

1.3.5.1 Confirmed Metabolic Exopeptidase Substrates of FAP

One possible role for FAP in the endocrine pancreas is cleavage of the incretins, GLP-1 and GIP. These peptide hormones are known to stimulate insulin release from beta cells of the pancreas through activation of their cognate receptors [30, 123]. Increases in incretin signaling in the FAP KO mouse as a result of decreased proteolysis of the incretin hormones, could potentially explain some of the FAP KO mouse phenotype. However, FAP's contribution to incretin degradation may be masked by DPPIV, which hydrolyzes GLP-1 and GIP considerably faster than FAP.

FAP also cleaves peptide YY and neuropeptide Y, two peptides involved in regulation of food intake [178, 179, 207]. Though these substrates are cleaved by FAP, they are also cleaved by DPPIV, calling into question their relevance *in vivo*. This raises the possibility that FAP may have other relevant substrates that contribute to the knockout mouse phenotype.

1.3.5.2 Potential Metabolic Substrate of FAP: Enterostatin

One such possible FAP substrate is the peptide enterostatin. In humans, the sequence of enterostatin, APGPR could potentially be subject to both the exopeptidase and/or

endopeptidase activity of FAP. Enterostatin is a pentapeptide formed from the cleavage of pancreatic procolipase. The resulting pancreatic colipase is critical for digestion of fat by pancreatic lipase, while the pentapeptide regulates fat consumption [70]. Enterostatin suppresses fat intake in a mechanism dependent on both peripheral and central CCKA receptors [79]. In addition, enterostatin appears to increase thermogenesis in brown adipose tissue and suppress reward pathways through interactions with F_1F_0-ATP synthase and u-opioid receptors [78]. If FAP has a role in inactivating enterostatin, potentiation its metabolic effects in the FAP KO mouse could potentially explain part of the observed phenotype.

1.3.5.3 Potential Metabolic Substrate of FAP: Leptin

The hormone leptin is another possible FAP substrate based on the presence of an N-terminal Val-Pro sequence. Leptin is recognized as a key regulator of satiety [215]. Inactivating mutations in the leptin or leptin receptor genes are responsible for monogenic obesity both in humans and mice [199]. Leptin is an adipokine secreted mainly by white adipose tissue. It mediates its effects through binding of leptin receptors (ObRs) that are present in both peripheral tissues and the brain [132]. ObR engagement activates a number of intracellular signaling pathways that ultimately result in decreased food intake, and increased energy expenditure [176]. The FAP KO mouse is reported to have increased energy expenditure and slightly decreased food intake, therefore leptin could potentially be a relevant substrate of FAP.

1.3.5.4 Potential Metabolic Substrate of FAP: FGF-21

FGF-21 (fibroblast growth factor 21) is a member of the FGF super-family of proteins, but unlike many members of this family, its primary role is in the regulation of glucose and lipid metabolism [82, 97, 129]. FGF-21 is expressed primarily by the liver, but activates its target fibroblast growth factor receptors (FGFRs) in a variety of tissues including the liver, pancreas and adipose [111, 133, 170]. Engagement of the FGFRs together with beta-klotho (βklotho) on the surface of adipocytes results in glucose uptake dependent on the translocation of glucose transporter 1 (GLUT 1) [113].

FGF-21 administration improves glucose tolerance and insulin sensitivity in multiple mouse models of obesity and also decreases hyperlipidemia in db/db mice [5, 112]. The metabolic effects of FGF-21 are also conserved in humans and rhesus monkeys as FGF-21 or its analogs improve glucose and lipid homeostasis in both species [72, 160]. Additional effects of FGF-21 *in vivo* include decreased hepatic glucose output, enhanced fatty acid oxidation, reduced triglyceride synthesis and increased metabolic rate [156, 232].

The significant metabolic effects of FGF-21 have inspired a diverse set of drug design efforts. The recombinant protein itself is likely of little clinical value due to a limited half-life of 1-2 hours in animal models; therefore drug design programs have mainly focused on increasing the duration of FGF-21 action [97]. One such effort entailed fusing the N-terminus of FGF-21 to the Fc domain of human IgG [213]. As part of this effort, both the native and fused FGF-21 were tested for stability in plasma. C-terminal proteolysis by an unidentified protease was observed between P171 and S172, both in mice and monkeys. This proteolysis truncates FGF-21 10 amino acids from the C-terminus. Further

inspection of the C-terminal proteolytic site on FGF-21 revealed that glycine was in the P2 position and proline in the P1 position. This is the consensus site for FAP endopeptidase activity, therefore FAP is likely responsible for this proteolytic event.

Both the N and C-termini of FGF-21 are important for its function, with the N-terminal end binding FGFRs and the C-terminal end binding the co-receptor βklotho [96]. Previous work has demonstrated that removing these 10 amino acid residues from the C-terminus results in over a 400 fold reduction in adipocyte FGF signaling suggesting that cleavage of FGF-21 at this site is functionally significant [54]. However, the mouse form of FGF-21 lacks this FAP cleavage site. Therefore, FGF-21 cannot explain the FAP KO mouse phenotype and any biological relevance of FGF-21 will be unique to humans.

1.3.5.5 Potential Metabolic Substrate of FAP: Apelin

Apelin is a peptide hormone resulting from extensive post-translational processing of the 77 amino acid product of the *APLN* gene [150]. The largest apelin isoform found in circulation is apelin-36, which has 3 FAP endopeptidase consensus sites, while the shorter forms apelin-13 and pyroglutamate apelin-13 (Pyr1-apelin-13), have a single FAP endopeptidase consensus site.

Apelin signals through its receptor APJ, which is a G-protein coupled receptor found in a number of major organs including the brain, heart, adipose, kidney and intestines [166, 188]. While the apelinergic system has reported roles in many different processes including cardiac function, fluid homeostasis and the stress response, it also has a number of metabolic effects [14].

Apelin is produced in a number of different organs including adipose tissue, where it is an adipokine upregulated by insulin [16]. Apelin has recently received much attention recently as a potential treatment for metabolic syndrome based on the fact that Apelin KO mice have decreased glucose tolerance and are insulin resistant, suggesting a key role for this hormone in regulating glucose homeostasis [13]. In addition, injection of apelin normalizes blood sugar in obese mice [15]. Apelin is also reported to decrease weight gain and adiposity in mice without affecting food intake, indicating an increase in metabolic rate mediated by uncoupling proteins in the brown adipose tissue [17]. Apelin is therefore a prime candidate for a potential FAP substrate and reduced proteolysis of apelin by FAP in the FAP KO mouse could potentially explain may aspects of the diabetes and obesity resistant phenotype.

1.3.6 Aims of the Thesis: Part II

-Determine the role of FAP enzymatic activity in metabolism

-Investigate the role of FAP in the development or progression of diabetes and obesity

-Identify relevant substrate(s) of FAP related to metabolism

Methods

2.1 Investigation of the Role of FAP in Cancer: Methods

Post-Proline Cleaving Enzyme Assays

Compounds were dissolved at 100mM in DMSO (or .01M HCL for dipeptide boronic acids). These compound solutions were then diluted to 1mM in the appropriate assay

buffer and serial 1:10 dilutions were performed. Assay buffers were prepared as follows: DPPIV and DPP9, 25mM Tris, pH 8. FAP, 50mM Tris, 140mM NaCl, pH 7.5. DPP8, 50mM Tris, pH 7.5. DPP2, 25mM MES, pH 6. PREP, 25mM Tris, 250mM NaCl, pH 7.5. Recombinant enzyme (DPPIV, FAP, PREP, DPP2, DPP9 (R&D Systems), DPP8 (Enzo Life Sciences) was diluted in the appropriate assay buffer at the following concentrations: DPPIV, 0.1nM. DPP8, 0.8nM. DPP9, 0.4nM. FAP 1.2nM. PREP, 0.6nM. 180µl of enzyme solution was added to a 96 well plate, in triplicate, followed by the addition of 20µl of compound solution. Compounds were allowed to incubate with enzyme for 10 minutes at room temperature. Substrate, dissolved in the appropriate assay buffer, was then added to a final concentration of 25 µM. Substrates are as follows: DPPIV, DPP8 and DPP9: H-Gly-Pro-AMC (Bachem). FAP and PREP: Z-Gly-Pro-AMC (Bachem). DPP2: H-Lys-Pro-AMC (VWR). After 15 minutes, the fluorescence intensity was read using SpectraMax M2 plate reader (Molecular Devices) at ex.380nm/em.460nm. IC50's were determined using sigmoidial dose response non-linear regression function on GraphPad Prism software.

Intracellular DPP8/9 and PREP Assays

Human embroyonic kidney cells transformed with adenovirus 5 DNA (HEK293) were grown in RPMI1640 supplemented with 10% FBS, 4.5g/L glucose, 10mM HEPES, 1mM sodium pyruvate, 2mM L-glutamine and 1% Pen-Strep. In preparation for the assay, cells were trypsinized and plated in supplemented RPMI1640 without phenol red. Cells were plated at 100,000 cells/well for DPP8/9 assay and 25,000 cells/well for PREP assay in black 96 well plates and allowed to equilibrate overnight in a humidified incubator (37°C, 5% CO_2). Compounds were prepared as 100mM stocks in DMSO (or .01M HCL

for dipeptide boronic acids) then diluted to 4mM in media. The 4mM stock was serial diluted 1:10 and added to the cell culture media at a 1:4 dilution. Cells were returned to the incubator for 2 hours at 37°C followed by addition of H-Ala-Pro-AFC for the DPP8/9 assay or Z-Gly-Pro-AMC for the PREP assay to a final concentration of 25µM. Plates were then incubated for 10 minutes at 37°C followed by a fluorescence measurement at ex.400nm/em.505nm (Ala-Pro-AFC) or ex.380nm/em.460nm (Z-Gly-Pro-AMC).

Cell Viability Assays

Mouse colon cancer cells cultured from chemically induced tumors in Balb/c mice (CT-26) cells were seeded approximately 18 hours prior to treatment at a density of 5,000 cells/well in RPMI 1640 supplemented with 1% human serum and 1% Pen-Strep. Compound stocks were prepared at 100mM in DMSO (or .01M HCL for dipeptide boronic acids). A 4mM stock was prepared by dilution in media and this was serial diluted 1:10 and added to the cell culture media at a 1:4 dilution in a final volume of 100µl. Cells were returned to the incubator for 48 hours followed by addition of 20µl Cell-TiterBlue reagent (Promega). Cells were returned to the incubator for an additional 2h. Fluorescence was measured at ex.560nm/em.590nm. Percent cell viability was determined in comparison to vehicle treated control cells.

FAP Activity in Cell Culture

CT-26 and HEK293 mFAP cells were seeded at a density of 25,000 cells per well in serum free RMPI 1640 in a final volume of 100 µL and allowed to attach overnight. ARI-3144 substrate was added to a final concentration of 25 µM. Fluorescence was measured at ex.380nm/em.460nm at 37°C every 30 seconds for 10 minutes for slope determination.

CT-26 Tumor Model: DPPIV, FAP and DPP8/9 Inhibitors

CT-26 WT cells were grown in RPMI1640 supplemented with 10% FBS, 4.5g/L glucose, 10mM HEPES, 1mM sodium pyruvate, 2mM L-glutamine, 1% Pen-Strep. Cells were trypsinized and prepared for inoculation by re-suspension in phenol-free RPMI1640 without supplements at $5x10^6$ cells/ml.

Female BALB/c mice at 10 weeks of age (Charles River Laboratories) were inoculated on day 0 with $5x10^5$ CT-26 cells via 100µl subcutaneous injection on the right flank. B.I.D. dosing began on day 2 and continued until the end of the experiment. All compounds were administered via oral gavage (P.O.) at the following doses: Glu-boroPro, 100µg/dose. Sitagliptin, 100µg/dose. 5057, 500µg/dose. 8J, 300µg/dose. Tle-Pro-CN, 200µg/dose. Val-boroPro, 20µg/dose. Tumors were measured with carbon fiber digital calipers (Fischer) by blinded investigative staff. Mice were weighed twice weekly.

On day 24, mice were sacrificed 1, 3 or 6 hours after dosing. Blood was collected via cardiac puncture and transferred into lithium heparin coated tubes. Tumors were excised and immediately flash frozen in liquid nitrogen. All animal experiments were approved by the Tufts University IACUC under protocol B2014-132.

DPPIV Activity Assay in Plasma

5µl plasma samples were added per well, in triplicate, to 384 well round bottom low volume plates (Corning). 5µl of 80mM $MgCl_2$ in DPPIV assay buffer (25mM HEPES, 140mM NaCl, 1% BSA) was added to the plasma samples followed by addition of 10µl of 100µM H-Gly-Pro-AMC substrate solution dissolved in DPPIV assay buffer.

Reactions were incubated at room temperature for 20 minutes followed by measurement of fluorescence at ex.380nm/em.460nm.

FAP Activity Assay in Plasma

Plasma samples were diluted in 1:60 in PBS and 180µl of diluted sample was added per well, in triplicate, to a 96 well plate. 20µl of 500µM ARI-3144 substrate solution was added to each well. Fluorescence was measured at ex.380nm/em.460nm every minute for 30 minutes at 37°C.

FAP Activity Assay in Tumor Homogenates

Lysis buffer (50mM Tris-HCL pH 7.5, 10% glycerol, 5mM EDTA, 1mM DTT) was added to tumors at 10ml/g of tissue. Tumors were homogenized using a polytron homogenizer (Kinematica) and then briefly sonicated. Protein concentrations of tissue homogenates were determined using BCA Assay (Pierce). Protein concentrations of tumor homogenates were normalized to 1mg/ml by dilution in PBS. 180µl of 1mg/ml tumor homogenate was added, in triplicate, to a 96 well plate. 20µl of 500µM ARI-3144 substrate solution dissolved in PBS was added to each well. Fluorescence was measured at ex.380nm/em.460nm at 37°C every minute for 30 minutes for slope determination.

Serum G-CSF Measurements

Male BALB/c mice (Charles River Laboratories) at 7-8 weeks of age were dosed subcutaneously with the indicated compounds dissolved in sterile saline. 5057 was dosed as a suspension in sterile saline. 6 hours after dosing, mice were sacrificed and serum was

collected. Serum G-CSF concentrations were determined by Mouse G-CSF Quantikine ELISA (R&D systems) according to the manufacturer's instructions.

Plasma Drug Concentrations

1.5µl of 1mg/ml d8-Val-boroPro was added to 15µl of plasma sample. 15µl of .6M TCA was added to each tube to precipitate proteins, followed by centrifugation at 20,000 RCF for 15 minutes. Supernatant was analyzed for presence of selected drugs by LC/MS using Agilent 2000 HPLC with Zorbax Eclipse Plus C18 column (4.6 x 50mm, 1.8µM) and Qtrap 4000 (Applied Biosystems, Grand Island, NY). All samples were measured in duplicate and averaged using d8-Val-boroPro as the internal standard.

Determination of DPP8/9 Inhibition in Tumors

Protein concentrations of tumor homogenates were determined by BCA assay and protein concentrations were normalized to 2mg/ml in PBS and divided into two separate groups. The first group was spiked with sitagliptin and 5057 so that the final concentration of each compound was 1µM. The second group was also spiked with sitagliptin and 5057, but also with compound M, to a final concentration of 1µM. 180µl of the 2mg/ml tumor homogenate with inhibitors from each group was added, in triplicate, to a 96 well plate. 20µl of 500µM H-Gly-Pro-AMC substrate solution dissolved in PBS was added to each well. Fluorescence was measured at ex.380/em.460 every minute for 15 minutes for slope determination. DPP8/9 activity was taken to be the difference in slopes between the control sample (sitagliptin+5057) and the DPP8/9 inhibitor sample (sitagliptin+5057+compound M). Percentage inhibition was determined by comparison to vehicle-treated tumor homogenates.

CT-26 Tumor Model: PREP Inhibitors

CT-26 WT cells were grown in RPMI1640 supplemented with 10% FBS, 4.5g/L glucose, 10mM HEPES, 1mM sodium pyruvate, 2mM L-glutamine, 1% Pen-Strep. Cells were trypsinized and prepared for inoculation by re-suspension in phenol-free RPMI1640 without supplements at $5x10^5$ cells/ml.

Female BALB/c mice at 10 weeks of age (Charles River Laboratories) were inoculated on day 0 with $5x10^4$ CT-26 cells via 100µl subcutaneous injection on the right flank. Once daily dosing began on day 2 and continued until the end of the experiment. All compounds were administered via oral gavage (P.O.) at the following doses: Val-boroPro, 10µg/dose. 5332, 75µg/dose. 3531, 75µg/dose. Tumors were measured with carbon fiber digital calipers (Fischer) by blinded investigative staff. Mice were weighed twice weekly.

On day 31, mice were sacrificed 1, 3 or 6 hours after dosing. Blood was collected via cardiac puncture and transferred into lithium heparin coated tubes. Tumors were excised and immediately flash-frozen in liquid nitrogen.

Plasma Z-Gly-Pro-AMC Activity Assignment

5 µl of plasma from vehicle-treated CT-26 tumor bearing mice was diluted 1:60 in PBS containing 3531 and/or 5057. 180µl of diluted sample was added per well, in triplicate, to a 96 well plate. 20µl of 250µM Z-Gly-Pro-AMC substrate solution was added to each well. Final inhibitor concentrations were 1µM. Fluorescence was measured at ex.380nm/em.460nm over a 30 minute kinetic run at 37°C.

PREP Activity Assay in Spiked Plasma

Recombinant PREP was diluted to .6nM in PBS. 10µl of enzyme solution was added to a 384 well low volume round bottom plate. 5µl of plasma samples were added to each well followed by a 10 minute incubation at room temperature. 2.5mM Z-gly-Pro-AMC dissolved in DMSO was diluted 1:25 in PBS to give a 100µM stock. Then 5µl of 100µM Z-Gly-Pro-AMC was added to each well to give a final concentration of 25µM. Reactions were incubated at room temperature for 20 minutes followed by measurement of fluorescence at ex.380nm/em.460nm.

PREP Activity Assay in Tumor Homogenates

Tumor homogenates were prepared as above. 180µl of 1mg/ml tumor homogenate was added, in triplicate, to a 96 well plate. 2.5mM Z-Gly-Pro-AMC dissolved in DMSO was diluted 1:10 into PBS. Then 20µl of the 250µM Z-Gly-Pro-AMC was added to 180µl of lysate to give a final concentration of 25µM. Fluorescence was measured at ex.380nm/em.460nm at 37°C every minute for 30 minutes for slope determination.

2.2 Investigation of the Role of FAP in Diabetes and Obesity: Methods

FAP Activity in Tissues and Plasma

Diet-induced obese mice (DIO) and lean controls (Jackson Labs) at 23 weeks of age (17 weeks HFD) were sacrificed and tissues were collected and immediately flash frozen in liquid nitrogen. Blood was collected in heparin coated tubes and centrifuged at 16RCF for 15 minutes to isolate plasma. Alternatively, lysis buffer (50mM Tris-HCL pH 7.5, 10% glycerol, 5mM EDTA, 1mM DTT) was added to tissues at 10ml/g of tissue followed

by homogenization using a polytron homogenizer and then briefly sonicated. Protein concentrations of tissue homogenates or plasma were determined using BCA Assay. Protein concentrations of tissues or plasma were normalized to 1mg/ml by dilution in PBS. 180µl of 1mg/ml homogenate or plasma was added, in triplicate, to a 96 well plate. 20µl of 500µM ARI-3144 substrate solution dissolved in PBS was added to each well. Fluorescence was measured at ex.380nm/em.460nm at 37°C every minute for 30 minutes for slope determination.

In Vitro Digests of Proteins and Peptides by FAP

Apelin-13 (Cayman Chemical), Pyr1-apelin-13 (abcam), human FGF-21 (Peprotech) or mouse FGF-21 (Abcam) was reconstituted in FAP assay buffer (50mM Tris, 140mM NaCl, pH 7.5). Reactions were carried out at a final concentration of 20µM substrate, 200nM FAP and 16µM 3099. At each time point, aliquots of the reaction were taken and quenched with 10% v/v .01M HCL. Reaction aliquots were run on LC/MS (Column: Zorbax C-18, 2.2 x 50 mm, 3.5 µM) and quantified by extracted ion chromatogram integration of the major ion observed. Alternatively, 3µg of protein from quenched aliquots was loaded onto a reducing 20% SDS-PAGE gel. Gels were stained with Gelcode Blue Stain Reagent (Thermo Scientific).

In vitro Digests of Proteins and Peptides by PREP

Human FGF-21 was reconstituted in PREP assay buffer (25mM Tris, 250mM NaCl, pH 7.5). Reactions were carried out at a final concentration of 20µM FGF-21, 200nM PREP. Aliquots of the reaction were taken and quenched with 10% v/v .01M HCL and 3µg of

protein was loaded onto a reducing 20% SDS-PAGE gel and stained by Gelcode Blue Stain Reagent.

Plasma FGF-21 Digests

Pooled human or cynomolgus monkey plasma (Innovative Research) or pooled mouse plasma from C57BL/6 mice (Jackson Laboratory) was incubated with recombinant FGF-21 in FAP activity buffer with or without 3099. Final concentrations are 1µM for FGF-21 and 16µM for 3099. Reactions were incubated at 37°C for 24 hours and aliquots were taken and quenched with 10% v/v .01M HCL. Levels of intact FGF-21 were assessed by Human Intact Fibroblast Growth Factor ELISA (Eagle Biosciences) according to the manufacturer's instructions. FAP digested FGF-21 is not recognized by this ELISA (Fig. 25). Plasma FAP activity in plasma from different species was assessed as described in section 2.1.

Clearance and Degradation of Human FGF-21 in Mouse

50mpk 3099 or PBS vehicle was administered to C57BL/6 mice subcutaneously, followed 1 hour later by I.P. injection of human FGF-21 at .5mpk in PBS. Blood samples were collected by tail vein nick. Levels of intact FGF-21 were assessed by human intact fibroblast growth factor ELISA.

FAP Inhibitor Plasma Pharmacodynamic Profiles

8 week old male C57BL/6J mice (Jackson Laboratory) were dosed P.O. with the chosen compounds dissolved in water at the indicated concentrations. 5057 was dosed as a

suspension in water. Blood samples were collected by tail vein nick and plasma was prepared for FAP activity assay as previously described.

5057 Adipose Pharmacodynamic Profile

8 week old male C57BL/6J mice were dosed P.O. with 5057 as a suspension in water. Animals were sacrificed at the indicated time points and adipose tissue was collected and immediately flash frozen. Homogenates were prepared as previously described and FAP activity was assessed by ARI-3144 following a 1:5 dilution of homogenate in PBS.

Chronic FAP Inhibition with 5057 in chow fed mice (Lean)

7 week old male C57BL/6J mice were allowed to acclimate for 1 week before the start of dosing. Mice were fed a standard chow diet (Telkad). Mice were treated P.O. with vehicle (water) or 5057 (20mpk) once per day for 8 weeks at which time a oral glucose tolerance test was performed. Mice were given a 4 day wash-out period during which time dosing continued. Mice were then sacrificed with blood collected via cardiac puncture for endpoint measurements.

Chronic FAP Inhibition with 5057 in high-fat fed mice (Obese)

7 week old male C57BL/6J fed a chow diet since birth were maintained on a chow diet during a 1 week acclimation period before the start of dosing. At 8 weeks of age dosing began and the mice were switched to a high-fat diet (Research Diets D12492, 60% kCal fat). Mice were treated P.O. with vehicle (water) or 5057 (20mpk) once per day for 8 weeks at which time a oral glucose tolerance test was performed. Mice were given a 4

day wash-out period during which time dosing continued once per day. Mice were then sacrificed with blood collected via cardiac puncture for endpoint measurements.

Chronic FAP Inhibition with 5057 in DIO Mice (Severely Obese)

23 week old male DIO C57BL/6 (17 weeks of HFD) were allowed to acclimate for 1 week before the start of dosing. Mice were maintained on the HFD for the duration of the experiment. Mice were treated P.O. with vehicle (water) or 5057 (20mpk) once per day for 4 weeks at which time an oral glucose tolerance test was performed. Dosing was then continued for another 2 weeks followed by a second OGTT. Mice were then given a 4 day wash-out period during which time dosing continued once per day. Mice were then sacrificed with blood collected via cardiac puncture for endpoint measurements.

Cholesterol and Insulin Measurements

Plasma cholesterol was measured by Total Cholesterol Flourometric Assay Kit (Cayman Chemical) according to the manufacturer's instructions. Serum insulin was measured by Rat/Mouse Insulin ELISA Kit (R&D Systems) according to the manufacturer's instructions.

Oral Glucose Tolerance Tests (OGTTs)

Blood glucose levels of mice were monitored by tail vein nick using Freestyle Lite Blood Glucose Monitors (Abbott). Mice were fasted for 6 hours prior to glucose administration. Compounds were administered 45 minutes before glucose challenge, unless otherwise indicated, with 50% glucose at 2.5 or 5g/kg. Blood glucose readings were taken at -60, -15, 20, 40, 60, 90 and 120 minutes post-glucose administration. All readings were

performed in duplicate. Baseline correction was accomplished by subtracting blood glucose levels immediately prior to glucose administration (-15 time point) from values obtained after glucose administration. Fasting blood glucose reading was taken after 5 hours of fasting. Area under the curve was calculated for each individual mouse using the AUC function on GraphPad Prism software.

Results

3.1 Investigation of the Role of FAP in Cancer

3.1.1 Glu-boroPro, but Not Specific Inhibitors of FAP and DPPIV Suppress Tumor Growth in the CT-26 Model

We first investigated the effects of specific pharmacological inhibition of FAP in BALB/c mice transplanted subcutaneously with the mouse-derived CT-26 colon cancer cell line. In the CT-26 model, FAP is not expressed on the tumor cells, but is expressed following inoculation on tumor-associated stromal cells [49, 248]. This pattern of FAP expression recapitulates that reported in human epithelial carcinomas, thereby enabling the role of FAP enzymatic activity to be investigated in the correct biological niche. Because the host mouse is immunocompetent, the CT-26 model also allows for testing of any possible contribution of FAP inhibition to the immune-stimulating dipeptide boro-proline mechanism, which requires an intact immune system for maximum tumor responses.

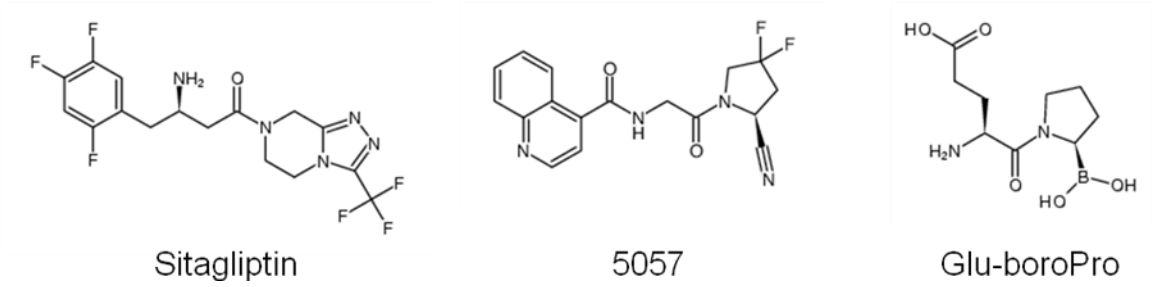


Figure 6. Chemical structures of DPPIV and FAP inhibitors. Sitagliptin ((3R)-3-amino-1-[3-(trifluoromethyl)-6,8-dihydro-5H-[1,2,4]triazolo[4,3-a]pyrazin-7-yl]-4-(2,4,5-trifluorophenyl)butan-1-one), 5057 ((S)-N-(2-(2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide) and Glu-boroPro ((4S)-4-amino-5-[(2R)-2-boronopyrrolidin-1-yl]-pentanoic acid).

Table 1. Inhibition of the PPCEs by FAP and DPPIV Inhibitors						
	IC50 (nM)					
Compound	DPPIV	FAP	DPPII	PREP	DPP8	DPP9
Sitagliptin	3	>100,000	>100,000	>100,000	8,300	13,000
5057	10,600	.8	>100,000	640	4,300	1,475
Glu-boroPro	1	4	12	340	26	13

The pan-DASH inhibitor Glu-boroPro was previously reported to suppress growth of CT-26 tumors, and the effect was attributed to the inhibition of FAP [248]. We sought to replicate this result using the same dose of Glu-boroPro (100µg) and dosing schedule (B.I.D. beginning on day 2), while also testing specific inhibitors of DPPIV and FAP (Fig. 6, Table 1). We chose sitagliptin as a specific DPPIV inhibitor because it is highly selective over all the other related PPCEs. For FAP inhibition, we selected the recently described compound 60, hereafter referred to as 5057 [91]. 5057 is a nitrile-based FAP-specific inhibitor that provides long-lasting inhibition of FAP *in vivo*. Because FAP and DPPIV potentially share substrates that are susceptible to dipeptidyl peptidase hydrolysis, we also tested whether both enzymes were simultaneously involved in the anti-cancer effects of Glu-boroPro by investigating the tumor response to a combination of specific

FAP and DPPIV inhibitors. In culture, CT-26 cell viability was unaffected by the addition of Glu-boroPro, 5057 or sitagliptin to the cell cultures at concentrations up to 100μM (Fig. 7).

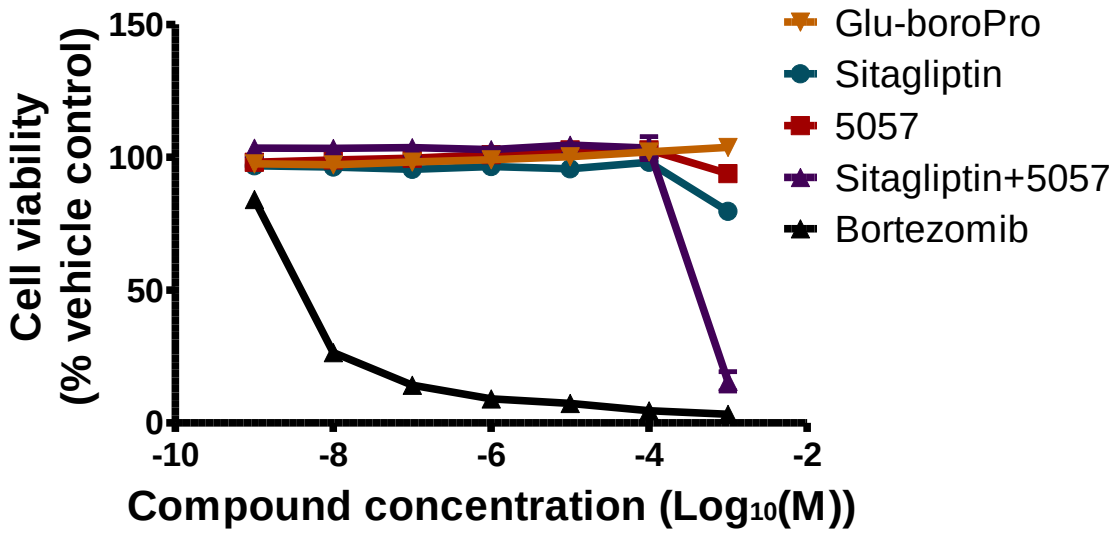


Figure 7. Cytotoxicity of selected FAP and DPPIV inhibitors. The selected FAP and DPPIV inhibitors are not directly cytotoxic to cells. CT-26 cells were treated with the indicated compounds at concentrations ranging from 1mM to 1nM. Viability was assessed after 48 hours by addition of fluorescent redox dye (n=3). Bortezomib was used as a positive control. Data expressed as mean ± SEM.

In vivo, the growth of CT-26 tumors was not altered by administration of either sitagliptin or 5057, but we found modest inhibition of tumor growth in mice administered Glu-boroPro (Fig. 8A, B). FAP activity was detectable in CT-26 tumor homogenates from vehicle-treated mice, but not in CT-26 cells in culture demonstrating the induction of FAP within the tumor (Fig. 8C, D). Pharmacodynamic investigation of the selected compounds in tumor-bearing mice revealed robust inhibition of plasma DPPIV activity with both Glu-boroPro and sitagliptin (Fig. 8E). Inhibition of plasma FAP activity in mice treated with 5057 was nearly complete at all time points tested (Fig. 8F).

Correspondingly, tumor FAP activity was significantly inhibited by 5057 and activity remained at less than 30% of vehicle-treated mice 6 hours after dosing (Fig. 8G). In contrast, Glu-boroPro transiently inhibited FAP both in the plasma and the tumor tissue.

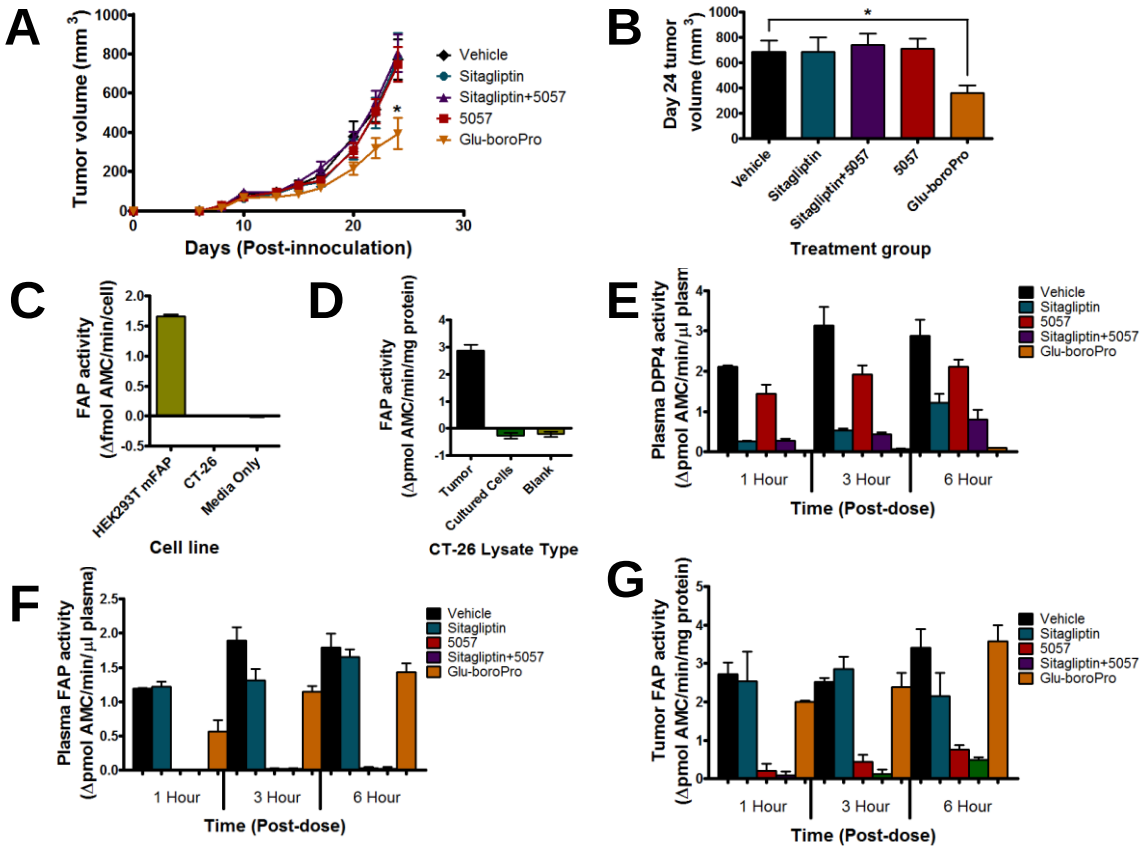


Figure 8. Effect of FAP and DPPIV inhibitors on tumor growth in the CT-26 model. (A, B) Glu-boroPro, but not sitagliptin or 5057 slows tumor growth. Female BALB/c mice were inoculated with $5x10^5$ CT-26 cells on day 0 followed by initiation of B.I.D. dosing on day 2 (n=25 in 2 independent experiments, *P<.05 by ANOVA). **(C, D)** FAP is expressed in CT-26 tumors, but not by CT-26 cells in culture. FAP activity of CT-26 cells in culture (n=6) and CT-26 homogenates (n=12 tumors, n=3 cell culture lysate) was assessed using ARI-3144. **(E)** Sitagliptin and Glu-boroPro inhibit DPPIV in the plasma of tumor-bearing mice. DPPIV activity was assessed in plasma of tumor-bearing mice on day 24 by H-Gly-Pro-AMC substrate (n=4-5). Sitagliptin, sitagliptin+5057 and Glu-boroPro were significant relative to vehicle at all time points measured (P<.05 by ANOVA). **(F)** 5057 and Glu-boroPro inhibit plasma FAP activity. FAP activity was assessed in plasma of tumor-bearing mice on day 24 by ARI-3144 (n=4-5). 5057 and 5057+sitagliptin were significant relative to vehicle at all time points measured (P<.001 by ANOVA). Glu-boroPro was significant relative to vehicle at 1 hour (P<.05 by ANOVA). **(G)** 5057 suppresses FAP activity in *ex vivo* tumor homogenates. FAP activity

in CT-26 tumor homogenates was assessed by ARI-3144 (n=4-5). 5057 and 5057+sitagliptin were significant relative to vehicle at all time points measured (P<.001 by ANOVA). All data expressed as mean ± SEM.

3.1.2 Treatment with Glu-boroPro, but Not Specific Inhibitors of FAP and DPPIV, Causes Substantial Increases in Serum G-CSF

The ability of Glu-boroPro to suppress tumor growth in the CT-26 model, whereas specific inhibition of FAP does not, suggests that FAP is not the sole anti-cancer target of the dipeptide boro-prolines. Therefore, alternative targets are likely to be involved in the anti-cancer effects of Glu-boroPro. Glu-boroPro is a dipeptide boro-proline that is structurally related to Val-boroPro and T-butyl-Gly-boroPro. One feature of this class of compounds is the ability to increase the serum concentration of various cytokines in mice, including IL-6, MCP-1 and G-CSF, the latter of which has been reported to be the greatest [126, 208]. Treatment of BALB/c mice with the beta-branched dipeptide boro-prolines Val-boroPro or T-butyl-Gly-boroPro can result in over 500-fold increases in serum G-CSF, with peak concentrations observed 6 hours after dosing [64]. G-CSF is a stimulator of hematopoiesis and a master regulator of neutrophil maturation and mobilization, and in earlier studies, the G-CSF response was reported to be closely associated with neutrophil infiltration of tumors and inhibition of tumor growth in Val-boroPro treated mice [36]. We hypothesized that Glu-boroPro may share this immune stimulatory mechanism, and this activity, rather than FAP inhibition alone, may explain its anti-cancer activity.

To determine if Glu-boroPro may exhibit anti-tumor effects through a similar mechanism, we investigated whether Glu-boroPro could produce cytokines using G-CSF

as a marker. At high doses, Glu-boroPro was capable of causing a greater than 75-fold increase in serum G-CSF, as measured by ELISA (Fig. 9). In contrast, we found that 5057 failed to produce an increase in serum G-CSF concentration, and sitagliptin only produced a minimal increase at the 2mg dose level. This result suggests that FAP and DPPIV inhibition are insufficient to replicate the cytokine stimulating effects of Glu-boroPro or the dipeptide boro-prolines.

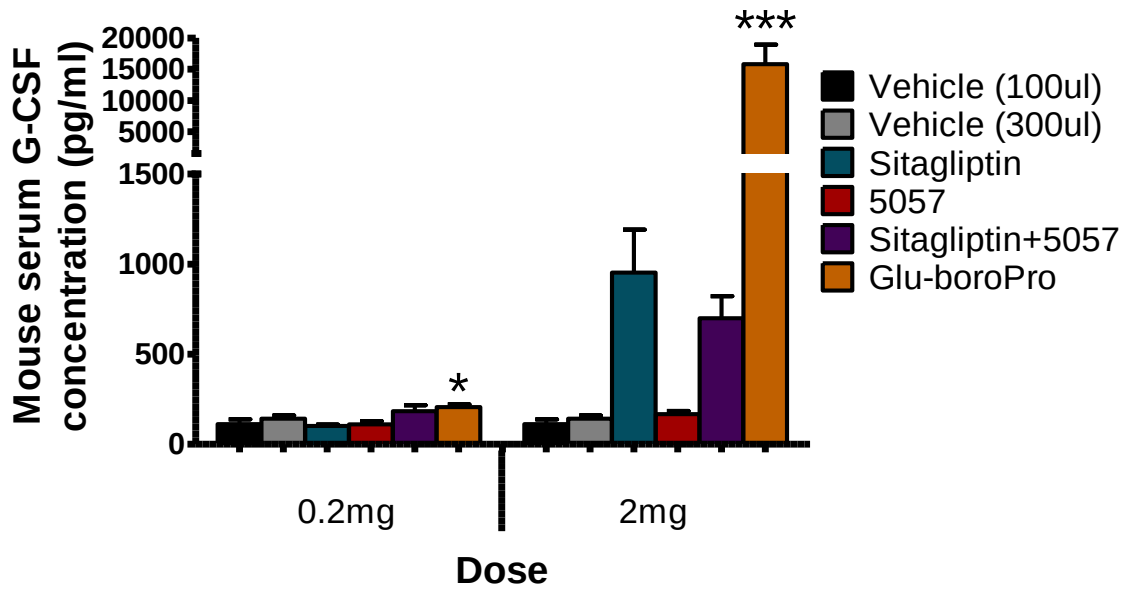


Figure 9. Effect of FAP and DPPIV inhibitors on mouse serum G-CSF concentrations. Glu-boroPro is capable of inducing a large increase in serum G-CSF, but selected specific inhibitors of DPPIV and FAP cannot. Male BALB/c mice were injected subcutaneously with 0.2mg or 2mg of the indicated compounds dissolved in 100µl of sterile saline. 5057 was dosed as a suspension in 300µl sterile saline. Blood was collected 6h after dosing and serum G-CSF concentration was assessed by ELISA (n=3-5, *P<.05, ***P<.0001 relative to vehicle by ANOVA). All data expressed as mean ± SEM.

3.1.3 Val-boroPro Suppresses Tumor Growth in the CT-26 Model, but Selected Inhibitors of DPP8/9 Do Not

We next chose to investigate the possible contribution of DPP8/9 inhibition to the anti-cancer effects of the dipeptide boro-prolines. As previously reviewed, inhibition of DPP8/9 is a putative cause of the induction of cytokines caused by the dipeptide boro-prolines. Selection of inhibitors for pharmacological inhibition of DPP8 and DPP9 *in vivo* is complicated by the intracellular location of the enzymes, so we chose inhibitors to study on the basis of both their potency and ability to penetrate cells. Cellular permeability was assessed by treatment of HEK293 cells with the desired compounds, followed by addition of the fluorescent substrate Ala-Pro-AFC to measure dipeptidyl peptidase activity. Because HEK293 cells do not express FAP or DPPIV, the activity of intracellular DPP8/9 (IC DPP8/9) can be assessed by this technique. We chose two compounds with the lowest intracellular IC_{50} values for DPP8/9 to test in the CT-26 model (Fig. 10, Table 2).

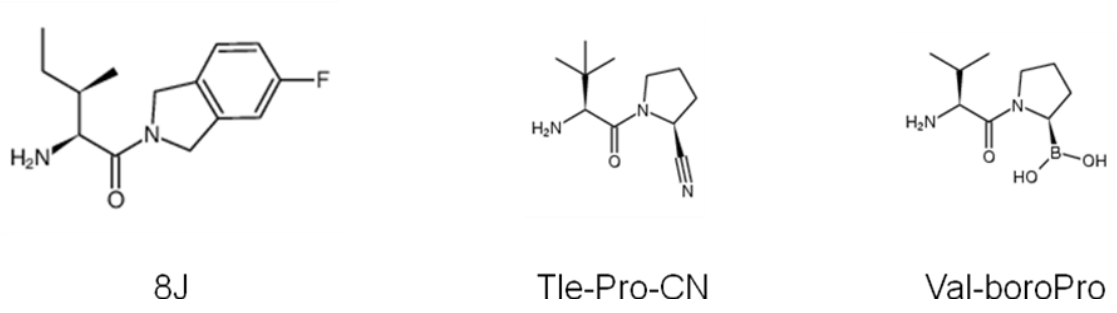


Figure 10. Chemical structures of DPP8/9 inhibitors. 8J ((2S,3R)-2-amino-1-(5-fluoroisoindolin-2-yl)-3-methylpentan-1-one), Tle-Pro-CN (1-[(2S)-2-amino-3,3-dimethyl-1-oxobutyl]-(2S)-2-Pyrrolidinecarbonitrile), Val-boroPro ([(2R)-1-[(2S)-2-Amino-3-methyl-1-oxobutyl]-2-pyrrolidinyl]boronic acid).

Table 2. Inhibition of the PPCEs by DPP8/9 Inhibitors

	IC50 (nM)						
Compound	DPPIV	FAP	DPPII	PREP	DPP8	DPP9	IC DPP8/9
8J	7,900	>100,000	31,000	>100,000	5	5	33
Tle-Pro-CN	1	700	>100,000	>100,000	42	19	120

Val-boroPro	.8	13	8	56	5	2	80

Compound 8J is an isoindoline derivative with selectivity for DPP8/9 versus the other PPCEs, and Tle-Pro-CN is a cyanopyrrolidine derivative that is a potent inhibitor of DPPIV in addition to DPP8/9 [147]. We tested both compounds alongside Val-boroPro, which unlike Glu-boroPro is readily cell permeable, for their effects on tumor growth in the CT-26 model. Like the DPPIV and FAP inhibitors, none of these compounds affected the viability of CT-26 cells in culture at concentrations up to 100μM (Fig. 11).

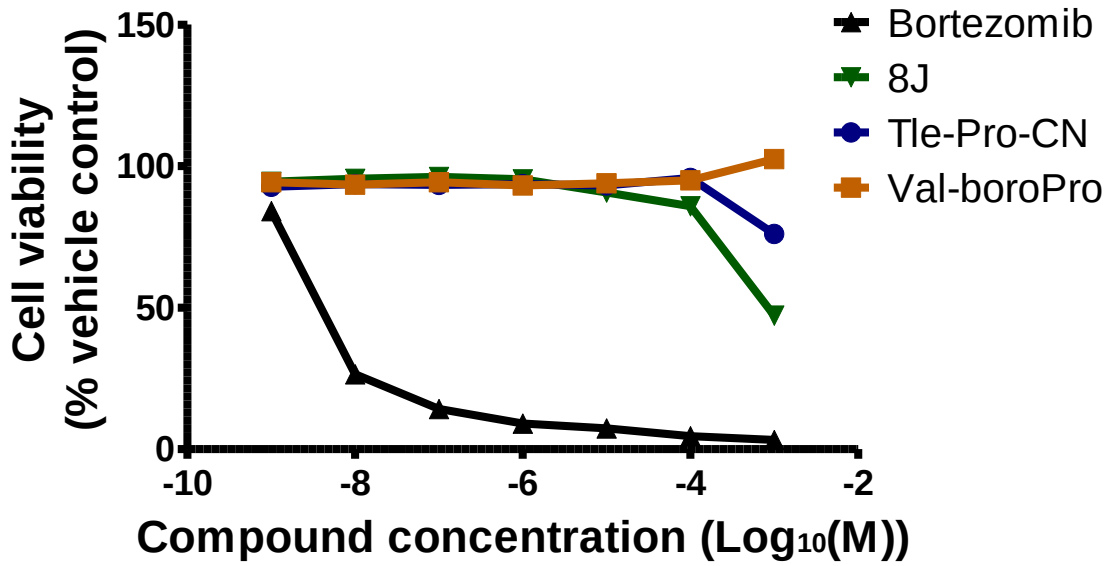


Figure 11. Cytotoxicity of selected DPP8/9 inhibitors. The selected DPP8/9 inhibitors are not directly cytotoxic to cells. CT-26 cells were treated with the indicated compounds at concentrations ranging from 1mM to 1nM. Viability was assessed after 48 hours by addition of fluorescent redox dye (n=3). Bortezomib was used as a positive control. Data expressed as mean ± SEM.

As expected, we found that Val-boroPro potently inhibited CT-26 tumor growth. No tumor responses were observed, however, in mice administered either of the other DPP8/9 inhibitors (Fig. 12A, B). In order to provide supportive evidence for the ability of

the compounds to inhibit the target enzymes *in vivo*, we examined their pharmacokinetics in the plasma of CT26 tumor-bearing mice. We confirmed that the compounds were orally available, and that for at least 6 hours following dosing, their plasma concentrations exceeded the intracellular DPP8/9 IC50's determined in cell culture (Fig. 12C).

DPP8/9 inhibition in the CT-26 tumors was determined *ex vivo* in tumor homogenates utilizing the previously described DPP8/9 inhibitor allo-ile-isoindoline hereafter referred to as compound M [61] (Table 3). This novel method utilizes the substrate H-Gly-Pro-AMC, which is cleaved by DPPIV, DPP8/9 and FAP. In order to isolate the DPP8/9 tumor activity, homogenates were spiked with sitagliptin and 5057, which inhibit DPPIV and FAP respectively. The remaining activity can largely be assigned to DPP8/9, although some residual activity is observed (Fig. 13A). Comparison of this remaining H-Gly-Pro-AMC activity in spiked tumor homogenates from treated mice with or without added compound M can then be used to determine the percentage of DPP8/9 inhibition (Fig. 13B). Using this technique, we demonstrate highly significant inhibition of DPP8/9 in the CT-26 tumors 1-h post-dose with all 3 inhibitors (Fig. 12D). Val-boroPro and 8J demonstrated superior inhibition of DPP8/9 even after 6 hours; but inhibition with Tle-Pro-CN was much shorter lived.

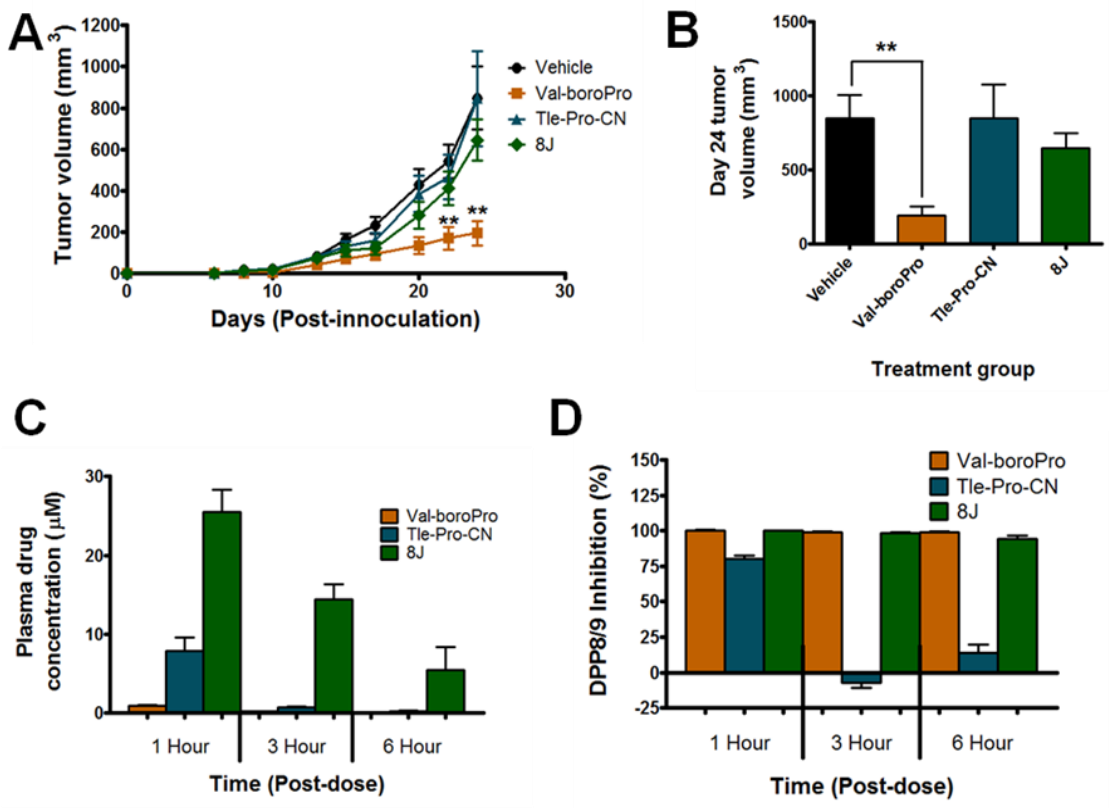


Figure 12. Effect of DPP8/9 inhibitors on tumor growth in the CT-26 model. (A, B) Val-boroPro, but not the other selected inhibitors of DPP8/9 suppress tumor growth. Female BALB/c mice were inoculated with $5x10^5$ CT-26 cells on day 0 followed by initiation of B.I.D. dosing on day 2 (n=15, **P<.01 by ANOVA). **(C)** Drug concentrations in the plasma of tumor-bearing mice exceed those needed for DPP8/9 inhibition in cell culture. Plasma concentrations of DPP8/9 inhibitors were assessed by LC-MS following blood collection in tumor-bearing mice on day 24 (n=3). **(D)** DPP8/9 inhibition in *ex vivo* tumor homogenates is nearly complete over the course of 6 hours with both Val-boroPro and 8J. DPP8/9 inhibition in CT-26 tumor homogenates was assessed by subtracting H-Gly-Pro-AMC activity of homogenate spiked with 1μM 5057 and 1μM sitagliptin from homogenate spiked with 1μM 5057, 1μM sitagliptin and 1μM compound M (n=3). Percent inhibition is relative to vehicle treated controls. All data expressed as mean ± SEM.

Table 3. Inhibition of the PPCEs by Compound M							
	IC50 (nM)						
Compound	DPPIV	FAP	DPPII	PREP	DPP8	DPP9	IC DPP8/9
Compound M	14,000	>100,000	24,000	>100,000	23	30	340

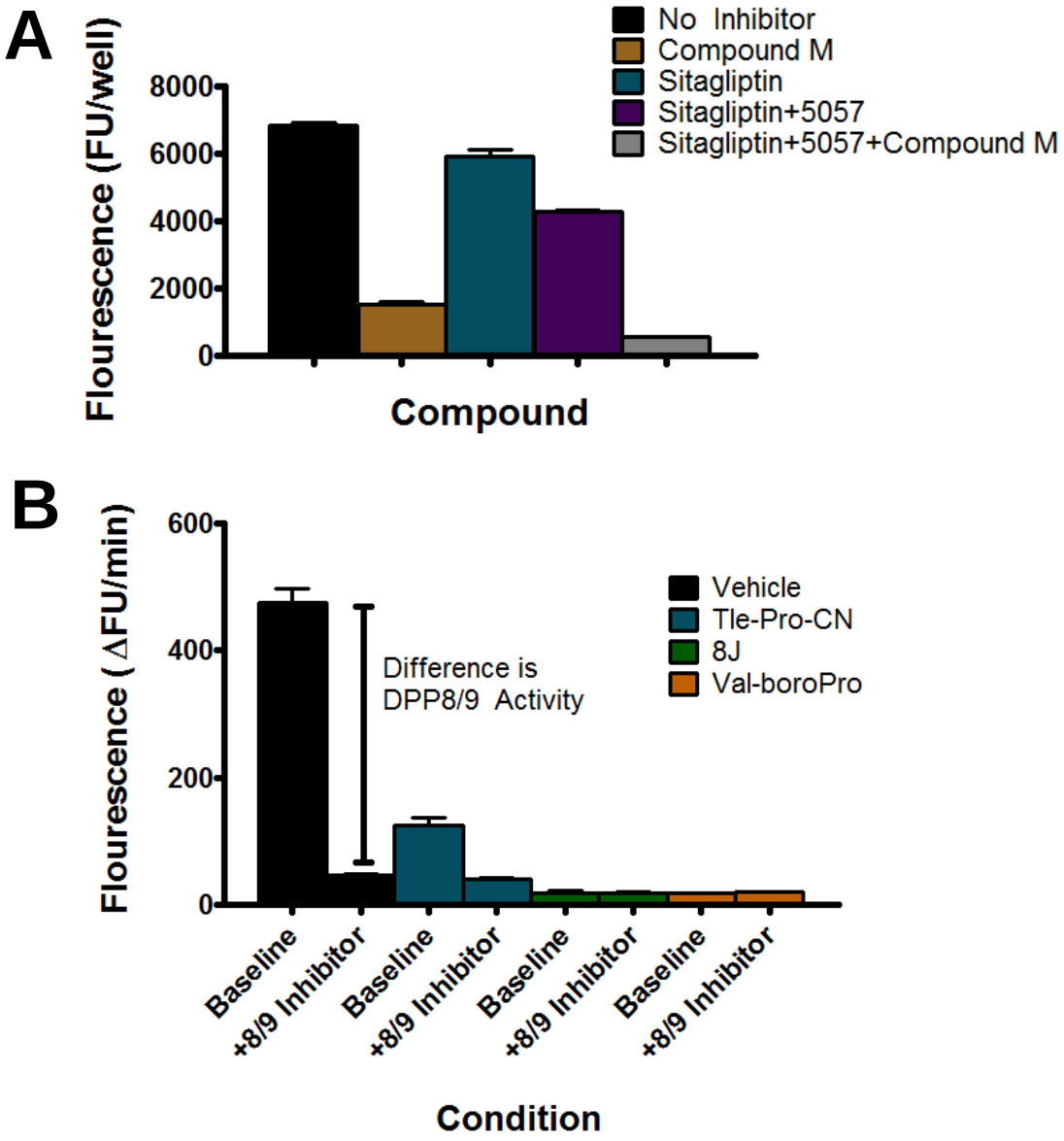


Figure 13. DPP8/9 inhibition assay. (A) The majority of H-Gly-Pro-AMC activity in CT-26 tumors can be assigned to DPP8/9. Vehicle treated tumor homogenates were spiked with the indicated inhibitors to a final concentration of 1µM and activity towards H-Gly-Pro-AMC substrate was assessed (n=3). **(B)** DPP8/9 inhibition assay. Homogenates from tumors harvested 1h post-dose (n=3) were spiked with both 1µM sitagliptin and 5057 (Baseline), or 1µM sitagliptin, 5057 and compound M (+DPP8/9 Inhibitor). DPP8/9 activity is the difference in activity between the Baseline group and the group with compound M (+DPP8/9 Inhibitor). All data expressed as mean ± SEM.

We also found that DPP8/9 inhibitors lack the ability to induce G-CSF, which is thought to be a marker for dipeptide boro-prolines capable of stimulating tumor immunity. This

finding appears consistent with the inability of the DPP8/9 inhibitors to produce tumor responses *in vivo*. In contrast, we found that Val-boroPro, at the relatively low dose of 5-μg, produced a large increase in serum G-CSF concentration (Fig. 14). Taken together, the data indicate that pharmacological inhibition of DPP8/9 is insufficient to induce either a G-CSF or even a marginal tumor response in the CT-26 model.

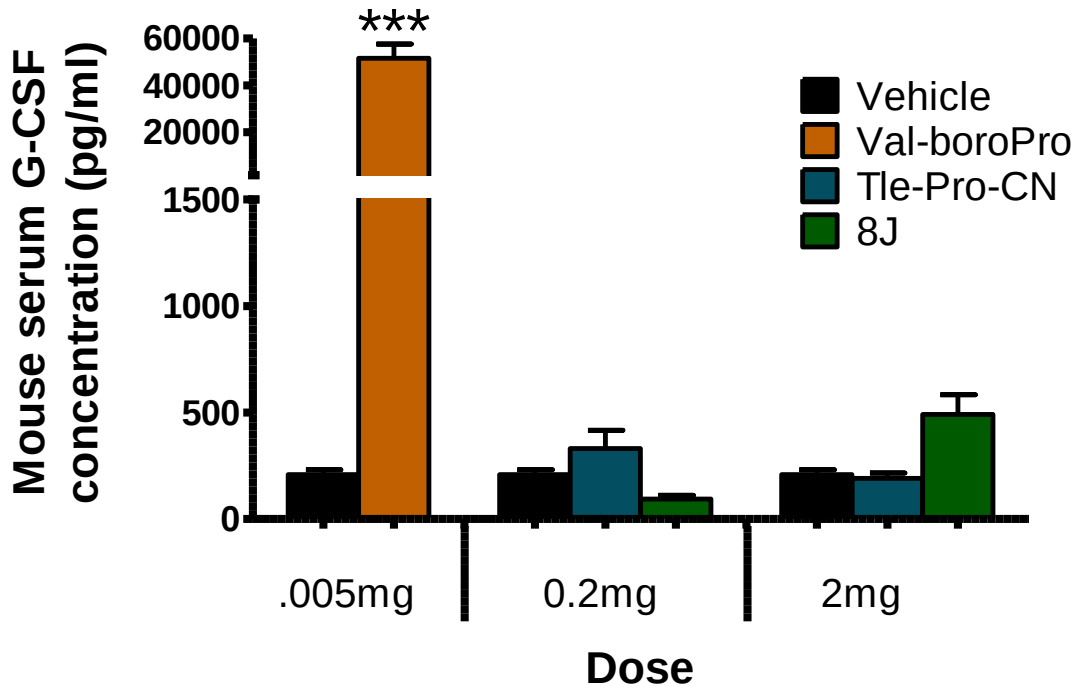


Figure 14. Effect of DPP8/9 inhibitors on mouse serum G-CSF concentrations. Administration of Val-boroPro, but not selected DPP8/9 inhibitors, causes a large increase in serum G-CSF concentrations. Male BALB/c mice were injected subcutaneously with .005, 0.2mg, or 2mg of the indicated compounds dissolved in 100μl of sterile saline. Blood was collected 6h after dosing and serum G-CSF concentration was assessed by ELISA (n=4-5, ***P<.0001 relative to vehicle by *t*-test). All data expressed as mean ± SEM.

3.1.4 Two PREP-Specific Inhibitors Have Opposing Effects on Tumor Growth in the CT-26 Model

PREP inhibition was then investigated as a possible contributory mechanism to the anti-cancer effects of the dipeptide boro-prolines. PREP was recently implicated as an anti-cancer target based on the ability of compound J94, hereafter referred to as 5332, to suppress growth of HCT116 xenografts [243]. Like DPP8/9, PREP is an intracellular enzyme, so the ability of compounds to enter cells, as well as potency, must be taken into account when selecting compounds for study. Cellular permeability was assessed by treatment of HEK293 cells with the selected compounds, followed by addition of the fluorescent substrate Z-Gly-Pro-AMC to measure PREP activity. Because HEK293 cells do not express FAP, the activity of intracellular PREP can be assessed by this technique.

We chose 3 compounds to test the anti-cancer effect of PREP inhibition. The first, compound 3531 was chosen due to its excellent potency and low intracellular IC_{50} values for PREP (Fig.15, Table 4). The second compound chosen was 5332, based on its documented anti-cancer effects in a previous study. In this report, the authors purposely developed compound 5332 to be poorly cell penetrant, based on a hypothesized mechanism for PREP in the regulation of the extracellular angiogenic peptide Ac-SDKP [191]. Our results confirm that this compound is poorly cell penetrant (Table 4). The final compound chosen was Val-boroPro for use as a positive control. Surprisingly, Val-boroPro was also a poor inhibitor of intracellular PREP, despite excellent potency against the recombinant enzyme and previously documented ability to potently inhibit intracellular DPP8/9 under similar assay conditions (Table 2). This suggests that there may be differences between recombinant and cellular PREP. Alternatively, DPP8/9 may be acting as a sink for Val-boroPro that has entered the cell. All of these compounds were

tested side by side for their effects on tumor growth in the CT-26 model. Unlike the previous experiments, once daily I.P. dosing was chosen. This route and frequency was chosen because it had been previously used for testing 5332 in the HCT116 xenograft model. None of these compounds affected the viability of CT-26 cells in culture at concentrations up to 100µM (Fig. 16).

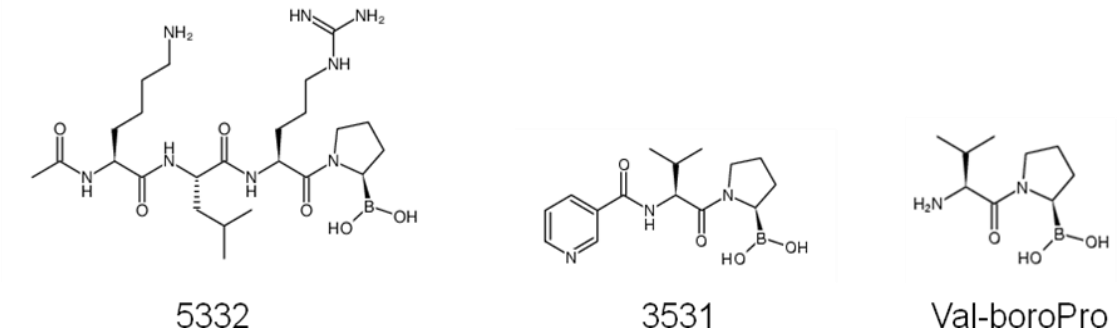


Figure 15. Chemical structures of PREP inhibitors. 5332 Acetyl-L-lysyl-L-leucyl-L-arginyl-L-boroproline, 3531 N-(Pyridine-3-carbonyl)-L-valinyl-L-boroproline, Val-boroPro ([(2R)-1-[(2S)-2-Amino-3-methyl-1-oxobutyl]-2-pyrrolidinyl]boronic acid).

Table 4. Inhibition of the PPCEs by PREP Inhibitors							
	IC50 (nM)						
Compound	DPPIV	FAP	DPPII	PREP	DPP8	DPP9	IC PREP
5332	>100,000	>100,000	21,000	4	27,000	5,500	>100,000
3531	>100,000	>100,000	>100,000	1	>100,000	>100,000	31
Val-boroPro	.8	13	8	56	5	2	15,000

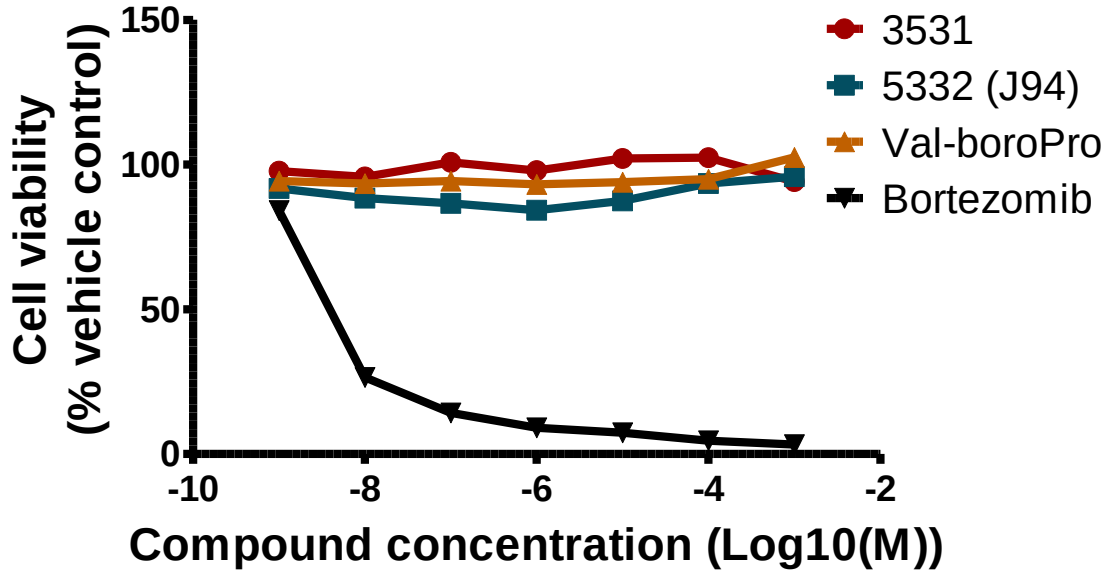


Figure 16. Cytotoxicity of selected PREP inhibitors. The selected PREP inhibitors are not directly cytotoxic to cells. CT-26 cells were treated with the indicated compounds at concentrations ranging from 1mM to 1nM. Viability was assessed after 48 hours by addition of fluorescent redox dye (n=3). Bortezomib was used as a positive control. Data expressed as mean ± SEM.

As expected, we found that Val-boroPro inhibited CT-26 tumor growth. 5332 was also found to suppress tumor growth, confirming that the anti-cancer efficacy of this compound extends to the CT-26 model (Fig. 17A, B). Interestingly, 3531 significantly increased growth of the CT-26 tumors and tumor volumes for mice treated with 3531 were twice as large on average on days 24 and 27 post-inoculation. By day 27, one fifth of mice treated with 3531 had exceeded the maximum allowable tumor size limit of 1,500 mm^3 and had to be sacrificed, which explains the drop off in tumor volumes at later time points.

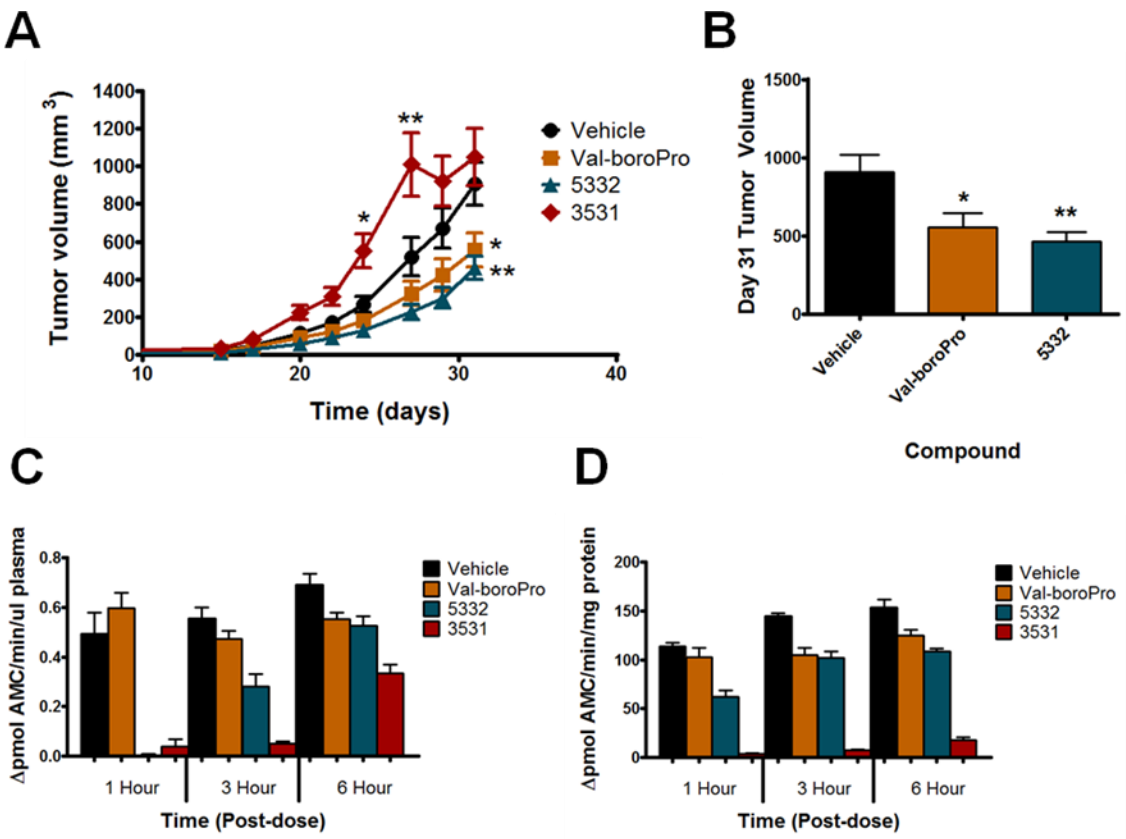


Figure 17. Effect of PREP inhibitors on tumor growth in the CT-26 model. (A, B) Val-boroPro and 5332 slow tumor growth, but 3531 accelerates tumor growth. Female BALB/c mice were inoculated with $5x10^4$ CT-26 cells on day 0 followed by initiation of B.I.D. dosing on day 2 (n=15, *P<.05, **P<.01 by ANOVA). **(C)** 3531 and 5332 inhibit PREP in plasma from tumor-bearing mice spiked with PREP. PREP activity was assessed by Z-Gly-Pro-AMC substrate (n=3). 3531 was significant relative to vehicle at all time points measured (P<.001 by ANOVA). **(D)** 3531 potently suppresses PREP activity in *ex vivo* tumor homogenates. PREP activity in CT-26 tumor homogenates was assessed by Z-Gly-Pro-AMC (n=3-5). 3531 was significant relative to vehicle at all time points measured (P<.001 by ANOVA). All data expressed as mean ± SEM.

In order to provide supportive evidence for the ability of the compounds to inhibit PREP *in vivo* we assessed PREP activity both in plasma and *ex vivo* tumor homogenates using the substrate Z-Gly-Pro-AMC. Though there was detectable Z-Gly-Pro-AMC activity in plasma from control tumor-bearing mice, at least part of this activity was attributable to FAP and the remaining activity, attributable to PREP, was barely detectable and highly

variable, increasing at a rate of only 1 RFU/minute (Fig. 18). Thus, to increase the dynamic range of our assay, we spiked plasma from tumor-bearing mice with recombinant PREP and assessed activity using Z-Gly-Pro-AMC. Using this technique, we demonstrate excellent inhibition of PREP one hour post-dose in plasma derived from mice treated with 5332 and 3531 (Fig. 17C). Inhibition of PREP in plasma was rapidly lost in the 5332 treated group, with PREP activity returning to 60% of controls at three hours and 80% at six hours post-dose. In comparison, 3531 potently inhibited PREP in the plasma at 3 hours and activity remained 50% below that of controls 6 hours after dosing. This result suggests that 3531 is better than 5332 at inhibiting extracellular PREP.

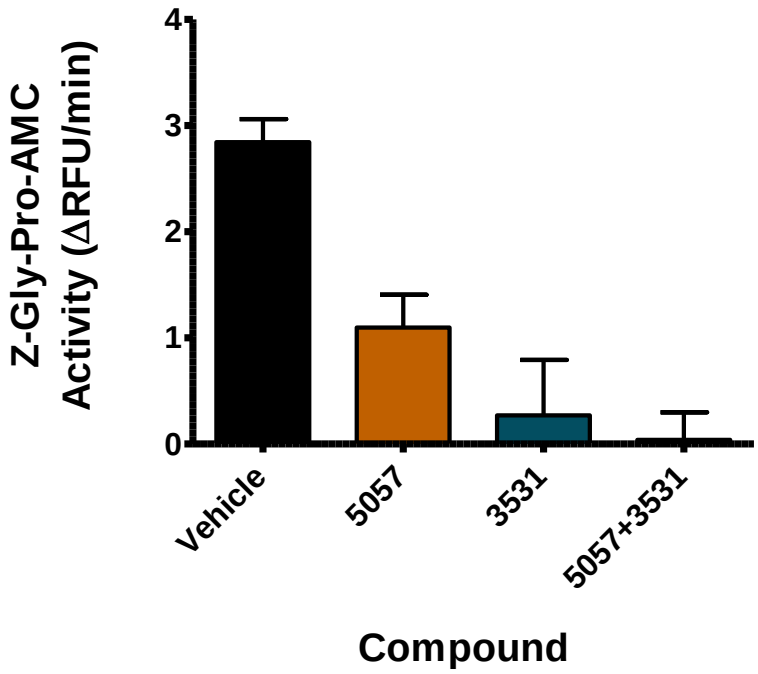


Figure 18. Effect of PREP and FAP inhibitors on plasma Z-Gly-Pro-AMC activity. Mouse plasma Z-Gly-Pro-AMC activity is inhibited by both PREP and FAP inhibitors. Plasma from vehicle treated tumor-bearing mice was spiked with the selected inhibitors to a final concentration of 1μM. Activity was assessed by Z-Gly-Pro-AMC (n=3). All data expressed as mean ± SEM.

PREP activity in *ex-vivo* tumor homogenates was also examined. In contrast to plasma, CT-26 tumors possess ample activity towards Z-Gly-Pro-AMC, which is nearly all attributable to PREP. As expected, mice treated with 3531 had potently suppressed tumoral PREP activity (Fig. 17D). This activity remained 85% below that of vehicle treated control mice even 6 hours after dosing. Consistent with the poor ability of Val-boroPro and 5332 to inhibit intracellular PREP in cell culture, both of these compounds only resulted in marginal inhibition of PREP activity in tumor homogenates. Together these results suggest that inhibition of intracellular PREP enhances CT-26 tumor growth. Conversely, this data suggests intracellular PREP enzymatic activity is important for tumor suppression.

3.1.5 The PREP-Specific Inhibitor 5332 is a Arg-boroPro Prodrug *in vivo*

The fact that 3531 results in accelerated tumor growth, while 5332 suppresses tumor growth is curious given they are both PREP-specific inhibitors. 5332, unlike 3531, is a large pseudopeptide inhibitor with the residues Lys-Leu-Arg flanked by a N-terminal acetyl group and C-terminal boro-proline residue. In our experience, large inhibitors or substrates with amino acid backbones can often be cleaved by proteases *in vivo*. Such proteolysis, if it occurs, may not necessarily be observable in cell culture. Therefore, we hypothesized that 5332 may be cleaved *in vivo*.

Of particular interest with respect to compound 5332 is the potential for release of Arg-boroPro. If this dipeptide boronic acid is released *in vivo,* it could potentially explain the

anti-cancer effects of this compound. Arg-boroPro, like other dipeptide boro-prolines is an excellent DPPIV inhibitor. Therefore, we first examined DPPIV activity in the plasma to look for evidence of Arg-boroPro release (Fig. 19). Plasma DPPIV activity was potently suppressed in the Val-boroPro treated mice as expected. However, the 5332 treated mice also had greatly reduced levels of plasma DPPIV activity. Given that 5332 is not an inhibitor of DPPIV *in vitro*, this data suggests that the parent compound is changed *in vivo*, such that it is now able to inhibit DPPIV.

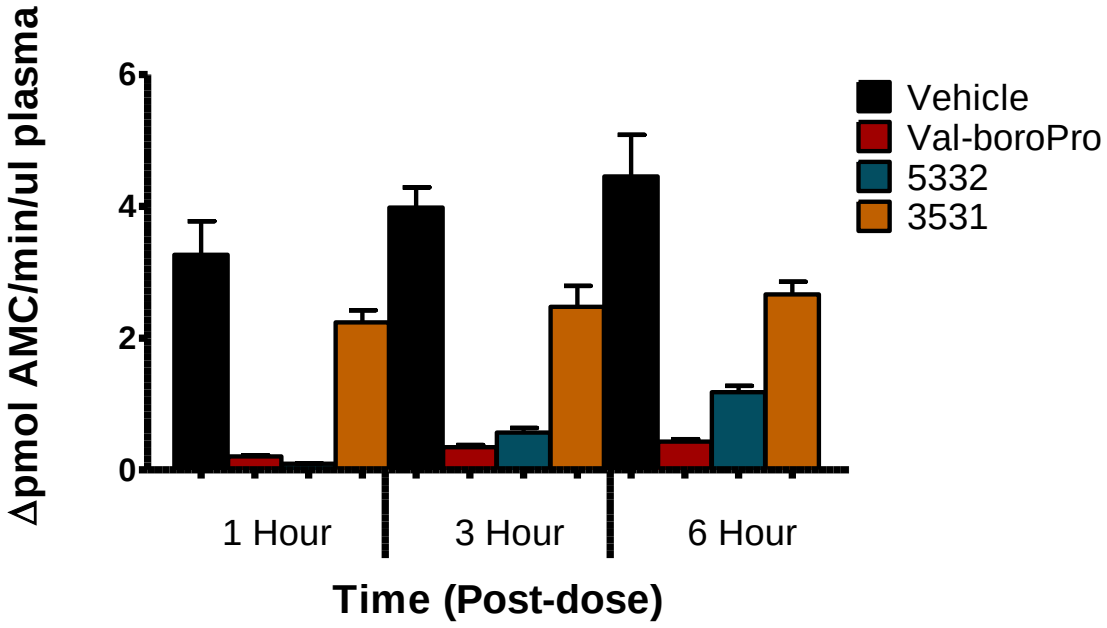


Figure 19. Effect of PREP-specific inhibitors on plasma DPPIV activity *in vivo*. Val-boroPro and 5332 inhibit plasma DPPIV activity *in vivo*. DPPIV activity was assessed in plasma of tumor-bearing mice by Gly-Pro-AMC. Val-boroPro and 5332 were significant relative to vehicle at all time points measured (n=3-5, P<.001 by ANOVA). All data expressed as mean ± SEM.

We then tested whether or not Arg-boroPro is the compound responsible for inhibition of DPPIV in plasma from mice treated with 5332. Using LC-MS, it was determined that Arg-boroPro is rapidly liberated from the parent compound, such that its concentration

exceeded that of 5332 by three fold just one hour after dosing (Fig. 20). Given the documented efficacy of other dipeptide boro-prolines in the CT-26 model, it is likely that the release of Arg-boroPro explains both the anti-cancer activity of this compound and the discrepancy versus 3531 with respect to their effects on tumor growth.

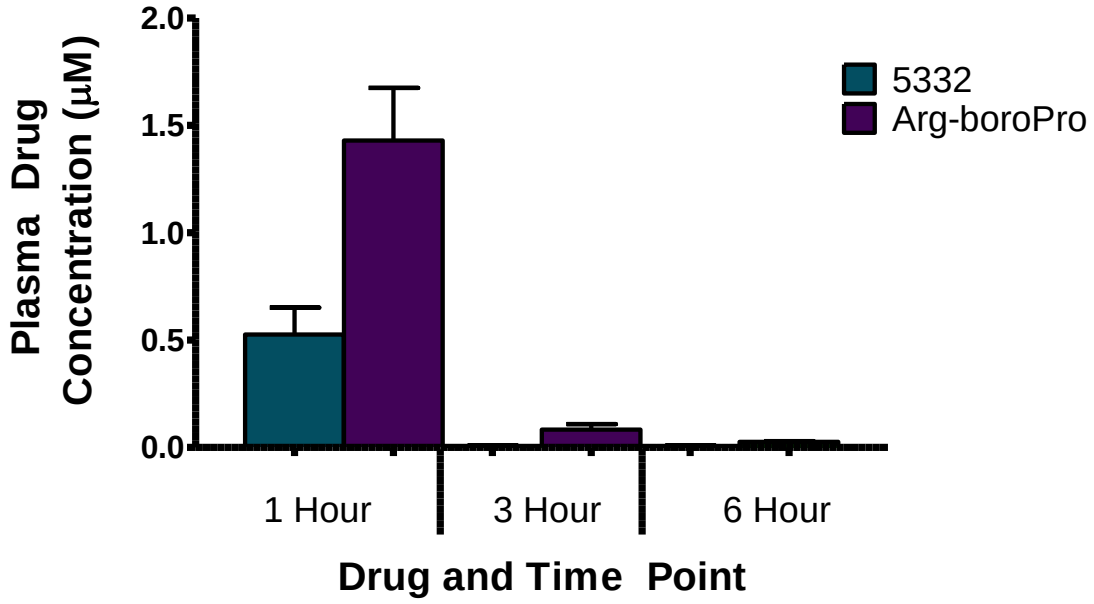


Figure 20. Pharmacokinetics of 5332 and Arg-boroPro in plasma. Arg-boroPro is released from 5332 *in vivo*. Drug concentrations in the plasma of tumor bearing mice were assessed by LC-MS following blood collection in tumor-bearing mice (n=3). All data expressed as mean ± SEM.

3.1.6 5332 has Reduced Toxicity Relative to Val-boroPro Despite Similar Efficacy

Val-boroPro is thought to possess the most potent anti-cancer effect of all the dipeptide boro-prolines. Unfortunately, this efficacy comes with a number of side effects, which likely led to its failure in the clinic. In the CT-26 model, prolonged exposure to Val-boroPro led to a number of adverse effects. Most notably, mice treated with Val-boroPro

exhibited a pronounced abdominal and ventral alopecia, thought to be a result of immune activation (Fig. 21A). Additionally, mice treated once a day with 10µg of Val-boroPro I.P. gained 10% less weight over the course of the experiment compared to vehicle-treated mice (Fig. 21B). While some of that reduced weight gain can be attributed to smaller tumor sizes, much can also be attributed to the toxicity of the compound. Val-boroPro administration resulted in noticeable lethargy and behavioral changes in the mice such as reduced tearing of available bedding. In contrast, mice treated with 75µg of 5332 I.P. exhibited no alopecia, lethargy or behavioral changes (Fig. 21C). Weight gain of 5332 treated mice was only 3% less than that of vehicle-treated mice and much, if not all, of that difference can be attributed to the larger tumors in the vehicle group. Despite the vast differences in toxicities, the anti-tumor effect of 5332 was as good, if not better, than Val-boroPro (Fig. 17A, B).

While a full characterization of the cytokine inducing capabilities of 5332 and 3531 have not yet been performed, our preliminary data suggests that compound 3531 is incapable of stimulating G-CSF in mice. In contrast, compound 5332 is capable of increasing serum G-CSF, albeit to levels well below that of Val-boroPro at equivalent dosages. The ability of 5332 to elevate serum G-CSF provides further evidence that the anti-cancer effects of this compound are likely due to Arg-boroPro release.

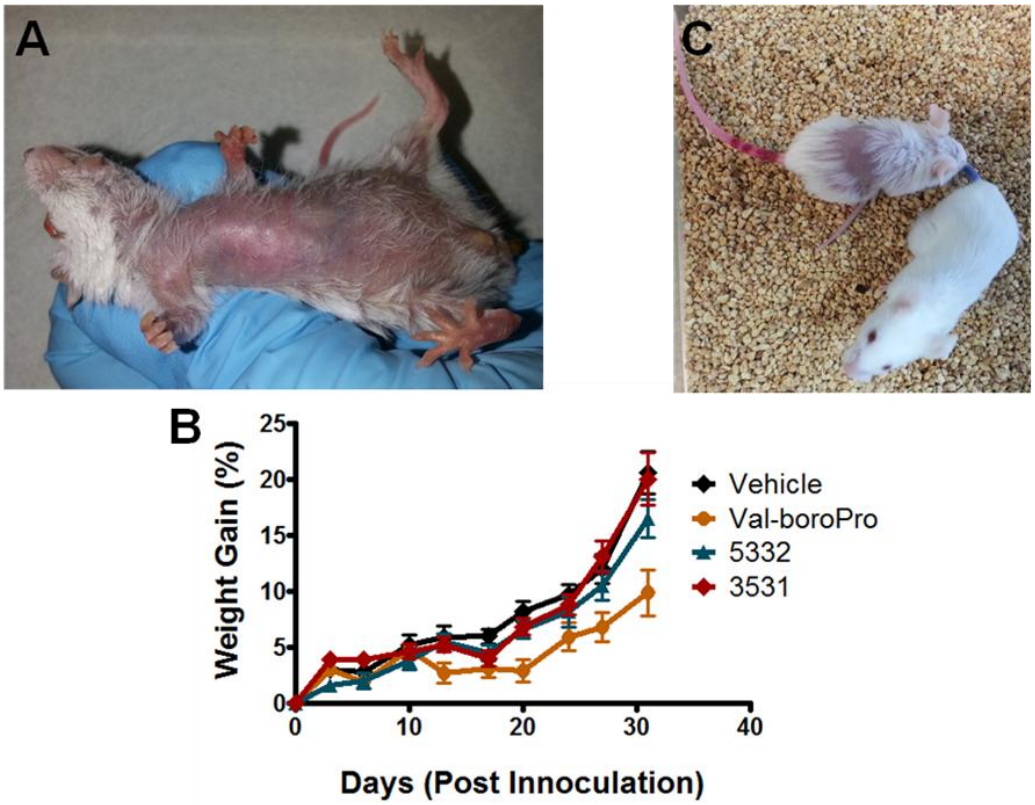


Figure 21. Adverse effects of Val-boroPro versus 5332. Toxicity of Val-boroPro is greater than that of 5332 at the chosen dosages. **(A)** Val-boroPro results in abdominal alopecia. Mouse pictured is representative of mice treated with 20µg of Val-boroPro B.I.D. on day 24. (**B)** Mice treated with 10µg Val-boroPro I.P. once daily have reduced weight gain relative to vehicle-treated mice, but 5332-treated mice do not (n=15). Data expressed as mean ± SEM. (**C**) 5332 treated-mice do not exhibit alopecia. Mice pictured are representative of mice treated with 10µg Val-boroPro I.P. once daily on day 31 (Top, red tail mark) and mice treated with 75µg 5332 I.P. once daily on day 31 (Bottom, blue tail mark).

3.2 Investigation of the Role of FAP in Diabetes and Obesity

3.2.1 FAP Activity is Upregulated in the White Adipose Tissue of DIO Mice

The reported diabetes and obesity resistant phenotype of the FAP KO mouse led us to investigate the function of FAP in these processes. Given that the FAP KO mouse is only phenotypically different from WT mice upon high-fat feeding, it was hypothesized that FAP or its relevant substrate may be upregulated in the obese state. Such an upregulation might explain why phenotypic differences between the FAP and WT mice can only be observed when the animals are fed a high-fat diet.

To test this hypothesis, FAP activity was compared in the tissue and plasma of obese and lean mice. Many mouse models of obesity and diabetes exist, the majority of which rely on genetic mutations to drive the obesity process. Rapid onset models of obesity include the db/db mouse and ob/ob mouse that are compelled to eat by inactivating mutations in the leptin receptor gene and leptin gene respectively [81, 199]. Late-onset genetic models of obesity include the tubby and agouti mouse [95, 222]. However, we chose to utilize the same C57BL/6 diet-induced obese (DIO) model used in the FAP KO mouse study by Gorrell et. al [180]. In the C57BL/6 DIO model, male mice on a high-fat diet become obese and develop a phenotype similar to human type II diabetics characterized by elevated fasting blood glucose, increased serum insulin and decreased glucose tolerance [52, 53, 124]. Utilization of the same C57BL/6 DIO model used in the FAP KO mouse study by Gorrell et. al. allows us to study the distribution of FAP activity in a model where phenotypic differences between WT and FAP KO mice have already been established.

FAP activity in the plasma and tissues of lean and DIO mice were examined using the FAP-specific substrate ARI-3144. Prior to sacrifice, lean mice were maintained on a

chow diet with 10% of calories represented by fat (10kcal% fat), while DIO mice were maintained on a high-fat diet consisting of 60kcal% fat. Plasma FAP activity levels were not significantly different between lean and diet-induced obese mice, although the measurements for the obese animals exhibited more variance (Fig. 22A). A panel of tissues from lean and DIO mice were also examined for FAP activity and only epididymal adipose tissue was significantly upregulated in the DIO mice versus lean controls (Fig. 22B). This upregulation was approximately three fold. A similar increase in adipose FAP activity was also observed in a second group of DIO mice compared to lean controls (Fig. 22C). Based on this result, we hypothesized that FAP upregulation in the adipose tissue might contribute to the development of diabetes and obesity induced by high-fat feeding. In particular, it is tempting to speculate that FAP may regulate adipokines or other bioactive peptides produced by, or directed to, the adipose tissue via proteolysis.

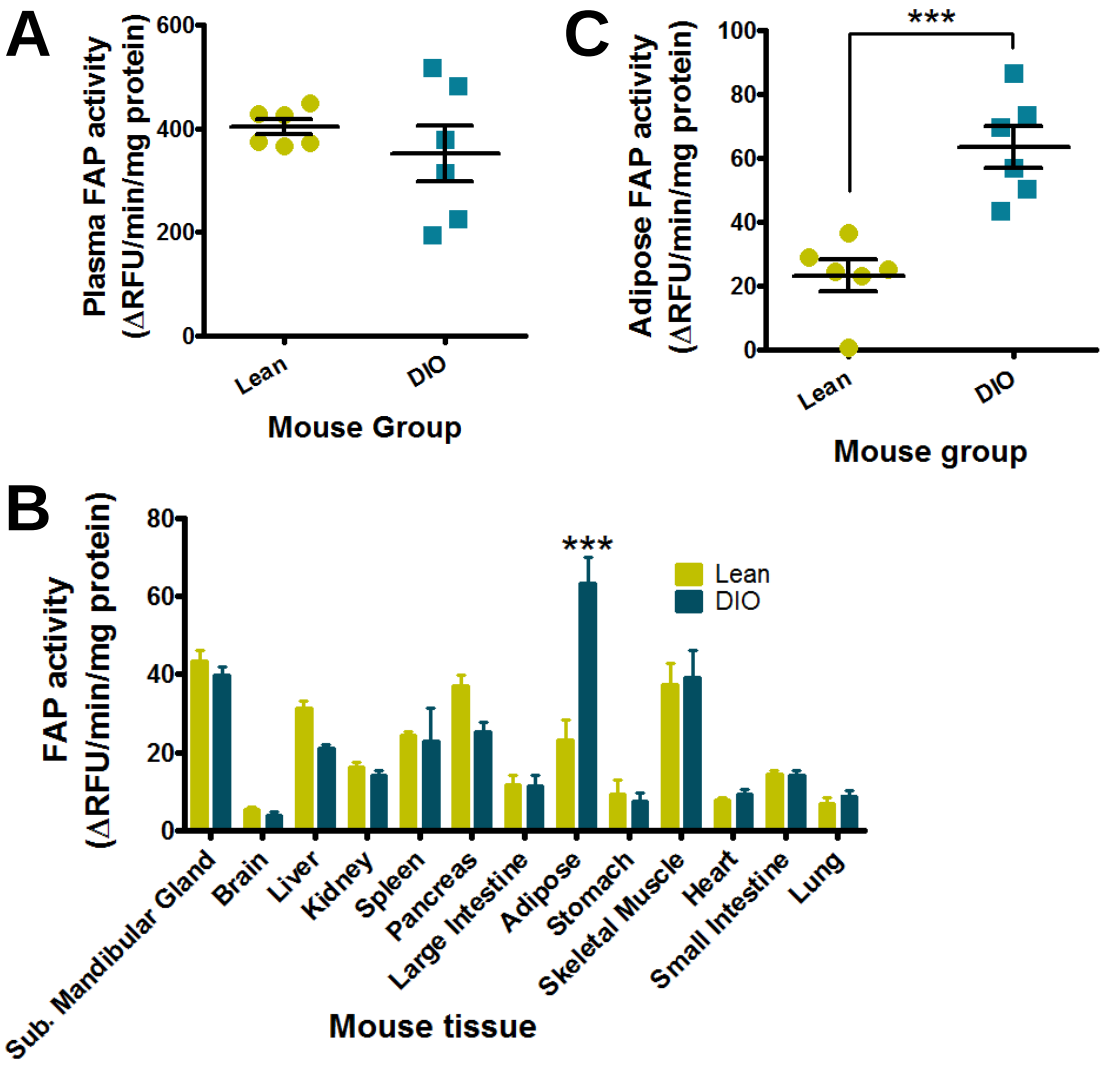


Figure 22. Comparison of FAP activity in lean and DIO mice. (A) Plasma FAP activity is not significantly different between lean and DIO mice. Blood was collected from mice at 23 weeks of age (17 weeks of HFD or chow diet) and plasma FAP activity was assessed by ARI-3144 (n=6). (**B)** FAP activity is upregulated in the white adipose tissue of DIO mice. FAP activity was assessed *ex vivo* in tissue homogenates using ARI-3144 (n=6, ***P<.001 by ANOVA). (**C**) FAP activity is upregulated in the adipose tissue of a second independent set of mice (n=6, ***P<.001 by *t*-test). All data expressed as mean ± SEM.

3.2.2 FAP Cleaves Metabolically Active Peptides and Proteins

With the knowledge that FAP is upregulated in the adipose tissue of DIO mice, we then investigated potential substrates of FAP. FAP possesses both dipeptidyl peptidase activity and endopeptidase activity. The dipeptidyl peptidase activity of FAP is shared by DPPIV, also an extracellular enzyme that is much more ubiquitously expressed than FAP [226]. The dipeptidyl peptidase substrate specificity of FAP completely overlaps with that of DPPIV, so that no described FAP exopeptidase substrate is not also a DPPIV substrate. However, the reverse is not true because DPPIV is capable of cleaving peptides that FAP cannot [179]. In addition, DPPIV also appears to be a superior dipeptidyl peptidase compared to FAP [237]. Combined, these facts suggest that the net contribution of DPPIV to proteolysis of dipeptidyl peptidase substrates is likely to dwarf that of FAP, although locally important effects on exopeptidase substrates cannot be ruled out.

Where FAP enzymatic activity differs from DPPIV is in its ability to cleave endopeptidase substrates [195]. FAP endopeptidase activity has a strict requirement for glycine in P2 followed by proline in P1. FAP appears to prefer endopeptidase substrates as it more readily hydrolyzes the fluorescent substrate Ac-Gly-Pro-AMC than the same substrate without N-terminal acetylation [179, 237]. Therefore, we attempted to identify substrates of FAP, with a particular focus on endopeptidase substrates.

Candidate substrates for FAP were first determined by literature search for metabolically active peptides or proteins. Potential exopeptidase substrates were selected base on the presence of an N-terminal penultimate proline or alanine residue. Potential endopeptidase substrates were identified by the presence of an FAP consensus Xaa-G-P-Xaa sequence near the N or C-termini. The search for FAP endopeptidase consensus sequences was

focused on the termini of larger proteins based on the fact that access to the FAP active site is restricted to less than 24 angstroms by the interface of the propeller and hydrolase domains [237]. Therefore, FAP is unlikely to be able to cleave areas of peptides or proteins with significant secondary or tertiary structure. Evidence for this can be seen with the FAP substrate α2AP, which contains four Xaa-G-P-Xaa sequences, yet FAP is only able to cleave at the consensus site 12 amino acids from the N-terminus in a region predicted to be disordered [181]. Substrates fitting these criteria were synthesized or purchased to test for FAP hydrolysis and four new substrates for FAP were discovered.

Table 5. Primary Sequences and Cleavage Sites of Novel FAP Substrates

Peptide or Protein	**Amino Acid Sequence**
Enterostatin	APGPR
Leptin (22-56)	VP|IQKVQDDTKTLIKTIVTRINDISHTQSVSSKQK
Human FGF-21	MHPIPDSSPL LQFGGQVRQR YLYTDDAQQT EAHLEIREDG TVGGAADQSP ESLLQLKALK PGVIQILGVK TSRFLCQRPD GALYGSLHFD PEACSFRELL LEDGYNVYQS EAHGLPLHLP GNKSPHRDPA PRGPARFLPL PGLPPALPEP PGILAPQPPD VGSSDPLSMV GP|SQGRSPSY AS
Apelin-13	QRPRLSHKGP|MPF
Pyr1-Apelin-13	(Pyr)-RPRLSHKGP|MPF

*FAP cleavage sites denoted by red bar (|)

Table 6. Cleavage of Novel FAP Substrates								
	Intact Peptide or Protein				**Cleaved Peptide or Protein**			
Peptide or Protein	Predicted MW (g/mol)	Observed Major MS Ion	MW of Major MS Ion (g/mol)	Observed MW (g/mol)	Predicted Peptide MW (g/mol)	MS Ion	MW of MS Ion (g/mol)	Predicted MW of MS Ion (g/mol)
Enterostatin	496.6	+1	497.3	496.3	N/A	N/A	N/A	N/A
Leptin (22-56)	3950.6	+4	988.5	3950.0	3754.3	+4	939.4	939.7
FGF-21	19542.3	+11	1777.3	19539.3	18521.2	+11	1685.4	1684.5
Apelin-13	1550.86	+1	1551.2	1550.2	1175.4	+1	1175.5	1175.8
Pyr1-Apelin-13	1533.8	+2	767.4	1532.8	1158.3	+2	579.6	579.7
*Column shaded in grey indicates observed MW of the MS ion from the cleaved peptide								

3.2.2.1 Novel FAP Substrate: Enterostatin

Enterostatin incubated with FAP was rapidly digested as assessed by LC-MS (Table 5 and 6). Although the MS peak corresponding to the intact peptide disappeared when FAP was added, the corresponding cleavage products were likely too small and hydrophilic to be detected by our instruments under the reaction conditions or were during washing of the column. Nevertheless, it is reasonable to assume enterostatin is cut after both proline residues given the small and disordered nature of this peptide.

If enterostatin is a substrate of FAP *in vivo*, increasing levels of intact enterostatin-1, through inhibition of FAP or genetic deletion, could potentially reduce food intake and increase energy expenditure. However, it is unlikely that enterostatin is a significant substrate of FAP or can explain the differences observed between WT and FAP KO mice. We have consistently found very low levels of FAP in the gut including the large and

small intestines where enterostatin is produced (Fig. 22). In addition, enterostatin is also cleaved by DPPIV, an enzyme known to be present at very high concentrations both in the gut and circulation [161]. Thus if proteolysis is a regulatory component of enterostatin-1 signaling, DPPIV is more likely to be the critical protease.

3.2.2.2 Novel FAP Substrate: Leptin

Leptin is a secreted 167 amino acid protein with an N-terminal sequence of valine followed by proline. This sequence is potentially susceptible to FAP exopeptidase activity. However, proteins of this size cannot be synthesized chemically, so they are typically produced in *E. coli*. As a result the N-terminal methionine, required for production in *E. coli*., is not removed. FAP and DPPIV do not have tripeptidyl peptidase activity, so this N-terminal methionine prevents evaluation of full-length leptin as a substrate for FAP. However, we were able to obtain a chemically synthesized N-terminal leptin peptide consisting of residues 22-56 (leptin 22-56). These residues encompass the first of four anti-parallel alpha helices present in full length leptin. When incubated with FAP, the N-terminal Val-Pro sequence of this leptin peptide was removed (Table 5 and 6).

It is important to note that FAP cleavage of this truncated form of leptin does not mean FAP is capable of cleaving the full length protein, especially given its stacked alpha helical structure, which may prevent the N-terminal residues from entrance into the active site of FAP [44]. Additionally, while leptin is an adipokine, it is unlikely that FAP cleavage of leptin could explain the difference in phenotype between wild-type and FAP KO mice.

Leptin's primary function is to regulate and promote satiety; however satiety appears to be unaffected in FAP KO mice relative to WT mice, except for a small decrease in food consumption when on a high-fat diet [180].

Additionally, removal of the two N-terminal amino acids is not predicted to have any effect on leptin signaling to its receptor, as these amino acids are not thought to be at the interface of this interaction [155]. Finally, like enterostatin, DPPIV can also cleave leptin (22-56), potentially overwhelming any contribution of FAP to proteolysis *in vivo*. Combined, this evidence suggests that leptin is not likely to be a biologically relevant substrate of FAP.

3.2.2.3 Novel FAP Substrate: FGF-21

Human FGF-21 is a candidate substrate for FAP based on the presence of Xaa-Gly-Pro-Xaa sequence near the C-terminus of this ~20kDa protein. We first tested if FAP could hydrolyze FGF-21 *in vitro* and found that FGF-21 incubated with FAP resulted in a truncated version of FGF-21 with an approximate shift of 1kDa in a reducing SDS-Page gel (Fig. 23A). Cleavage of FGF-21 by FAP after proline 171 was demonstrated by LC-MS and this truncation could be prevented by addition of the FAP-specific inhibitor 3099 (Fig. 23B, Table 5). The other PPCE with endopeptidase activity, PREP, failed to cleave FGF-21 even after 24 hours of incubation (Fig. 23C)

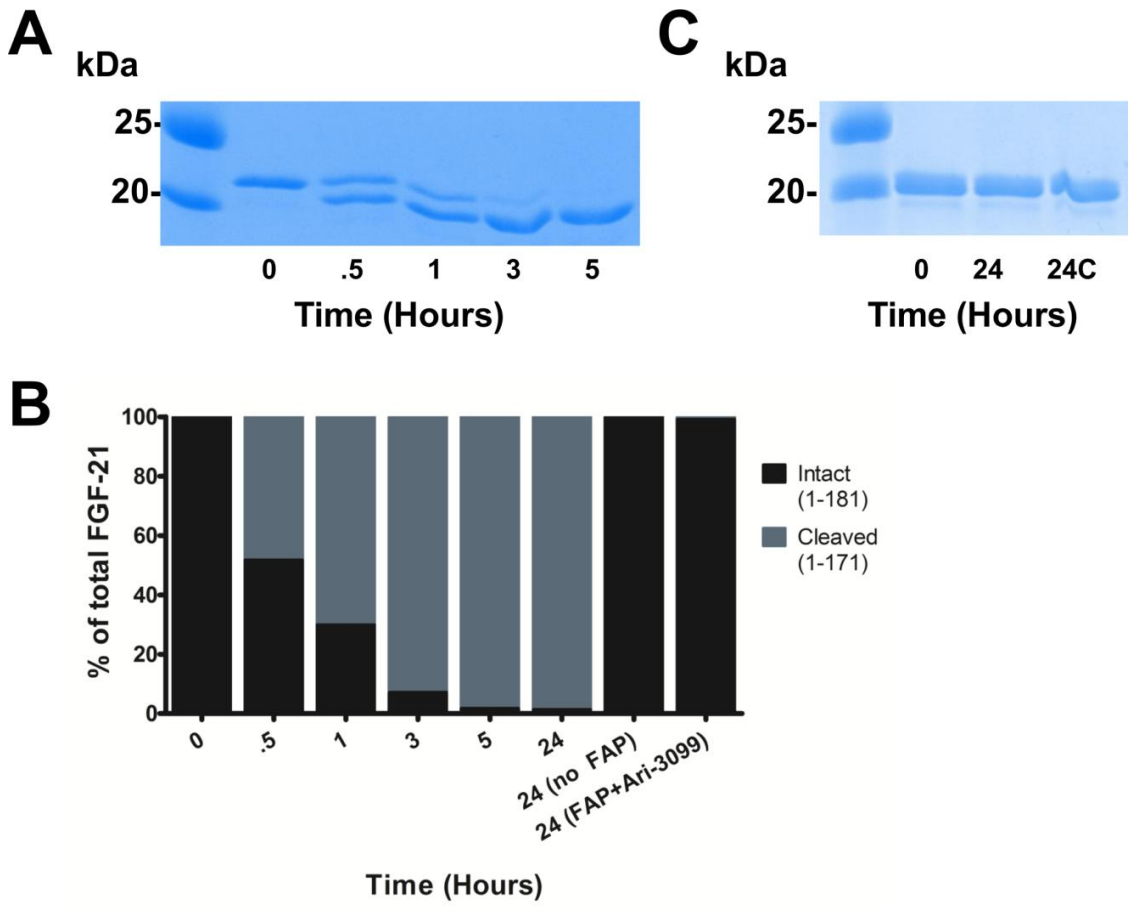


Figure 23. FGF-21 digestion by FAP. (A) Human FGF-21 is cleaved by FAP. Recombinant FGF-21 was digested by FAP and visualized by Coomassie staining of SDS-Page gel. **(B)** Time course of FGF-21 digestion by FAP quantified by LC/MS extracted ion integration **(C)** PREP does not cleave human FGF-21. PREP was added to recombinant FGF-21 and visualized by coomassie staining of SDS-Page gel.

Although the human form of FGF-21 is cleaved by FAP following proline 171, the mouse form is not (Fig. 24). This is likely due to the presence of a glutamic acid residue in the P2 position of the mouse protein in place of glycine in the human form, which prevents cleavage by FAP. Because FAP cannot cleave mouse FGF-21, this substrate is not responsible for any aspect of the obesity resistant phenotype of the FAP KO mouse and suggests that any biological relevance FAP cleavage of FGF-21 will be unique to humans.

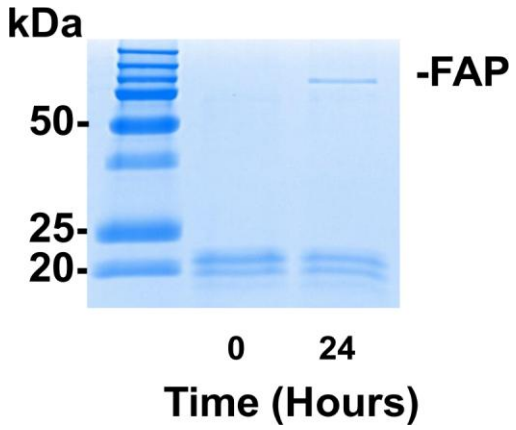


Figure 24. Mouse FGF-21 is not cleaved by FAP. 20µM FGF-21 was incubated with or without 200nM FAP at 37 ºC for 24 hours and visualized by coomassie staining of SDS-Page gel.

We next investigated if FGF-21 is cleaved by FAP in plasma. FGF-21 was added to mouse, monkey and human plasma and levels of intact FGF-21 were assessed by ELISA. This sandwich ELISA has antibodies directed to the N and C-termini of FGF-21 and does not recognize FAP cleaved FGF-21 (Fig. 25). FGF-21 was cleaved in the plasma of all three species and this could prevented by the addition of 3099 (Fig. 26A). Additionally, the degree of FGF-21 cleavage correlated with the relative FAP activity in plasma from each species (Fig. 26B).

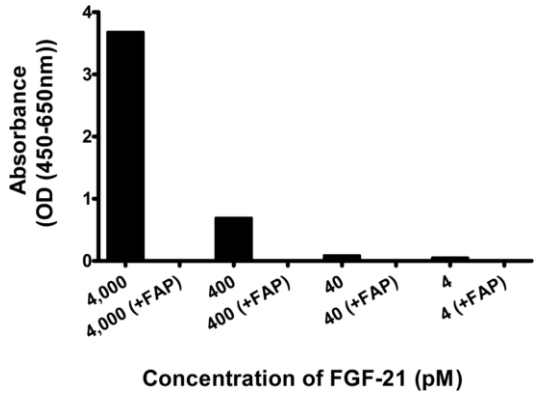


Figure 25. Intact FGF-21 ELISA does not recognize FAP cleaved FGF-21. 10µM FGF-21 was incubated with or without 3µM FAP at 37 ºC for 3 hours and then assayed for intact FGF-21 by sandwich ELISA.

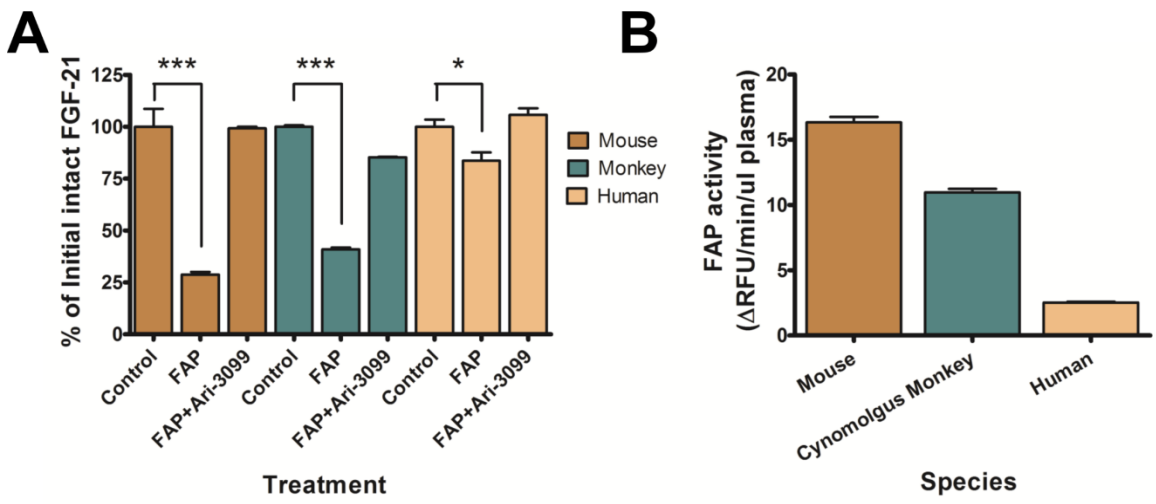


Figure 26. Effect of FAP inhibition on FGF-21 digestion in plasma. (A) FAP cleaves human FGF-21 in mouse, monkey and human plasma. Recombinant FGF-21 was added to plasma to a final concentration of 1µM in the presence or absence of 16µM 3099 followed by assessment of intact FGF-21 by sandwich ELISA (n=3 per group, *P<.05 ***P<.001 by ANOVA). **(B)** Relative plasma FAP activity between species as assessed by the FAP-specific fluorescent substrate ARI-3144. All data expressed as mean ± SEM. Finally, the potential for FAP to regulate levels of intact FGF-21 *in vivo* was examined.

FGF-21 is primarily thought to be cleared from the circulation by kidney filtration, however previous work on long-lived FGF-21 analogs suggest that proteolysis may also contribute to the short half-life of FGF-21 [213, 229]. To test the role of FAP in regulating intact FGF-21 levels *in vivo*, mice were administered vehicle or the FAP inhibitor 3099 followed by injection with human FGF-21. Pre-treatment with 3099 prolonged the half-life of intact human FGF-21 from 48 to 79 minutes, demonstrating that proteolysis by FAP contributes to clearance of FGF-21 in mice, at least at pharmacological doses (Fig. 27).

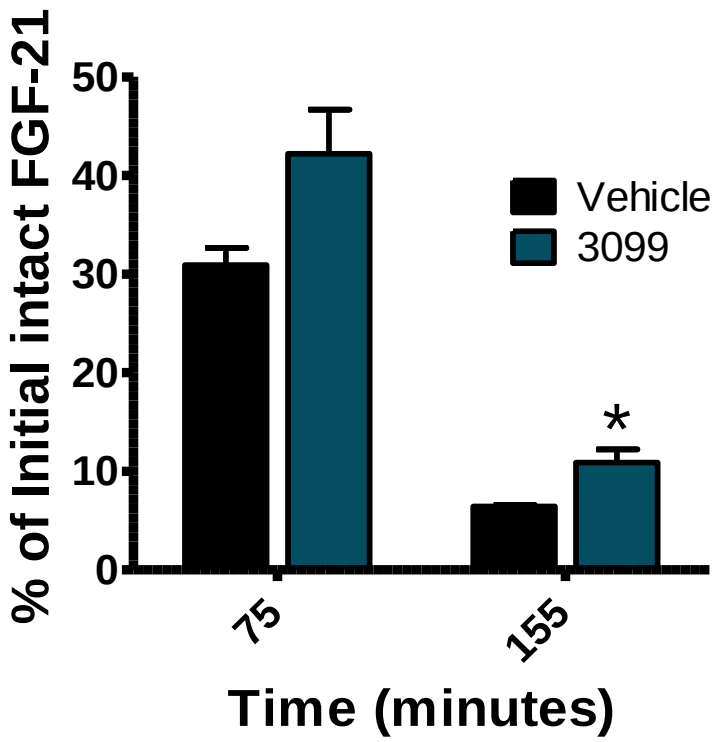


Figure 27. Inhibition of FAP prolongs the half-life of human FGF-21 in mice. Mice were pre-treated with vehicle or 50mpk 3099 followed by injection of 0.5mpk human FGF-21 (n=4 per group, *P<.05 by *t*-test). Plasma samples were assessed for intact FGF-21 concentrations by sandwich ELISA. Values are mean ± SEM.

3.2.2.4 Novel FAP Substrate: Apelin

The primary sequence of apelin has a number of Xaa-G-P-Xaa FAP consensus sites therefore, we hypothesized that this peptide would be cleaved by FAP. A number of different isoforms of the apelin hormone are reported to exist in humans, the largest of which is apelin-36 [32]. However the shorter isoforms, apelin-13 and Pyr1-apelin-13, are thought to be the major forms of apelin in circulation. Unlike FGF-21, the FAP consensus site in apelin-13 is conserved between mice and humans. Apelin reportedly lacks significant secondary structure suggesting that this peptide may be accessible to the

active site of FAP [236]. As expected, apelin-13 and Pyr1-apelin-13 were cleaved by FAP after proline 10 (Fig. 28, Table 5 and 6).

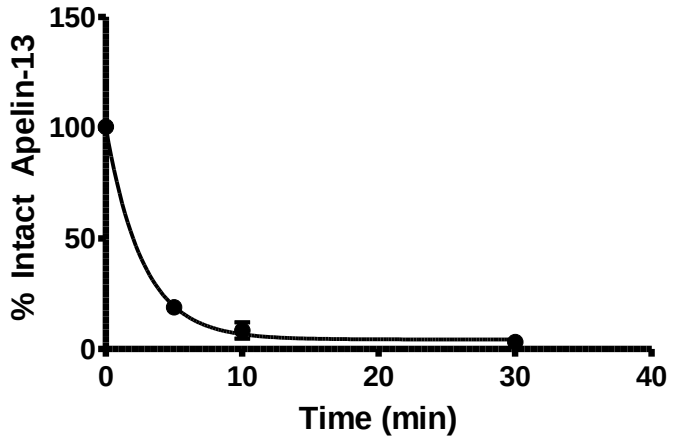


Figure 28. Apelin-13 digestion with FAP. Apelin-13 at 20µM was added to 200nM FAP. Percentage of intact apelin-13 was determined by LC/MS extracted ion integration (n=3 per time point). Values are mean ± SEM.

Studies of apelin agonism of the APJ receptor have demonstrated that the C-terminal section of Apelin-13 is important for receptor activation and these residues are highly conserved [18]. Particularly critical is the C-terminal phenylalanine, a residue that is removed by FAP cleavage [165]. Our preliminary data confirms previous findings that removal of the C-terminal residues of apelin-13 results in significantly reduced potency at the APJ receptor [165]. Therefore, FAP cleavage of apelin-13 is likely to be functionally significant.

However, another protease, angiotensin-converting enzyme-related carboxypeptidase (ACE2), also removes this N-terminal phenylalanine in a proteolytic event thought to be partially responsible for the exceptionally short half-life of apelin in circulation [130]. Yet a recent study with Pyr1-apelin-13, demonstrated that cleavage at the FAP endopeptidase

consensus site after proline-10 was in fact, the half-life limiting proteolytic event in rats [230]. As FAP is the only known extracellular PPCE possessing endopeptidase activity, this cleavage event is almost assuredly a result of FAP proteolysis. This suggests that FAP may regulate the degradation of apelin *in vivo*.

The profile of apelin makes it an attractive candidate for a biologically relevant substrate of FAP. Increased apelin signaling in the FAP KO mouse, resulting from a lack of FAP proteolytic activity, could potentially explain much of the observed obesity resistant phenotype. Additionally, apelin upregulation as a consequence of obesity suggests that it may play a more important metabolic role in obese rather than lean individuals, perhaps supporting insulin to reduce blood sugar. Thus differences between the WT and FAP KO mice may only be revealed upon high-fat diet feeding when insulin sensitivity is compromised.

3.2.3 Pharmacologic Inhibition of FAP Does Not Recapitulate the Diabetes and Obesity Resistant Phenotype of the FAP KO Mouse

3.2.3.1 Long-lasting inhibition of FAP can be Achieved in Mouse Plasma and Adipose

The diabetes and obesity resistant phenotype of the FAP KO mouse and the discovery of novel FAP substrates related to metabolism led us to investigate FAP inhibition as a potential therapeutic strategy for diabetes and obesity. Interrogation of the role of FAP enzymatic activity has thus far been limited by a lack of specific pharmacological inhibitors. However, two excellent FAP inhibitors have recently been described [91, 135].

Although these inhibitors are potent at inhibiting FAP *in vitro*, their *in vivo* pharmacodynamics have not yet been determined (Table 7).

Table 7. Inhibition of the PPCEs by FAP-Specific Inhibitors

	Enzyme IC50's (nM)					
Compound	DPPIV	FAP	DPPII	PREP	DPP8	DPP9
3099	>100,000	32	>100,000	2,100	6,400	2,100
5057	10,600	.8	>100,000	640	4,300	1,475

To identify which of these compounds would be amenable to examining the effect of pharmacologic inhibition of FAP on the diet induced obesity process, we first gauged the ability of these compounds to inhibit plasma FAP *in vivo*. Both compounds were dosed via oral gavage and FAP activity in the plasma was assessed by the FAP-specific substrate ARI-3144. The first compound tested, 3099, inhibited plasma FAP only transiently at both 20 and 80mpk, with activity completely returning to baseline levels 24 hours after dosing (Fig. 29A). In comparison, 5057 potently inhibits FAP enzymatic activity at both doses tested. With 5057, plasma levels of FAP are suppressed by at least 75% even 24 hours after dosing. Although the 80mpk dose provides slightly better inhibition of FAP, the amount of compound required to do long term experiments at this dosage level was prohibitive. Therefore, the 20mpk dose was chosen for future experiments. Importantly, 5057 at a 20mpk dose does not result in any inhibition of plasma DPPIV (Fig. 29B)

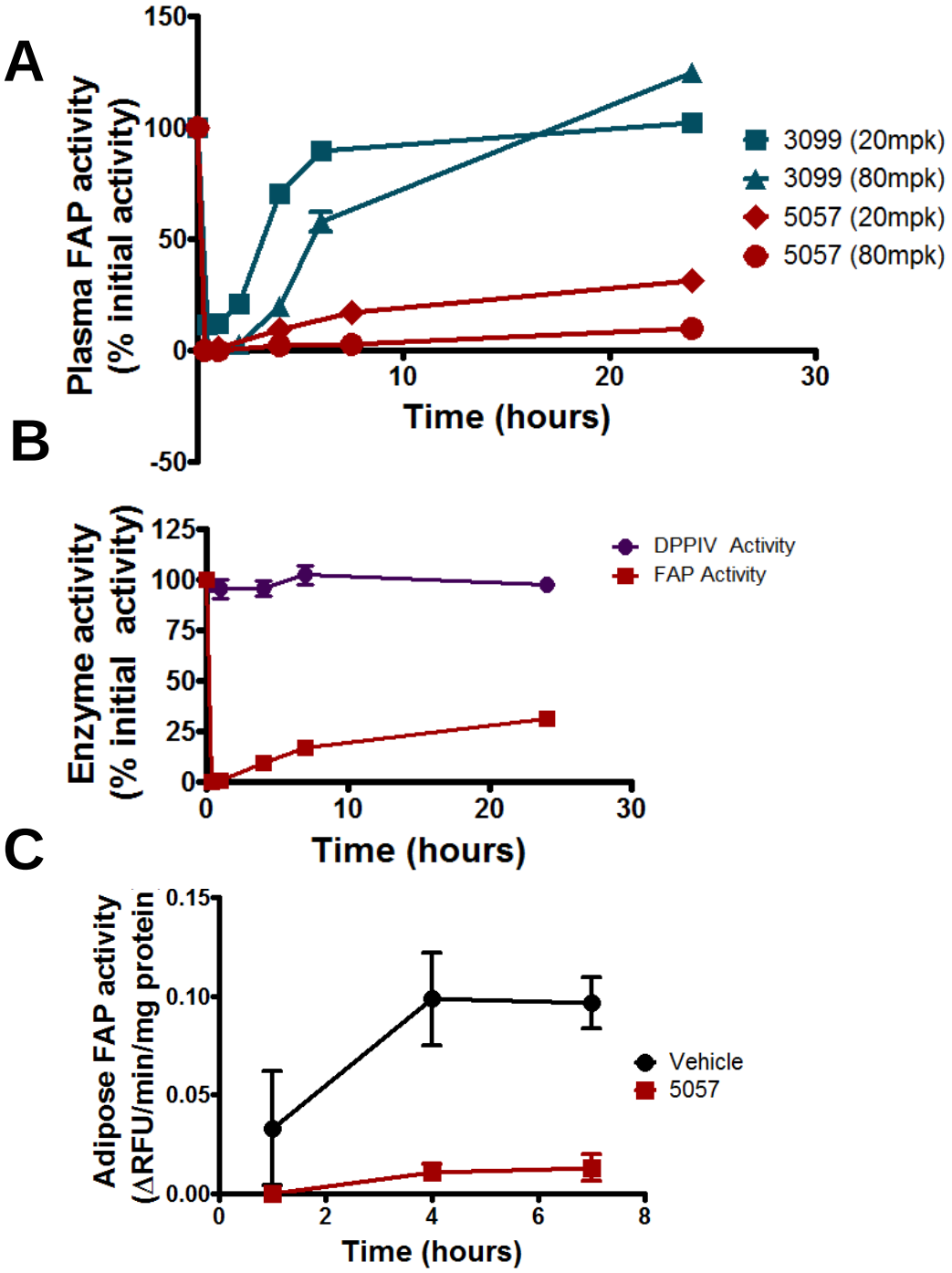


Figure 29. Pharmacological inhibition of plasma FAP *in vivo*. (A) 5057 is a superior inhibitor of plasma FAP compared to 3099 *in vivo*. Mice were treated with the indicated compounds via oral gavage. Plasma FAP activity was assessed by ARI-3144 (n=3 per time point). **(B)** 5057 does not inhibit plasma DPPIV. Mice were treated with 5057 at 20mpk via oral gavage. Plasma DPPIV activity was assessed by H-Gly-Pro-AMC (n=3 per time point). **(C)** 5057 inhibits adipose FAP activity in *ex vivo* tissue homogenates (n=3 per time point). All data expressed as mean ± SEM

While sustained inhibition of the plasma enzyme is potentially important, our findings that FAP is upregulated in the adipose tissue of DIO mice suggest that FAP activity may be most important in the adipose tissue during the development of diabetes and obesity. To determine if 5057 is capable of inhibiting FAP in the adipose tissue, we assessed FAP activity in adipose tissue homogenates following a 20mpk oral dose of 5057 (Fig. 29C). Adipose FAP activity was found to be significantly inhibited at all time points tested. This data demonstrates that 5057 is capable of sustained inhibition of FAP activity in both plasma and adipose, making this inhibitor suitable for *in vivo* studies on the effect of pharmacological inhibition of FAP on the DIO process.

3.2.3.2 Chronic Pharmacological Inhibition of FAP Has No Effect on the DIO Process

The FAP KO mouse is protected from the development of diabetes and obesity, so we hypothesized that sustained pharmacological inhibition of FAP may protect WT mice from these pathologies. We expected chronic dosing of an FAP inhibitor to be required to observe any effects of FAP inhibition based on the FAP KO mouse phenotype and because it takes time for mice to develop diet-induced obesity. In order to investigate the effect of FAP inhibition on the DIO process three groups of mice were utilized (Fig. 30). In the first group (lean), chow-fed mice were randomized to either 5057 or vehicle groups at 8 weeks of age. These mice were maintained on the standard chow diet for 8 weeks during once daily dosing. A second group (obese), were also randomized to 5057 or vehicle at 8 weeks, but were then switched to a high-fat diet for 8 weeks during dosing. The final group (severely obese), were older and already obese due to HFD-feeding when

randomized onto 5057 or vehicle, and this group was maintained on the HFD during 6 weeks of dosing.

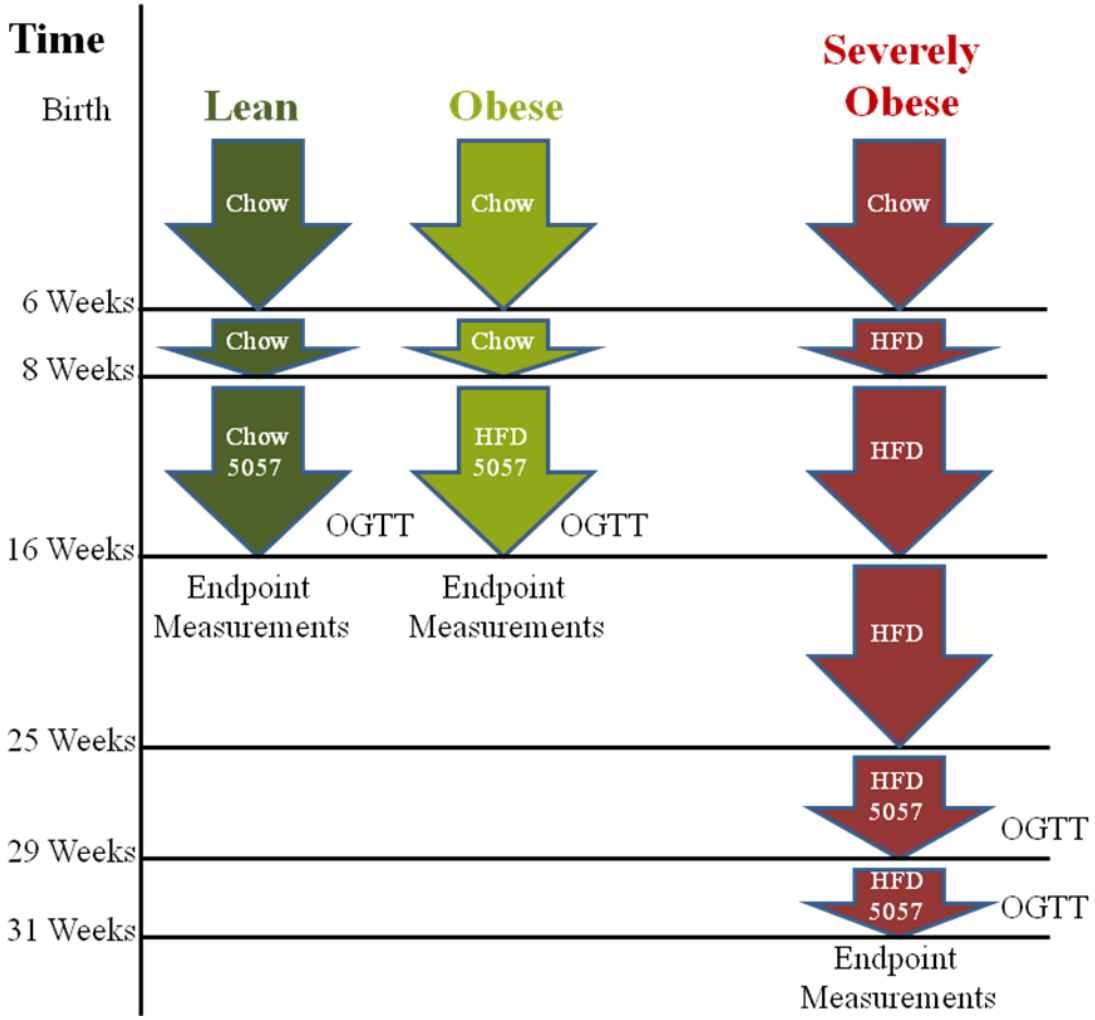


Figure 30. Effect of chronic inhibition of FAP on the DIO process: experimental design. Mice are divided into three groups, lean (green), obese (yellow) and severely obese (red). The diet fed to the animals is indicated by either chow (10% fat) or HFD (60% fat). Dosing of FAP inhibitor or vehicle is indicated by "5057". "OGTT" indicates the animals underwent an oral glucose tolerance test. "Endpoint Measurements" indicates sacrifice of the animals and blood collection for insulin and cholesterol measurements.

The third group was designed to test the effect of FAP inhibition on mice that are already obese to determine if 5057 was capable of ameliorating any aspects of pre-existing diabetes and obesity, while the first two groups were designed to determine if FAP

inhibition could prevent the transition from the normal to diabetic state. Being able to observe chow and HFD-fed mice side by side is important for two reasons. First, it allows for confirmation that the HFD-fed mice do indeed develop a DIO phenotype during the course of the experiment. Second, the phenotype of the FAP KO mouse suggests that the 5057 treated mice should only vary from the vehicle treated mice when on a HFD. Such a result would provide further evidence that FAP inhibition is responsible for the observed differences. Oral gavage was chosen as the dosing technique because it is minimally invasive and known to only minimally suppress weight gain of mice on a high-fat diet [217]. Therefore, this technique is amendable for chronic dosing of pharmacological inhibitors of FAP to assess their effects on DIO mice.

All groups of mice were monitored for plasma FAP inhibition, weight gain and food consumption throughout the course of the experiment. Blood was collected via tail vein nick and baseline plasma FAP activity was assessed before the start of dosing with ARI-3144. During the experiment, blood was collected once every two weeks for measurement of plasma FAP activity. Different time points were chosen each week to create a pharmacodynamic profile for plasma FAP inhibition (Fig. 31A). As expected plasma FAP inhibition was continuously suppressed in all 5057-treated groups. Weight gain was also monitored daily. The group of lean mice placed on the HFD diet (obese) gained approximately 30% more weight than mice maintained on the chow diet (lean), demonstrating the ability of the HFD to greatly increase weight gain. However, no differences in weight gain were observed between the vehicle and 5057-treated mice (Fig. 31B). In the severely obese group, weight gain was also unaffected by treatment

with 5057. Correspondingly, food consumption was unaltered by 5057 in any of the three groups (Fig. 31C).

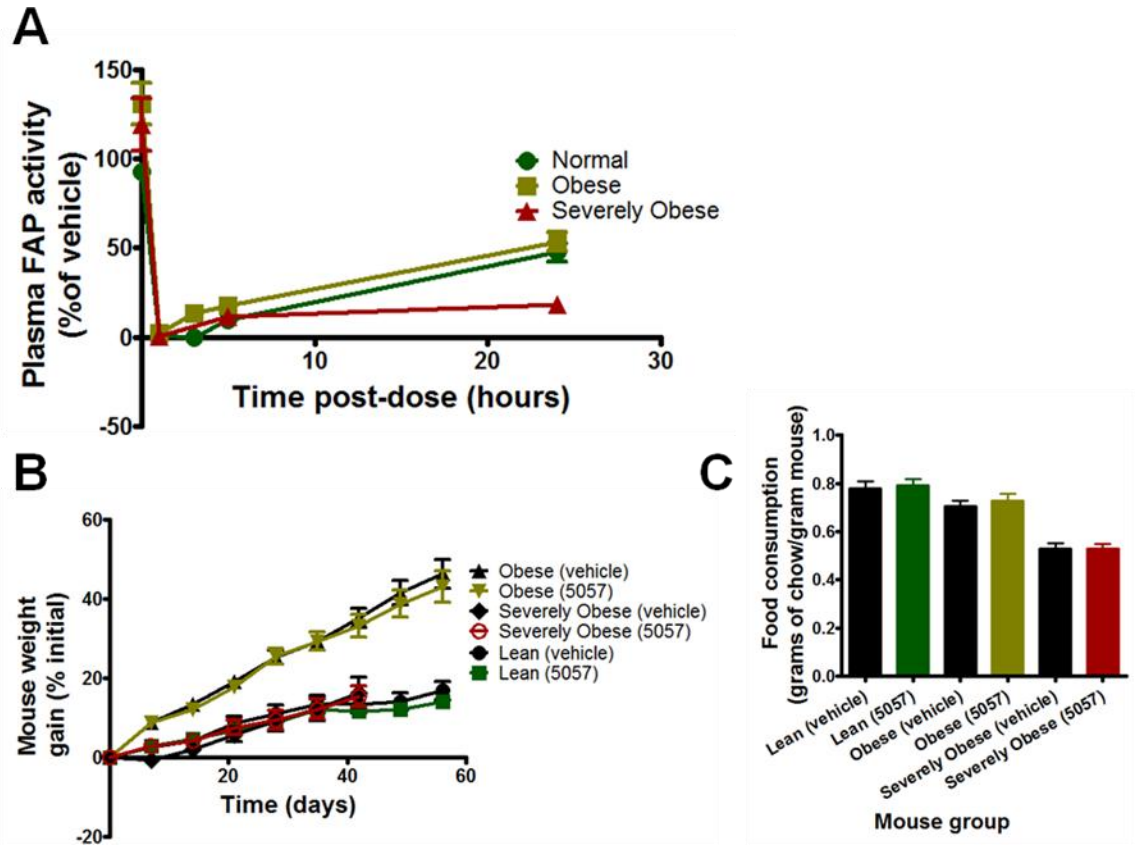


Figure 31. Effect of FAP inhibition on weight gain and food consumption in lean and obese mice. (A) 5057 suppresses plasma FAP activity. Blood was collected once every two weeks at the indicated time points. FAP activity was assessed by ARI-3144. **(B)** 5057 has no effect on weight gain of lean and obese mice. **(C)** 5057 has no effect on food consumption of lean and obese mice. Total food weight was measured by cage once weekly. All data expressed as mean ± SEM (n=8 per group for lean mice, n=12 per group for obese mice, n=10 per group for severely obese mice).

3.2.3.3 Chronic Pharmacological Inhibition of FAP Does Not Alter the Glucose and Lipid Metabolism of Lean and DIO Mice, with the Exception of an Effect on Glucose Tolerance

Though weight gain and food consumption was unaltered by chronic FAP inhibition, the FAP KO mouse on a HFD also has improved glucose and lipid homeostasis relative to WT mice. To examine the effect of pharmacological FAP inhibition on these parameters, a number of measurements were taken upon cessation of chronic 5057 dosing. Levels of total plasma cholesterol under fasting conditions were examined and 5057 had no effect on this lipid measure. The mice maintained on a HFD had significantly higher levels of plasma cholesterol than their chow fed counterparts, further confirming the induction of the DIO phenotype (Fig. 32A). Fasting serum insulin levels were also examined because elevated fasting serum insulin is a marker of peripheral insulin resistance. Again, 5057 had no significant effect on this parameter in any of the groups (Fig. 32B). As expected, fasting serum insulin was significantly increased in the severely obese group and there was a trend towards higher fasting serum insulin the obese group. Furthermore, fasting blood glucose was not affected by treatment with 5057 in any of the mouse groups, although increased fasting blood glucose was observed in both groups maintained on the high-fat diet (Fig. 32C).

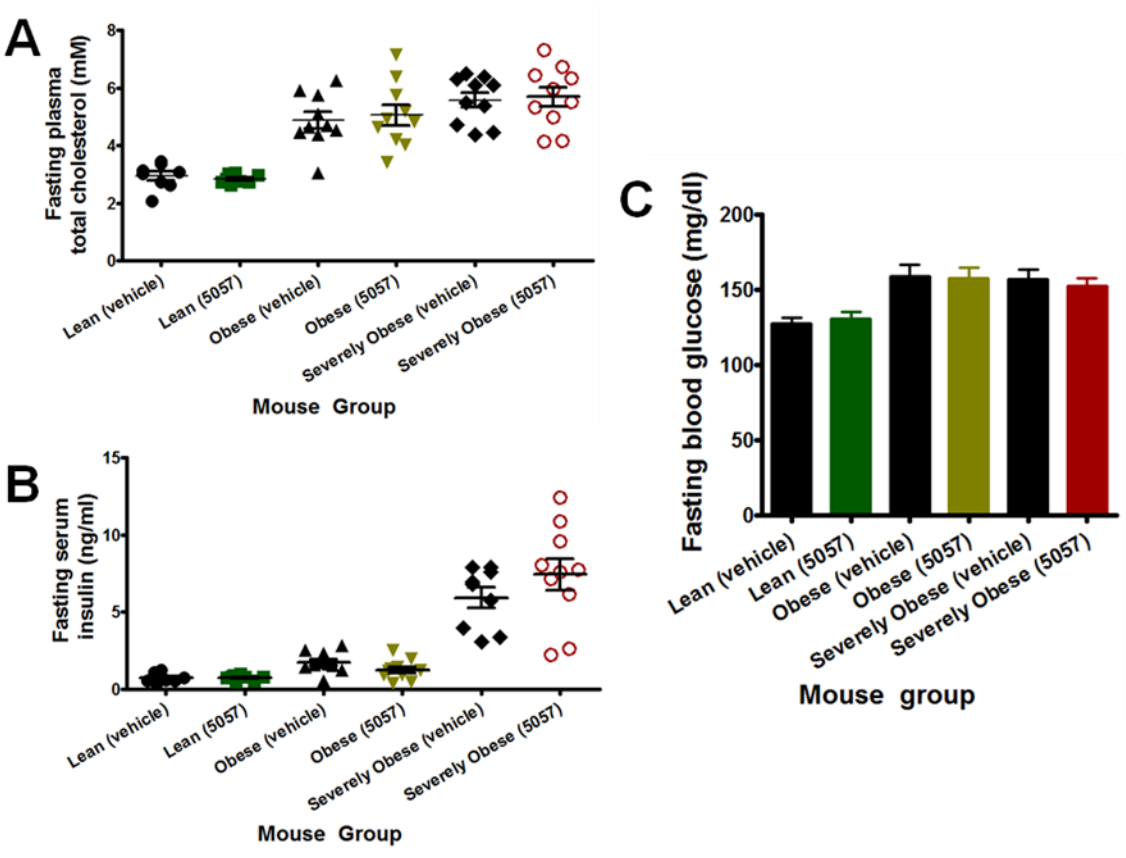


Figure 32. Effect of chronic FAP inhibition on cholesterol, insulin and fasting blood glucose. (A) Chronic administration of 5057 has no effect on total cholesterol in lean and obese mice. Total fasting plasma cholesterol levels were determined by colorimetric assay. **(B)** Chronic administration of 5057 has no effect on fasting serum insulin levels in lean and obese mice. Serum insulin levels were assessed by ELISA. **(C)** Chronic 5057 administration has no effect on fasting blood glucose. Blood glucose concentrations were assessed, using handheld glucometers. Mice were fasted for six hours before all measurements. All data expressed as mean ± SEM (n=8 per group for lean mice, n=12 per group for obese mice, n=10 per group for severely obese mice).

3.2.4 The FAP Inhibitor 5057 Improves Oral Glucose Tolerance

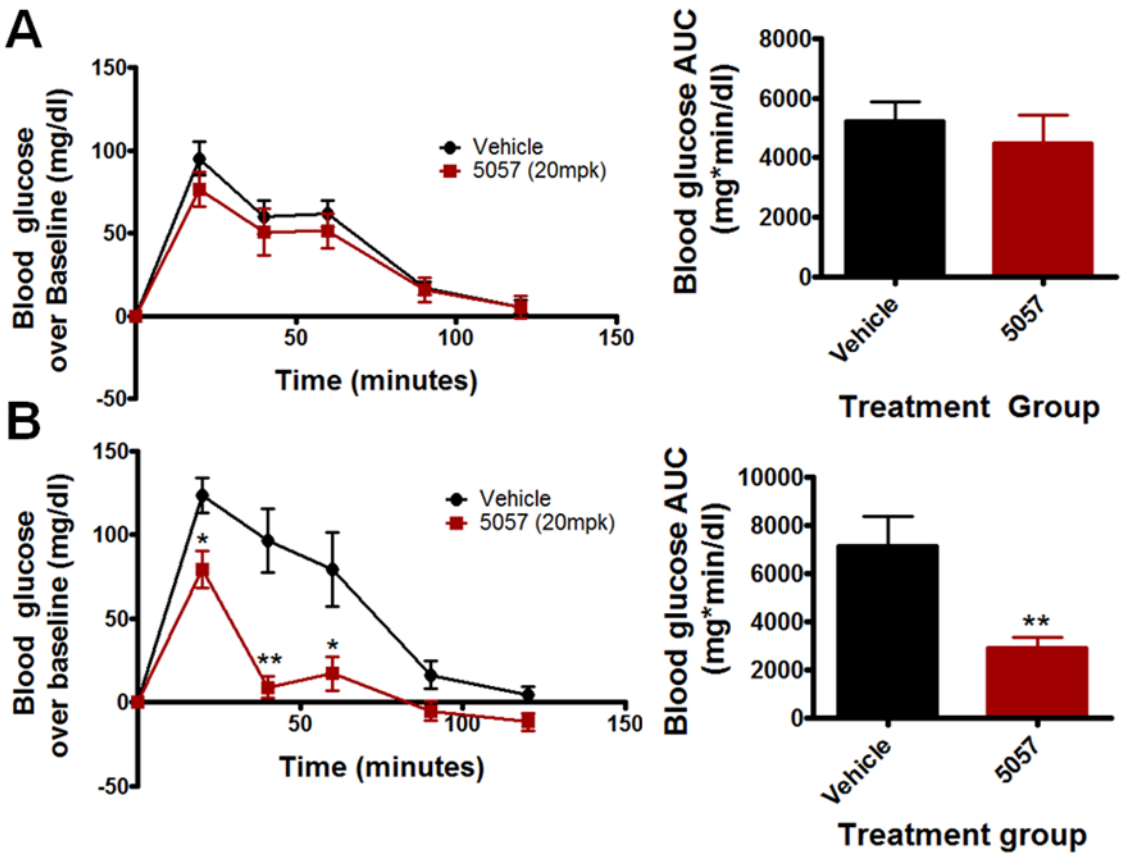
3.2.4.1 5057 Improves Oral Glucose Tolerance after Chronic Administration

The final measurement performed on these three groups of mice chronically dosed with 5057 was an oral glucose tolerance test (OGTT). During this procedure, fasted mice are given 5057 or vehicle via oral gavage, followed by an oral bolus injection of glucose. Blood glucose levels are then monitored by handheld glucometers [231]. This test was chosen because it is an excellent measure of the incretin effect, driven by the peptide hormones GLP-1 and GIP. The DPPIV inhibitors work primarily by enhancing the incretin effect and it is possible, given that GLP-1 is also a substrate of FAP, that 5057 could function in a similar manner.

To test this idea we first examined the effect of 5057 on glucose tolerance in the lean mice at the end of 8 weeks of chronic dosing. In this experiment, 5057 or vehicle was administered 2 hours prior to glucose injection and no difference was observed between vehicle and 5057 treated mice (Fig. 33A). This result was expected, given no differences in glucose tolerance reportedly exist between WT and FAP KO mice maintained on a chow fed diet [180].

Next, an OGTT was performed on the obese group of mice. This time, the drug was administered 45 minutes before the glucose challenge to maximize the chances of observing an effect. In this instance, glucose tolerance was considerably improved, with an approximately 45% reduction in blood glucose area under the curve (AUC) (Fig. 33B). A similar improvement in glucose tolerance was observed in an OGTT in the severely obese mice after only 4 weeks of chronic dosing with 5057 (Fig. 34C).

To determine if the timing of compound dosing relative to glucose injection is important, dosing of the severely obese mice was continued for another 2 weeks. At the end of these 2 weeks, another OGTT was performed, this time with the compound administered 4 hours before the glucose challenge. Using this new timing, no effect of 5057 on glucose tolerance was observed (Fig. 33D). This result suggests that 5057 must be dosed close to the time of glucose administration to have an effect and chronic dosing may not be required.



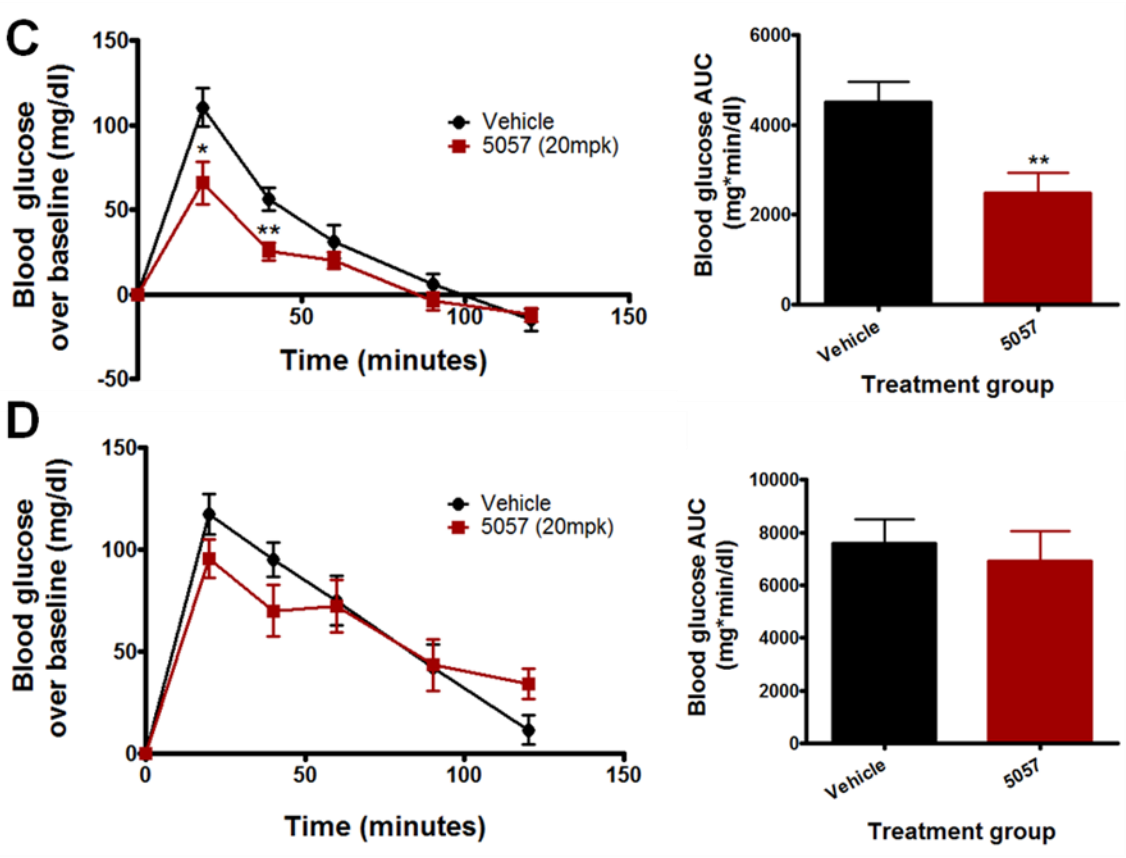


Figure 33. Effect of 5057 on oral glucose tolerance after chronic dosing. (A) 5057 has no effect on glucose tolerance in lean mice when administered 2 hours before glucose challenge at 5g/kg. Lean mice were chronically dosed with 5057 for 8 weeks prior to the OGTT. **(B)** Chronic administration of 5057 significantly reduces glucose excursion in obese mice after 8 weeks. 5057 was administered 45 minutes before glucose challenge at 2.5g/kg (**P<.01 by *t*-test). **(C)** Chronic administration of 5057 significantly reduces glucose excursion in severely obese mice after 4 weeks. 5057 was administered 45 minutes before glucose challenge at 2.5g/kg (**P<.01 by *t*-test). **(D)** Chronic administration of 5057 has no effect on glucose tolerance when administered 4 hours before a 2.5g/kg glucose challenge. Severely obese mice were chronically dosed with 5057 for 6 weeks prior to the OGTT. All data expressed as mean ± SEM (n=8 per group for lean mice, n=12 per group for obese mice, n=10 per group for severely obese mice).

3.2.4.2 5057 Improves Oral Glucose Tolerance on a Single Dose

After this result, the effect of 5057 on oral glucose tolerance on a single dose was investigated. In treatment naive subjects, 5057 significantly reduced glucose excursion in

both lean and DIO mice when administered 45 minutes before glucose injection. Fasting blood glucose concentrations were not significantly reduced after inhibitor dosing (Fig. 34A) Reductions in area under the curve were 63% for the lean mice and 45% for DIO mice (Fig. 34A, 34B). This data demonstrates that chronic administration of 5057 is not required to observe an OGTT effect. Additionally, the OGTT effect can be observed in WT mice that are not obese.

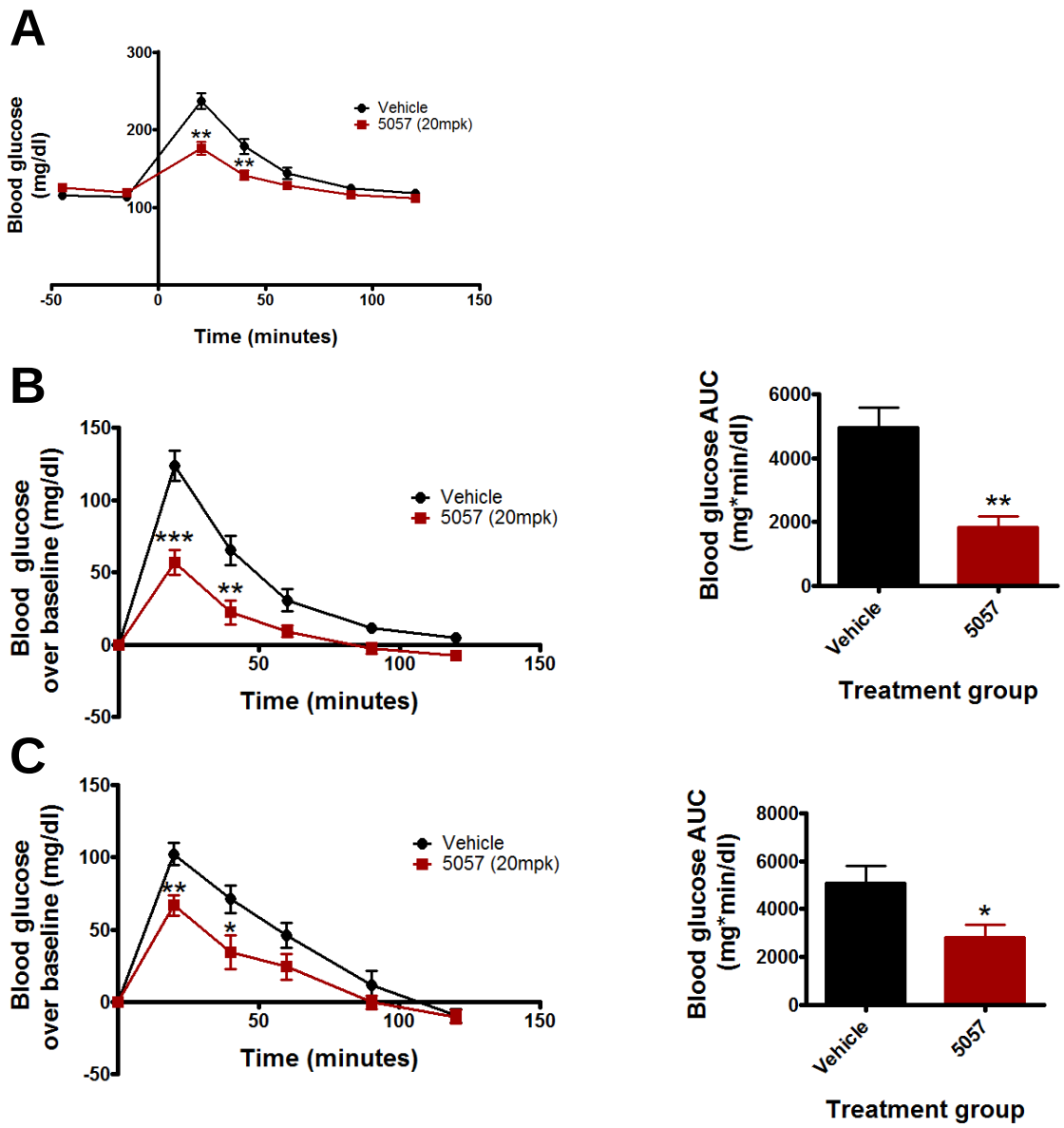


Figure 34. Effect of 5057 on oral glucose tolerance after a single dose. (A) 5057 reduces blood glucose concentrations during an OGTT in lean mice (16 weeks of age) on a single dose. Data expressed in absolute blood glucose terms. **(B)** 5057 reduces OGTT glucose excursion in lean mice (16 weeks of age) on a single dose. Glucose was administered at 2.5g/kg, 45 minutes after 5057. Data expressed in baseline-corrected terms. **(C)** 5057 reduces glucose excursion in DIO mice (16 weeks of age) on a single dose. Glucose was administered at 2.5g/kg, 45 minutes after 5057. All data expressed as mean ± SEM (*P<.05, **P<.01 by t-test).

3.2.4.3 The Glucose Tolerance Enhancing Effects of 5057 may be due to Interactions with Non-FAP Targets

The fact that the glucose tolerance of chow-fed lean mice is affected by 5057 is surprising given that no phenotypic differences are observed between FAP KO and WT mice when on a chow diet. Additionally, the less than 2 hour window of time between drug and glucose administration required to observe a significant glucose tolerance effect with 5057 does not appear consistent with FAP as a target due to the fact that the plasma enzyme is 90% inhibited for at least 4 hours after dosing with 20mpk of 5057. Together this data suggested that 5057 may be hitting an off-target.

The most obvious potential off-target for 5057 is DPPIV, given its well characterized effects on oral glucose tolerance and the fact that 5057 can inhibit DPPIV at high concentrations *in vitro*. While DPPIV is not inhibited in the plasma following 5057 dosing at 20mpk, it is possible that local inhibition of DPPIV in the gut could cause the observed effects on glucose tolerance. To test this, we administered 5057 via subcutaneous injection in lieu of oral gavage. Using this dosing strategy, 5057 did not have a significant OGTT effect, although there was a trend towards a reduced glucose excursion (Fig. 35).

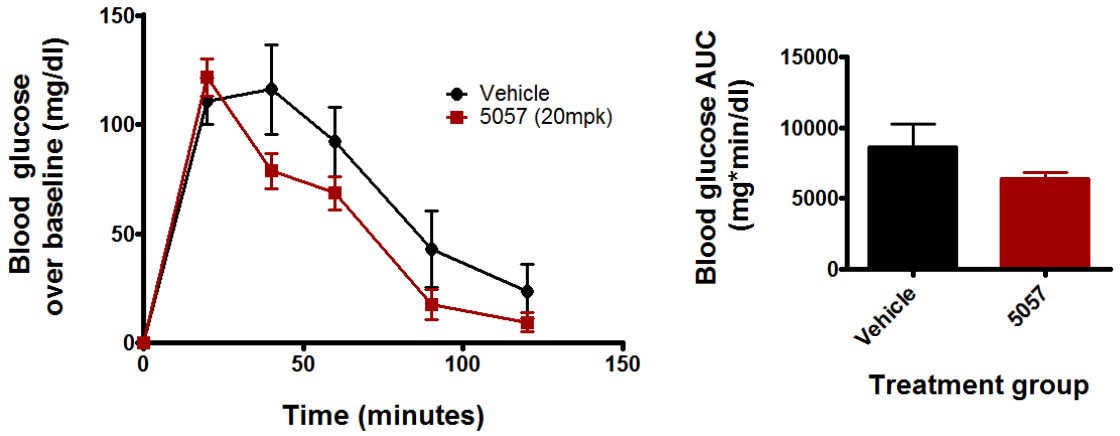


Figure 35. Effect of subcutaneously administered 5057 on oral glucose tolerance. (A) The OGTT effect of 5057 is less when dosed subcutaneously in comparison to oral dosing. Glucose was administered at 2.5g/kg, 45 minutes after 5057 to 23 week old DIO mice (17 weeks HFD, n=9 per group). All data expressed as mean ± SEM.

To further explore the possibility of gut DPPIV as an off-target of 5057, we compared the effect of the DPPIV inhibitor sitagliptin on glucose tolerance, both alone, and together with 5057. The 20mpk dose of sitagliptin is above what is required to observe a maximal OGTT effect and was chosen to ensure complete inhibition of DPPIV. Interestingly, we found that the effect of 5057 on glucose tolerance was additive to DPPIV inhibition with sitagliptin in WT chow-fed mice (Fig. 36A). Furthermore, this experiment allowed for a comparison of the size of OGTT effects of both compounds. The effect on glucose excursion between 5057 and sitagliptin alone was not significantly different at -63% and -74% respectively, suggesting that 5057 works equally well as sitagliptin to reduce glucose excursion (Fig. 36B). However, the combination of sitagliptin and 5057 resulted in a much larger 90% reduction in blood glucose AUC, with blood glucose concentrations peaking at only 20mg/dl above baseline at the 20 minute time point,

before returning below baseline. This result strongly suggests that DPPIV inhibition is not responsible for the OGTT effect of 5057.

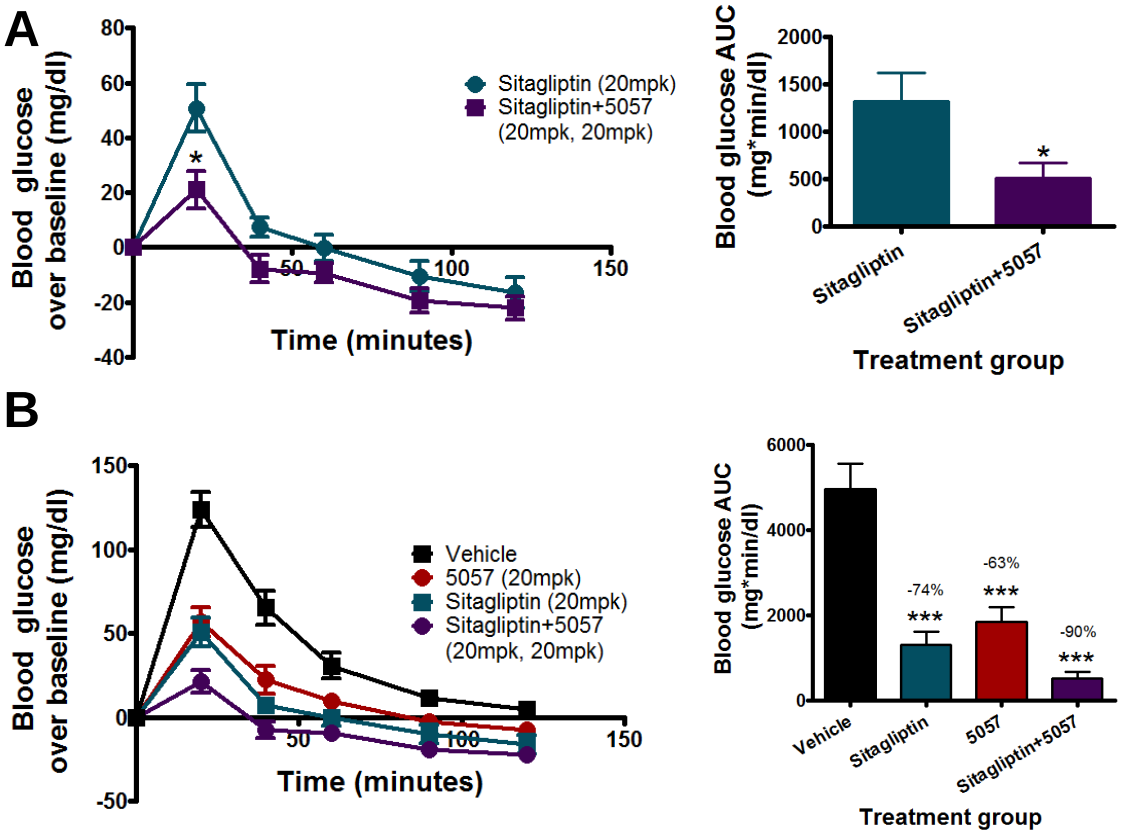


Figure 36. The OGTT effect of 5057 together with sitagliptin. (A) The OGTT effect of 5057 is additive to sitagliptin in lean mice (16 weeks of age) on a single dose (n=8 per group). **(B)** The OGTT effect of 5057 as a single agent in lean mice (16 weeks of age) is comparable to sitagliptin. Glucose was administered at 2.5g/kg, 45 minutes after compound administration. All data expressed as mean ± SEM (*P<.05, **P<.01, ***P<.001 by *t*-test).

Though these experiments investigated the potential of DPPIV as an off-target, the potential for other non-FAP targets remain. To address this, the ability of 3099 to affect glucose tolerance was tested in an OGTT in WT mice. Given that 5057 and 3099 are somewhat structurally dissimilar, with different electrophilic groups, it was reasoned that a positive result with 3099 would support FAP as the relevant target of the OGTT effect.

However, even at an extremely large 200mpk dose, 3099 failed to cause a significant reduction in glucose excursion (Fig. 37). Together, these results suggest 5057 is likely, but not definitively, mediating its effects on glucose tolerance through an unidentified off-target.

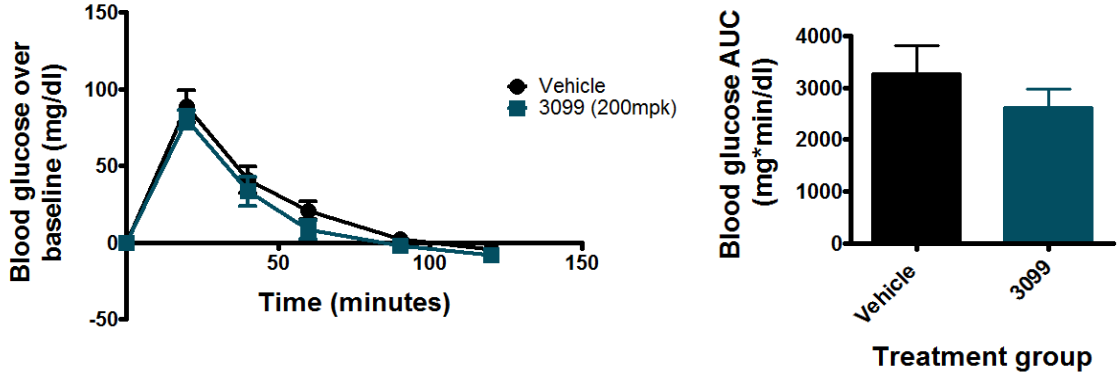


Figure 37. The FAP inhibitor 3099 does not affect oral glucose tolerance. 3099 has no OGTT effect at a 200mpk dose in lean mice (16 weeks of age). Glucose was administered at 2.5g/kg, 45 minutes after compound administration (n=8 per group). All data expressed as mean ± SEM

Discussion and Future Directions

4.1 The Role of FAP in Cancer

4.1.1 FAP Enzymatic Activity as an Anti-Cancer Target

FAP enzymatic activity has previously been reported to be an anti-cancer target in the same CT-26 model utilized in this work. In this model, FAP expressing stromal cells develop in the mouse during tumor growth, but are not present on the tumor cells. For this reason, the CT-26 model provides a proxy for human carcinomas, where FAP is observed on CAFs. Santos et al. demonstrated that the compound Glu-boroPro reduced CT-26 tumor growth, an effect attributed to its ability to inhibit FAP and this conclusion

was strengthened based on the fact that Glu-boroPro appeared to have no effect on CT-26 tumor growth in FAP KO mice.

Our data confirms the ability of Glu-boroPro to inhibit CT-26 tumor growth, but refutes the conclusions of Santos et. al. that FAP inhibition is responsible for the efficacy of this compound. We demonstrate that the FAP-specific inhibitor 5057 fails to suppress tumor growth in the CT-26 model, despite excellent inhibition of FAP both in the plasma and tumor microenvironment, as assessed by our FAP fluorescent substrate ARI-3144. In fact, at the dosages used, Glu-boroPro was a poor inhibitor of FAP making it even more unlikely that FAP is a relevant target. By comparison, the ability of 5057 to inhibit FAP in a long-lasting and specific manner presents a clear utility for this compound in studies investigating the role of FAP enzymatic activity *in vivo*.

Our conclusion that pharmacological inhibition of FAP has no effect on CT-26 tumor growth appears to conflict with the results of Santos et al. who show that Glu-boroPro has no anti-tumor effect in the FAP KO mouse. This result argues that FAP is the relevant target for Glu-boroPro. However, there are a number of possible explanations for the seemingly contradictory results. First, in the work by Santos et al., vehicle group tumors were allowed to grow to 150mm^2 when examining the effect of Glu-boroPro on CT-26 tumors in WT mice. However, in a separate experiment comparing the effect of Glu-boroPro in both WT and FAP KO mice with CT-26 tumors, the WT vehicle group tumors were only allowed to grow to 1/3 that size, suggesting this experiment was ended earlier with respect to tumor progression. In our hands the effect of Glu-boroPro on tumor

growth is quite modest, so it is possible any effect of Glu-boroPro may not yet have been detected at the smaller tumor sizes.

Additionally, in the Santos study, the FAP KO mice have substantially smaller tumors than their WT counterparts regardless of treatment with Glu-boroPro or vehicle. At the end of this experiment, tumors in both the Glu-boroPro and vehicle groups averaged only $25mm^2$. In our hands, there is no detectable difference between vehicle-treated and Glu-boroPro-treated WT mice at that tumor size. Had these tumors in the Santos et al. study been allowed to grow to larger sizes in the FAP KO mice perhaps the anti-cancer effect of Glu-boroPro would have been detectable. While the smaller size of tumors in the FAP KO mice may seem to support FAP as a therapeutic target, it does not directly implicate its enzymatic activity as a relevant target, especially given the large body of literature on non-enzymatic functions of FAP and FAP expressing cells. Further work on the role of FAP enzymatic activity in cancer would be greatly aided by generation of a transgenic mouse that expresses FAP with the catalytic serine substituted with alanine. Such a mouse could allow for investigation of FAP while separating the enzymatic and non-enzymatic functions of this protease.

Our findings with the FAP-specific inhibitor 5057 in the CT-26 model suggest that FAP enzymatic activity may not be a relevant therapeutic target for human carcinomas. This result argues that future work on FAP-directed oncology therapeutics should focus on FAP-activatable prodrugs and FAP-targeted antibodies as opposed to enzyme inhibitors and both of these alternative strategies have reported efficacy in preclinical models [196, 211,

[246]. However, our results revealed that while CT-26 tumors do have detectable FAP, FAP is not elevated to the same extent as seen in human tumors such as pancreatic adenocarcinoma (unpublished data). Given the relative paucity of FAP in CT-26 tumors compared to human tumors, it is possible that the effect of inhibiting FAP in this model might not reflect the typical clinical situation.

An alternative model is the HEK293 mFAP model used by Cheng et al. where the authors report that tumors consisting of HEK293 cells over-expressing FAP grew substantially faster than the same cells over-expressing a catalytically dead FAP [2]. This result directly implicates FAP enzymatic activity in tumor growth, however the FAP levels of these cells were likely well above what would be observed in human tumors. In addition, in this model, FAP is expressed by the tumor cells themselves, rather than exclusively on CAFs as is typical in the vast majority of human carcinomas. For these reasons this model may not be ideal for studying the role of FAP in tumor growth. Nevertheless, some cancer cells, particularly sarcomas, do express FAP. Investigation of the anti-cancer properties of 5057 in the HEK293 mFAP model may further our knowledge on the role of FAP enzymatic activity in these select cancers.

4.1.2 The PPCEs as Targets of the Dipeptide Boro-Prolines

4.1.2.1 FAP as a Target of the Dipeptide Boro-Prolines

Paralleling its hypothesized role in tumor growth, FAP has also been posited as the relevant anti-cancer target of the dipeptide boro-prolines [208]. Our results demonstrate that, at the chosen dosages, 5057 is a far superior inhibitor of FAP than Glu-boroPro *in vivo*,

yet 5057 is incapable of suppressing tumor growth in the CT-26 model. In comparison, Glu-boroPro does suppress tumor growth in this model. Given the superior inhibition of FAP with 5057, both in the tumor and plasma, we conclude that FAP in not a relevant anti-cancer target of the dipeptide boro-prolines in the CT-26 model.

FAP inhibition also does not appear to be the source of the cytokine-stimulating effects of the dipeptide boro-prolines as evidenced by the fact that 5057 cannot re-create the large increases in plasma G-CSF concentrations observed after dosing with both Val-boroPro and Glu-boroPro. This is important, as the cytokine response is thought to be a key driver of the anti-cancer effects of the dipeptide boro-prolines. Future work examining the cytokine response of the dipeptide boro-prolines in FAP KO mice may further eliminate FAP as a single target for the cytokine response, although its contribution in the context of inhibition of the other PPCEs cannot be ruled out.

Given that FAP inhibition does not appear responsible for either the anti-cancer or cytokine stimulating effects of Glu-boroPro, we suggest that this compound is instead engaging an immune-stimulatory mechanism common to the dipeptide boro-prolines. Though the 100µg dose of Glu-boroPro chosen for the CT-26 efficacy experiment is unlikely to result in a significant increase in serum G-CSF, this cytokine is simply a proxy for the overall cytokine response. It is possible that other serum cytokines typically observed after dosing with the dipeptide boro-prolines are induced at this dosage. Additionally, Val-boroPro has been reported to upregulate expression of a myriad of cytokines within the tumor microenvironment. Therefore, at the 100µg dose, Glu-boroPro

may upregulate the cytokine response within the tumor microenvironment, even if those cytokines are not reflected in the serum. Examination of the immune component of the anti-cancer effect of Glu-boroPro could be accomplished by comparing CT-26 tumor responses in immune-deficient mice to those from immune-competent mice.

4.1.2.2 DPPIV as a Target of the Dipeptide Boro-Prolines

DPPIV is the enzyme most closely related to FAP and because Glu-boroPro is an excellent DPPIV inhibitor it is reasonable to hypothesize that any biological effects of this compound may arise from inhibition of DPPIV activity. In fact, DPPIV and FAP share considerable substrate specificity as both have dipeptidyl peptidase activity with a preference for proline in the P1 position. Given this overlap, we thought it critical to test both a DPPIV inhibitor and combination of DPPIV and FAP inhibitors in the CT-26 model. Dual inhibition of these enzymes not only better mimics the inhibition profile of Glu-boroPro, but also addresses a situation in which any relevant substrate(s) may be cleaved by both enzymes. However, we observe no anti-cancer effect with the DPPIV-specific inhibitor sitagliptin, alone or in combination with FAP inhibition. While it is possible that the long-lived inhibition of DPPIV alone by Glu-boroPro *in vivo* is responsible for the observed anti-cancer effect, this is unlikely given that neither sitagliptin in our hands, nor the previously reported results with vildagliptin had any significant effect on CT-26 tumor growth [248].

DPPIV inhibition with sitagliptin is also unable to replicate the G-CSF inducing properties of the dipeptide boro-prolines. Although a 2mg dose of sitagliptin did appear to increase serum G-CSF concentrations, this upregulation pales in comparison to that

observed with Val-boroPro or Glu-boroPro. Additionally, the cytokine and anti-tumor response of Val-boroPro is reported to be unaffected in DPPIV KO mice, suggesting that DPPIV alone is not the relevant target [126].

4.1.2.3 DPP8/9 as Targets of the Dipeptide Boro-Prolines

Having excluded the extracellular PPCEs enzymes FAP and DPPIV as being responsible for the anti-cancer or cytokine inducing effects of dipeptide boro-prolines we then turned our attention to the intracellular PPCEs. Of these enzymes, DPP8 and 9 appear to have the best case for being relevant targets of the dipeptide boro-prolines. DPP8 and 9 are highly expressed in immune cells including macrophages and monocytes, which is consistent with an immune-based mechanism [58]. Additionally, DPP8/9 specific inhibitors share the ability of Val-boroPro to cause IL-1β release from THP-1 macrophages, an event which could potentially trigger the release of other cytokines such as G-CSF [163]. Finally, the DPP8/9 inhibitor compound M, shows some similar toxicities to Val-boroPro, including abdominal alopecia [61].

However, as with specific inhibitors of FAP and DPPIV, inhibition of DPP8 and DPP9 had no effect on CT-26 tumor growth. Our results also demonstrate that plasma levels of the chosen compounds in tumor-bearing mice are sufficient to expect inhibition of DPP8/9 based on permeability of these compounds to cells in culture. However, to confirm inhibition we developed a novel method for assessing DPP8/9 activity in tumor homogenates and found that 8J potently inhibited DPP8/9 activity while Tle-Pro-CN did so only transiently. This result emphasizes the importance of assessing DPP8/9 inhibition in tissues rather than assuming inhibition from plasma concentrations.

Interestingly, the DPP8/9 inhibitors used in our study caused no observable adverse effects in the mice. In contrast, Val-boroPro at 20μg/mouse B.I.D. caused a reduction in weight gain, noticeable lethargy and pronounced abdominal alopecia. Val-boroPro also potently stimulates G-CSF release in BALB/c mice, an effect not shared by the other DPP8/9 inhibitors. This result is consistent with the idea that the side effects of Val-boroPro are related to immune activation, although it is worth noting that Glu-boroPro has anti-cancer efficacy at doses which give negligible G-CSF release or side effects. This highlights the fact that the anti-cancer and cytokine inducing mechanism of Val-boroPro may not be inextricably linked. Thus, further exploration of these two aspects of the Val-boroPro mechanism may yield compounds which have a broader therapeutic window.

4.1.2.4 PREP as a Target of the Dipeptide Boro-Prolines

PREP is another intracellular PPCE that could be a potential target for the dipeptide boro-prolines. Though there is no evidence that PREP may contribute to the immune-stimulating aspects of the dipeptide boro-prolines, there is literature evidence that PREP inhibition may suppress tumor growth. In particular, the PREP inhibitor J94, referred to in this work as 5332, has shown anti-cancer effects in the HCT116 mouse model of cancer [243]. While we confirm the anti-cancer effect of 5332 in the CT-26 model, we also demonstrate that the PREP specific inhibitor 3531, actually results in accelerated tumor growth. This result was unexpected and suggests that PREP enzymatic activity may help to suppress tumor growth.

While 3531 accelerates tumor growth, it is unclear whether this is a result of intracellular and/or extracellular PREP inhibition. Though PREP is ostensibly an intracellular enzyme, there are reports of PREP in the extracellular space [151]. Our results demonstrate that 3531 is capable of inhibiting some of the Z-Gly-Pro-AMC activity present in plasma, suggesting that there may be very low levels of PREP in plasma of tumor-bearing mice. This extracellular PREP is hypothesized to play a role in angiogenesis through the generation of Ac-SDKP [202]. Whether inhibition of extracellular PREP might prevent tumor-associated angiogenesis and suppress tumor growth is a question that remains unanswered due to the fact that 3531 inhibits both extracellular and intracellular PREP. It is possible, that inhibition of intracellular PREP with 3531 may accelerate tumor growth such that it overrides any possible growth-limiting effect of extracellular PREP inhibition. Separation of the contribution of intracellular and extracellular PREP inhibition could be accomplished by examining the anti-cancer effect of a poorly cell permeable PREP inhibitor.

The fact that PREP inhibition appears to accelerate tumor growth suggests that PREP may be an anti-target of the dipeptide boro-prolines. While the dipeptide boro-prolines are all excellent inhibitors of DPPIV, DPP8 and DPP9, their affinity for PREP is somewhat more variable. In our experience PREP IC50's for the dipeptide boro-prolines range from the low nM to low μM range. Future development of the dipeptide boro-prolines may therefore be directed to compounds that are less potent PREP inhibitors, to ensure that this growth enhancing mechanism is avoided.

Surprisingly, at the doses we tested, Val-boroPro does seem to avoid inhibition of PREP despite its documented potency against the recombinant enzyme. We demonstrate that while Val-boroPro is able to enter cells and inhibit intracellular DPP8/9 activity, intracellular PREP activity is poorly inhibited by Val-boroPro. This discrepancy could potentially be explained by differences between endogenous and recombinant PREP or the existence of a physiological sink for Val-boroPro. If the latter is true, DPPIV, DPP8 and DPP9 are lead candidates for reducing the pool of available Val-boroPro. Nevertheless, it is notable that Val-boroPro, a dipeptide boro-proline with potent anti-cancer effects, appears to avoid PREP inhibition *in vivo*.

4.1.3 Dipeptide Boro-Proline Prodrugs

Compound 5332 (J94) was originally designed by Jackson et al. to test the anti-cancer activity of a PREP inhibitor relegated to the extracellular space, however our results demonstrate that this compound is actually cleaved into the dipeptide boro-proline Arg-boroPro in mice. While it could be argued that PREP inhibition could still be a potential mechanism by which this compound exerts its anti-cancer effects, this is highly unlikely given that 5332 is a poor inhibitor of PREP *in vivo*. In fact both plasma and tumor PREP activity is only transiently inhibited following a 75µg I.P. dose of 5332. Therefore, the most likely explanation for the anti-cancer activity of 5332 is release of Arg-boroPro.

Compound 5332 is thus acting as a prodrug releasing Arg-boroPro *in vivo*. However, whether this prodrug is useful in the sense that it can improve the anti-cancer activity or therapeutic window of Arg-boroPro remains to be determined. Future work should be

aimed at determining the contribution, if any, of the 5332 prodrug to the anti-cancer efficacy and therapeutic window of Arg-boroPro. Such an experiment could be done by comparing the anti-cancer efficacy of molar equivalent doses of Arg-boroPro both alone and in prodrug form. If the prodrug form does indeed provide advantages over Arg-boroPro, then work should focus on indentifying the cause, such as alterations in the Arg-boroPro pharmacokinetics, or concentration of Arg-boroPro in the tumor.

Irrespective of the reason, it is interesting that this Arg-boroPro-based drug lacks all the detectable toxicities of Val-boroPro, while retaining the anti-cancer efficacy. This result immediately suggests that the toxicities of the dipeptide boro-prolines may be uncoupled from their anti-cancer effects. Val-boroPro failed in the clinic, in large part, due to dose-limiting toxicities, so the ability to expand the therapeutic window of the dipeptide boro-prolines could provide new life for this mechanism in the clinic. While the anti-cancer efficacy of 5332 and Val-boroPro in the CT-26 model was not particularly impressive, combining the dipeptide boro-prolines with other immune-activating oncology therapies in the clinic may prove to have synergistic effects and/or provide a route for taking advantage of the remarkable ability of these compounds to induce immunological memory and prevent tumor recurrence.

4.1.4 The Link Between Cytokines and Anti-Tumor Effects

Ultimately however, the clinical utility of the dipeptide boro-prolines will depend on the mechanism by which these compounds exert both their anti-tumor effects and the mechanism by which they induce immunological memory. Clinical trials with Val-

boroPro demonstrated that at adverse events were detected at doses where only marginal increases in serum cytokines were observed. However in our work, large increases in G-CSF can be observed without acute toxicity in mice. This indicates that humans may be particularly sensitive to Val-boroPro induced immune inactivation. In particular, elevated levels of IL-6, a cytokine induced by Val-boroPro have been associated with cytokine release syndrome in humans. Therefore, the clinical utility of the dipeptide boro-prolines may depend on the need, or lack thereof, for elevated systemic cytokines to stimulate either the anti-tumor or immunological memory responses. If elevated serum cytokines are required to engage both mechanisms, the therapeutic window of the dipeptide boro-prolines may not be sufficient for further clinical development.

Alternatively, if either the anti-cancer or immunological memory-inducing facets of the dipeptide boro-proline mechanism can be accomplished in the absence of increased serum cytokines, it is likely a larger therapeutic window may exist for these compounds. Our data suggests that at least a marginal anti-cancer effect can be obtained with Glu-boroPro at a dose level not expected to give elevations in serum cytokines. If this anti-cancer effect is observed due to elevated levels of cytokines within the tumor microenvironment, this suggests concentrating these drugs in the tumor may provide a route for limiting toxicity while maximizing anti-tumor activity. Indeed evidence already exists suggesting that ARI-4175 may act directly to sensitize tumor cells to T-cell killing through upregulation of Fas and I-CAM1 [193]. Though these molecules are likely to enhance T-cell mediated apoptosis, it is tempting to speculate that the dipeptide boro-prolines may alter the way in which tumor cells are killed, leading to greater or enhanced

tumor antigen presentation. Such “immunological death” might explain the ability of the dipeptide boro-prolines to induce immunological memory. Whether induction of immunological memory requires serum or tumor cytokine release remains to be determined. Given the promise of these compounds, the mechanism by which the dipeptide boro-prolines exert their anti-cancer effects requires additional investigation.

4.1.5 Relevant Target(s) of the Dipeptide Boro-Prolines

Our examination of the contribution of the individual PPCEs to the mechanism of the dipeptide boro-prolines indicates that neither the anti-cancer, nor the cytokine-inducing effects of the Xaa-boroPros can be assigned to any of the enzymes investigated. However, despite our demonstration of *in vivo* pharmacodynamics for each of the inhibitors used in this study, it is still possible that differences in tissue distribution and pharmacokinetics could account for a failure of these compounds to induce cytokines or have measurable anti-cancer effects in the CT-26 model. Another possible explanation for these results is that inhibition of one or more of the PPCEs are needed. We have demonstrated that inhibition of the extracellular PPCEs is insufficient, but other combinations have not been investigated. Specific inhibitors of all the PPCEs are now available and these inhibitors could be used in a drug-cocktail to try and recreate both the anti-cancer or cytokine inducing effects of the dipeptide boro-prolines.

It is also possible that the dipeptide boro-prolines are engaging an unidentified target. This would explain why specific inhibition of each of the PPCEs individually fails to recapitulate the biological effects of this compound class. Further work directed at

identifying the relevant target(s) of the dipeptide boro-prolines could be accomplished by the synthesis of a tagged dipeptide boro-proline. In our experience, the structure of the P2 amino acid side chain can be greatly varied without the complete loss of cytokine inducing activity. Therefore, biotinylated or alkyne derivatives could potentially be synthesized and if these compounds retained cytokine-inducing and anti-cancer properties, they could be used to pull out potential binding partners via avidin binding or click-chemistry approaches.

Finally, the possible contribution of DPP2 to this mechanism remains unexplored. DPP2 is a vesicular enzyme, active at acidic pH, and is thought to be mainly involved in the general degradation of proline containing peptides [59]. However there is evidence that DPP2 may be involved in immune regulation. Specifically, T-cell knockdown of DPP2 is reported to result in T-cells that are hyperactive upon TCR stimulation and preferentially adopt the Th17 lineage [252]. However, it is worth noting that the anti-cancer effects of Val-boroPro are retained in IL-17 KO mice, indicating this cytokine is not critical [263]. Also interesting is the fact that mice with DPP2 knocked out in the T-cell lineage have increased numbers of peripheral T-cells [252]. Val-boroPro is thought to function, in part, by accelerating the T-cell response and inhibition of DPP2 could potentially be responsible [263]. Further work identifying the relevant targets of the dipeptide boro-prolines is critical to potentially harnessing this mechanism for the treatment of cancer.

4.2 The Role of FAP in Diabetes and Obesity

4.2.1 FAP Enzymatic Activity as a Therapeutic Target in Diabetes and Obesity

FAP has been implicated as a potential therapeutic target for diabetes and obesity based on the resistance of FAP KO mice to the development of metabolic abnormalities associated with high-fat diet feeding. The phenotype of FAP KO mice is very similar to DPPIV KO mice, the latter of which helped to define the role of DPPIV in regulation of post-prandial glucose homeostasis [76]. Therefore we explored enzymatic inhibition of FAP as a potential therapeutic strategy for diabetes and obesity. Our data suggests that pharmacologic inhibition of FAP is unable to reproduce any aspect of the diabetes and obesity resistant phenotype of FAP KO mice, with the exception of a short-lived effect on glucose tolerance. Additionally, inhibition of FAP in mice that were already obese and diabetic was unable to positively influence any of the metabolic parameters tested, again with a short-lived effect on glucose tolerance as the lone exception.

These results suggest that FAP may not be a critical regulator of the novel metabolic substrates described in this work, however this data does not refute the possibility that FGF-21 is a relevant substrate of FAP in humans. In mice, it remains possible that FAP cleavage of apelin could explain the OGTT effect observed with 5057 and additional investigation of FAP as a regulator of the effects of the apelinergic system, both metabolic and otherwise, is warranted. Such a study could be accomplished by examining levels of intact apelin-13 or Pyr1-apelin-13 following FAP inhibitor administration. Though potentiation of the apelinergic system by 5057 could potentially enhance glucose tolerance, it would also likely lead to other metabolic effects, such as decreased weight

gain that were not observed in our experiments. Therefore, FAP is most likely to be involved in the diabetes and obesity process through non-enzymatic mechanisms.

However, it cannot be ruled out that pharmacological inhibition with 5057 did not reduce FAP activity to an extent significant enough to observe an effect on the metabolic parameters we examined. Though plasma FAP activity was continuously and potently suppressed in our experiments, 100% inhibition was likely achieved only for brief periods and less than complete inhibition is also expected in various tissues. By comparison, the FAP KO mouse has no FAP activity in either tissue or plasma. Greater inhibition of FAP could potentially be obtained by dosing 5057 twice a day, but the increased stress put on the mice may prevent the development of the DIO phenotype [217]. Another possible method is using implantable osmotic pumps to continuously deliver drug. However in our experience, this method also causes a delay or prevention of diet-induced obesity due to the invasive nature of the technique. While there are other methods that could be explored for enhancing the duration of FAP inhibition by pharmacological means, the role of FAP enzymatic activity in diabetes and obesity would be best investigated with the aid of a transgenic mouse expressing catalytically dead FAP. Comparison of the DIO process in these mice to WT mice would allow for isolation of the enzymatic and non-enzymatic effects of the protein.

4.2.2 Potential Non-Catalytic Functions of FAP in Metabolism

FAP is present in a number of metabolically active adult mouse tissues, albeit at low levels. In particular, FAP+ stromal cells are found in skeletal muscle, adipose and

pancreas [49]. Depletion of these cells causes cachexia and anemia, indicating a role for FAP+ stromal cells in maintaining muscle mass [49, 138]. It is possible that the absence of FAP protein in the FAP KO mice may alter the function of this subset of stromal cells. Though a muscle wasting phenotype is not observed in FAP KO mice, these mice do exhibit reduced adiposity [180]. Thus, it is possible that a similar role for FAP+ stromal cells exists in maintaining or expanding adipose tissue mass and a lack of FAP in FAP KO mice alters the function of these cells, preventing proper adipose tissue expansion when these mice are put on a high fat diet. If FAP has such a function, this could potentially explain the FAP KO mouse phenotype. FAP is also reported to have other non-enzymatic functions including coordinating cell migration together with α3β1 integrin and the uPAR signaling complex in the HO-8910PM cell line [98]. Given the large, 90kDa, size of FAP, it is certainly possible that other unidentified binding partners exist and loss of these interactions in the FAP KO mouse may be important.

One such interaction that could be important is the ability of FAP to form heterodimers with DPPIV [206]. This complex is reported to be significantly more efficient than either enzyme alone at degrading ECM. Interestingly, ectopic expression of a catalytically dead DPPIV has previously been shown to cause FAP expression in melanoma cells, suggesting expression of these proteins might be linked through non-enzymatic mechanisms [219]. Whether this relationship is reciprocal and FAP expression can drive DPPIV expression is unknown. The FAP KO mouse has lower plasma levels of H-Gly-Pro-AMC activity than WT mice, but this molecule is also a substrate of FAP making it impossible to determine if the decrease is due to loss of FAP or reduced plasma levels of

DPPIV [210]. If loss of FAP does indeed reduce DPPIV expression, either in its soluble or membrane bound form, this could certainly explain the phenotypic similarities between the FAP KO and DPPIV KO mice.

4.2.3 The Role of FAP in Adipose Tissue

Our observation of elevated FAP activity in the adipose tissue of obese mice is consistent with the induction of FAP in other tissue remodeling and inflammatory processes [100]. Obesity results in a variety of pathological changes in the adipose tissue where initial adipocyte hypertrophy is followed by immune cell infiltration, particularly by macrophages as well as CD8+ and regulatory T-cells [173]. Infiltrating macrophages adopt an M1 phenotype characterized by secretion of the pro-inflammatory cytokine TNFα [185].

This expansionary and inflammatory process that occurs in adipose tissue as a consequence of obesity could theoretically lead to upregulation of FAP in a number of different ways. TNFα is reported to upregulate FAP on smooth muscle cells in culture and a similar effect could occur within the adipose tissue [105]. Alternatively, FAP could be expressed on infiltrating macrophages as has been documented in human breast cancer [103]. Another possibility is that FAP is expressed on adipocyte progenitor cells. Recent work has indicated that FAP is expressed on de-differentiated adipocytes in cell culture [34]. High-fat feeding has been shown to increase the formation of crown-like structures in the adipose consisting of lipid-laden macrophages surrounded by dead adipocytes [7]. These structures are eventually cleared and replaced with new adipocytes. In fact, during high-fat feeding, up to 80% of adipocytes in murine epididymal deposits may die and be

replaced in only a few weeks [8]. Thus elevated expression of FAP in the epididymal adipose of DIO mice may reflect increased number of precursor cells required to sustain the increased adipocyte turnover. Further work using immunohistochemistry or FACs analysis could potentially shed light on the types of cells expressing FAP in adipose tissue.

Additional investigation into the functional role, if any, of FAP in the adipose tissue is also warranted. While it is possible that FAP upregulation in the adipose tissue of DIO mice is simply a marker of the changes that occur in adipose tissue as a result of obesity, it is more likely that FAP is an active participant given the phenotype of the FAP KO mouse. The dynamic adipose tissue remodeling that occurs during the DIO process is known to induce other proteases involved in ECM remodeling [9]. Interestingly, the expression pattern of the protease cathepsin K is similar to that of FAP as the expression of both increase in the adipose tissue as a result of obesity [23]. Furthermore, cathepsin K, as well as cathepsin L and MMP2 KO mice display reduced adiposity and improved glucose metabolism, a phenotype also shared by FAP KO mice on a high-fat diet [24, 46, 115]. A lack of these proteases presumably affects the plasticity of the adipose ECM and it is possible that a lack of FAP would have a similar effect. For instance, FAP enzymatic activity could be required for proper collagen metabolism, needed for supporting adipocyte expansion during the DIO process. However, our data suggests that a lack of FAP enzymatic activity is likely not responsible for the FAP KO mouse phenotype. Therefore, FAP might instead be important as a binding partner for coordination of other protease systems. Future work utilizing adipose tissue-specific knockout of FAP could

help determine the contribution, if any, of adipose-derived FAP to the phenotype of the FAP KO mouse.

4.2.4 The OGTT Effect of 5057

Though FAP inhibition with 5057 failed to recapitulate many of the aspects of the FAP KO mouse, it did have a very clear effect on oral glucose tolerance. This decrease in glucose excursion could be observed in both lean and obese mice following 5057 administration. This result diverges from the FAP knockout phenotype, which only differs from WT mice upon high-fat feeding. The effect of 5057 on glucose tolerance was also short-lived, requiring dosing of the inhibitor less than 2 hours before glucose challenge for an effect to be observed. Additionally, a high dose of a second FAP inhibitor did not lead to a statistically significant improvement in glucose tolerance. Together this data suggests that 5057 may be hitting an off-target.

Though DPPIV is perhaps the most obvious potential off-target of 5057, our data demonstrates that this is not the case, first and foremost, based on fact that 5057 does not significantly inhibit plasma DPPIV activity at the chosen dosages. This result does not eliminate the possibility of DPPIV inhibition in gut, but the observation that the OGTT effect of the DPPIV inhibitor sitagliptin is additive to that of 5057 suggests that 5057 alone does not significantly inhibit gut DPPIV. Nevertheless, the short time window required for the OGTT effect, and the fact that no effect on oral glucose tolerance is observed with subcutaneous dosing of 5057, seems to implicate the gut as a possible site of action.

One possibility is that the compound is simply slowing glucose absorption in the gut, however the 20mpk dose of 5057 is between 125 and 250 times smaller, by weight, relative to the glucose that is administered during the OGTT procedures. This fact makes it highly unlikely that the bulk of 5057 could significantly affect glucose absorption. Additionally, preliminary data from our collaborators demonstrates that 5057 is also efficient in reducing glucose excursion when administered orally prior to an intraperitoneal glucose tolerance test (IPGTT). Because the absorption of glucose in an IPGTT occurs in the peritoneal cavity, separate from the absorption of 5057 in the gut, this result all but eliminates the possibility of 5057 slowing glucose absorption.

A second possibility is that 5057 is acting in the gut to stimulate the release of incretin hormones. A number of nutrients including fats and sugars have been shown to stimulate GLP-1 release from the L-cells of the intestines and it is possible that 5057 could mimic one or more of these nutrients [184]. The induction of GLP-1 secretion from nutrients is thought to be dependent on direct contact with the L-cells, which requires these molecules to reach the distal intestine [50, 121]. It is possible that 5057, which must be dosed as a suspension due to its poor solubility in aqueous solvents, is able to reach the distal intestine and act as a GLP-1 or GIP secretagogue. If 5057 is able to reach the distal intestine before being absorbed due to its poor solubility, this could potentially explain why 3099, which is readily water soluble, has no OGTT effect. Another indication that the effect of 5057 on glucose tolerance may be due to enhancement of the incretin effect is the fact that compound administration appears to have no effect on fasting blood

glucose and no ability to cause hypoglycemia. This is consistent with an incretin based mechanism, where B-cell-induced insulin secretion is dependent on elevated blood glucose concentrations.

Finally, it cannot be ruled out that 5057 is exerting its effects on glucose tolerance through inhibition of FAP. Nearly complete FAP inhibition in the plasma may be required to observe an OGTT effect with 5057 and this could explain the short-lived nature of its blood glucose-lowering effects. Alternatively, 5057 may only be capable of inhibiting FAP in the relevant tissue for a brief period. The fact that 3099 fails to improve glucose tolerance could also be due to shortcomings of this compound in its ability to inhibit FAP activity in the relevant tissue or fluid.

All of these questions surrounding the OGTT effect of 5057 can be addressed by further investigation of its mechanism. Plasma concentrations of GLP-1 and GIP could be assessed following dosing with 5057 to determine if 5057 is acting as an incretin secretagogue. Additionally, performing an OGTT in GLP-1 and GIP double-receptor knockout mice could provide further evidence as to the involvement of these incretins [67]. The most critical future experiment is determining if the OGTT effect of 5057 is lost or retained in FAP KO mice. If the FAP KO mice retain the OGTT effect, then this phenomenon can be assigned to an off-target. Alternatively, if the effect is lost in these mice, then FAP inhibition is likely to be responsible. If this is the case, methods to extend the duration of FAP inhibition should be explored and FAP inhibitors could potentially be advanced as a novel therapy for diabetes.

Regardless of the mechanism, the OGTT effect of 5057 is quite interesting simply due to the fact that it is additive to DPPIV inhibition. On its own, 5057 improves glucose tolerance by nearly the same degree as sitagliptin. However together, the compounds reduce glucose excursion by nearly 90%, which is substantially better than DPPIV inhibitors and is on par with injectable GLP-1 analogs [37]. Thus the combination of DPPIV inhibition and the mechanism of 5057 could potentially provide an oral alternative with similar efficacy to injectable GLP-1 therapies. However, the duration of action of 5057 would likely need to be substantially improved in order to make a clinically useful therapy.

4.3 Conclusions

This work has interrogated the role of FAP enzymatic activity in both diabetes and cancer. In our chosen models, FAP inhibition had no effect in either pathology, with the possible exception of a short-lived effect on glucose tolerance. This calls into question the validity of FAP enzymatic activity as a therapeutic target, particularly with respect to epithelial cancers. It remains possible that FAP inhibition could suppress tumor growth under different conditions, however, and while FAP was not an anti-cancer target in the CT-26 model, the work we have done characterizing the pharmacodynamics of various specific inhibitors *in vivo* should translate well to future studies.

Similarly, we report the characterization of DPP8/9 and PREP-specific inhibitors, and demonstrate the ability of these compounds to reach their respective targets in *ex vivo*

tumor homogenates. This work will also provide a blueprint for future use of these inhibitors *in vivo*. However, none of the specific inhibitors tested were able to recapitulate either the cytokine-inducing or anti-cancer effects of the dipeptide boro-prolines, suggesting additional or unidentified targets many exist that are required to engage this immune-stimulatory mechanism.

Our work also implicates PREP as an important enzyme in cancer progression based on the accelerated CT-26 tumor growth observed in mice treated with 3531. This is in contrast to 5332, a PREP-specific inhibitor *in vitro*, that we show is converted to Arg-boroPro *in vivo*. Both of these discoveries may help guide future development of the dipeptide boro-prolines as PREP is now implicated as an anti-target of the dipeptide boro-prolines, while 5332 may provide a pathway for developing dipeptide boro-proline inhibitors with an enhanced therapeutic window.

We also report the finding that FAP is upregulated in the adipose tissue of DIO mice and cleaves a number of metabolically active peptide and protein substrates, yet an FAP-specific inhibitor fails to recapitulate nearly all aspects of the obesity and diabetes resistant phenotype of the FAP KO mouse with the exception of the ability to improve glucose tolerance. Though future work remains to determine if this effect is due to inhibition of FAP or another target, this result is nonetheless interesting due to the OGTT effect of 5057 which is additive to DPPIV inhibitors.

In conclusion, the work presented here has largely served to dispute, rather than confirm, past assertions that loss of FAP catalytic activity has an effect on either tumor growth or the development of diabetes and obesity *in vivo*. While its unique distribution profile, particularly in tumor stroma and adipose tissue, certainly suggests that it likely has a critical role in these pathologies, the identity of that role remains elusive. At the very least, the detailed characterization of specific inhibitors carried out here should prove useful in future studies of this enzyme.

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