Canstatin, a Novel Matrix-derived Inhibitor of Angiogenesis and Tumor Growth*

(Received for publication, August 18, 1999, and in revised form, September 29, 1999)

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We isolated and identified an endogenous 24-kDa human basement membrane-derived inhibitor of angiogenesis and tumor growth, termed canstatin. Canstatin, a fragment of the α2 chain of type IV collagen, was produced as a recombinant molecule in Escherichia coli and 293 embryonic kidneys cells. Canstatin significantly inhibited human endothelial cell migration and murine endothelial cell tube formation. Additionally, canstatin potently inhibited 10% fetal bovine serum-stimulated endothelial cell proliferation and induced apoptosis, with no inhibition of proliferation or apoptosis observed on non-endothelial cells. Inhibition of endothelial proliferation was not concomitant with a change in extracellular signal-regulated kinase activation. We demonstrate that apoptosis induced by canstatin was associated with a down-regulation of the anti-apoptotic protein, FLIP. Canstatin also suppressed in vivo growth of large and small size tumors in two human xenograft mouse models with histology revealing decreased CD31-positive vasculature. Collectively, these results suggest that canstatin is a powerful therapeutic molecule for suppressing angiogenesis.

Vascular basement membranes are composed of macromolecules such as type IV collagen, laminin, heparan sulfate proteoglycans, fibronectin, and entactin (1). Type IV collagen is composed of six distinct gene products, namely α1–α6 (2). The α1 and α2 isoforms are ubiquitously present in human basement membranes (3), whereas the other four isoforms exhibit restricted distributions (4). Type IV collagen promotes cell adhesion, migration, differentiation, and growth (3) and via these functions may play a crucial role in angiogenesis, the process of formation of new blood vessels from pre-existing capillaries (5, 6). In the adult, new blood vessels arise via angiogenesis, a process critical for normal physiological events such as wound repair and endometrium remodeling (7). It is now well documented that angiogenesis is required for metastasis and growth of solid tumors beyond a few mm³ in size (6, 8). Several studies have shown that inhibitors of collagen metabolism have anti-angiogenic properties, supporting the notion that basement membrane collagen synthesis and deposition is crucial for blood vessel formation and survival (9, 10). Additionally, basement membrane organization is dependent on the assembly of a type IV collagen network, which is speculated to occur via the C-terminal globular non-collagenous (NC1) domain of type IV collagen (11, 27, 28). Based on these observations, we postulated that the NC1 domain of collagen type IV could disrupt tumor angiogenesis and thereby inhibit tumor growth. Our laboratory evaluated the anti-angiogenic potential of all six NC1 domains of type IV collagen, and in the present study, we demonstrate the ability of the NC1 domain of the α2 chain of type IV collagen, which we have named canstatin, to inhibit endothelial cell tube formation, migration, and proliferation and to induce apoptosis in vitro, as well as suppress tumor growth in vivo.

MATERIALS AND METHODS

Recombinant Production of Canstatin in Escherichia coli—The sequence encoding canstatin was amplified by PCR³ from the α2 NC1 (IV)pDBS vector (12) using a forward primer (5'-CGGGATCCTGTCAG- CATCGGCTACCTC-3') and a reverse primer (5'-CCAAAGCTTCAG- GTTCTG ATGCACAC-3'). The resulting cDNA fragment was digested with BamHIII and HindIIIIII and ligated into predigested pET22b (+) (Novagen, Madison, WI). This placed canstatin downstream of and in-frame with the pelB leader sequence, allowing for periplasmic localization and expression of soluble protein. Additional vector sequences encode the amino acids MDIGNSD. The 3' end of the sequence was ligated in-frame with the polyhistidine tag sequence. Additional vector sequence between the 3' end of the cDNA and the His tag encoded the amino acids KLAAALE. Positive clones were sequenced on both strands.

Plasmid constructs encoding canstatin were first transformed into E. coli HMS174 (Novagen, Madison, WI) and then transformed into BL21 for expression (Novagen, Madison, WI). An overnight bacterial culture was used to inoculate a 500-ml culture in LB medium. This culture was grown for approximately 4 h until the cells reached an A600 of 0.6. Then, protein expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM. After a 2-h induction, cells were harvested by centrifugation at 5,000 × g and lysed by resuspension in 6 M guanidine, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0. Resuspended cells were sonicated briefly and centrifuged at 12,000 × g for 30 min. The supernatant fraction was passed over a 5-ml of nickel-nitrotriacetic acid-agarose column (Qiagen) 4–6 times at a speed of 2 ml/min. Nonspecifically bound protein was removed by washing with 15

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* This work was supported in part by National Institutes of Health Grants DK-51711, DK-55001 (to R. K.), and CA-74401 (to J. W. M.), 1998 Hershey Prostate Cancer Research award (to R. K.), 1998 ASN Carl Gottschalk Research award (to R. K.), 1998 NKF Murray award (to R. K.), 1998 BIDMC Enterprise award (to R. K.), and research funds from the Beth Israel Deaconess Medical Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PCR, polymerase chain reaction; ERK, extracellular signal-regulated kinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CPAE, calf pulmonary arterial endothelial; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; FCS, fetal calf serum; MAA, mouse aortic endothelial; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; HUVEC, human umbilical endothelial cell; VEGF, vascular endothelial growth factor; HEK, human embryonic kidney.
ml of each of 10, 25, and 50 mm imidazole in 8 mM urea, 0.1 mM NaH2PO4, 0.01 mM Tris-HCl, pH 8.0. Canstatin protein was eluted from the column with two concentrations of imidazole (125 and 250 mM) in 8 mM urea, 0.1 mM NaH2PO4, 0.01 mM Tris-HCl, pH 8.0. The eluted protein was dialyzed twice against 2 liters of PBS at 4 °C. A portion of the total protein present after dialysis. Dialyzed canstatin was collected, precipitated during dialysis. Dialyzed protein was collected and centrifuged at approximately 3,500 × g and separated into pellet and supernatant fractions. Protein concentration in each fraction was determined by the BCA assay (Pierce) and quantitative (densitometric) SDS-PAGE analysis. The ratio of total protein in the pellet was approximately 40%, with the remaining 60% recovered as a soluble protein. The total protein yield was approximately 15 mg/liter.

Expression of Canstatin in 293 Embryonic Kidney Cells—We used the pDS plasmid containing α2(IV)NC1 (12) to PCR-amplify canstatin in a way that it would add a leader signal sequence in-frame into the α2(IV) chain was cloned into the lower chamber as a chemotactant. The cell-containing compartments were separated from the chemotactic polycarbonate filters (Poretics, CA) of 8-μm pore size. The chamber was incubated at 37 °C with 5% CO2 and 95% humidity for 4.5 h. After discarding the non-migrated cells and washing the upper wells with PBS, the filters were scraped with a plastic blade, fixed in 4% formaldehyde in PBS, and placed on a glass slide. By using a fluorescent high power field, several independent homogenous images were recorded by a digital SenSys™ camera operated with Image Processing Software PMIS (Photometrics, CO). Cells were counted by employing the OPTIMITE 6.0 software program (BioScan, WA) (16).

ERK Phosphorylation Assay—HUVEC cells were cultured overnight in McCoy's medium supplemented with 20% FBS, 1% penicillin/streptomycin, 50 μg/ml endothelial mitogen (Bio-Rad Laboratories). The following day, cells were treated as above, and then cells were counted using the method of trypsinization (Life Technologies, Inc.) at 37 °C for 5 min. A suspension of 12,500 cells in DMEM with 0.5% FCS was added to each well of a 24-well plate with 10 μl/ml fibronectin. The cells were incubated for 24 h at 37 °C with 5% CO2 and 95% humidity. Medium was removed and replaced with DMEM containing 5% FCS (unstimulated control cells). Additional following day, 60,000 cells were seeded on the upper chamber was incubated at 37 °C with 5% CO2 and 95% humidity for 4.5 h. After discarding the non-migrated cells and washing the upper wells with PBS, the filters were scraped with a plastic blade, fixed in 4% formaldehyde in PBS, and placed on a glass slide. By using a fluorescent high power field, several independent homogenous images were recorded by a digital SenSys™ camera operated with Image Processing Software PMIS (Photometrics, CO). Cells were counted by employing the OPTIMITE 6.0 software program (BioScan, WA) (16).

Inhibition of Endothelial Cell Proliferation—Bovine calf pulmonary aortic endothelial (CAE) cells were grown to confluence in DMEM with 10% fetal bovine serum (FBS) and kept confluent for 48 h. Cells were harvested by trypsinization (Life Technologies, Inc.) at 37 °C for 5 min. A suspension of 12,500 cells in DMEM with 0.5% FCS was added to each well of a 24-well plate coated with 10 μg/ml fibronectin. The cells were incubated for 24 h at 37 °C with 5% CO2 and 95% humidity. Medium was removed and replaced with DMEM containing 5% FCS (unstimulated control cells). Additional following day, 60,000 cells were seeded on the upper chamber was incubated at 37 °C with 5% CO2 and 95% humidity for 4.5 h. After discarding the non-migrated cells and washing the upper wells with PBS, the filters were scraped with a plastic blade, fixed in 4% formaldehyde in PBS, and placed on a glass slide. By using a fluorescent high power field, several independent homogenous images were recorded by a digital SenSys™ camera operated with Image Processing Software PMIS (Photometrics, CO). Cells were counted by employing the OPTIMITE 6.0 software program (BioScan, WA) (16).

ERK Phosphorylation Assay—HUVEC cells were cultured overnight in McCoy's medium supplemented with 20% FBS, 1% penicillin/streptomycin, 50 μg/ml endothelial mitogen (Bio-Rad Laboratories). The following day, growth factor-containing medium was removed and replaced with medium containing 20% serum alone (with or without 20 μg/ml canstatin) or medium with 20% serum and growth factors (with or without 20 μg/ml canstatin). All wells received polymyxin B to eliminate potential effects of endotoxin. Cells were lysed at 30, 60, and 180 min with passive lysis mix (Promega, Madison WI) plus leupeptin, phenylmethylsulfonyl fluoride, NaF, Na3VO4, β-glycerophosphate, and sodium pyrophosphate. Lysates were quantified for protein concentration and separated on 12% SDS-PAGE gels. Western blots of serum or serum + canstatin-treated HUVEC lysates were performed to detect levels of phospho-ERK using anti-phospho-ERK antibodies (New England Biolabs, Beverly, MA). Total ERK protein was detected on the same nitrocellulose membrane with anti-ERK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) illustrating equal amounts of total ERK protein.

FLIP Protein Level Quantification—HUVEC cells were cultured overnight in McCoy's medium supplemented with 20% FBS, 1% penicillin/streptomycin, 100 μg/ml heparin, and 50 μg/ml endothelial mitogen (Bio-Rad Laboratories). The following day, growth factor-containing medium was removed and replaced with medium containing 20% serum alone (with or without 20 μg/ml canstatin) or medium with 20% serum and growth factors (with or without 20 μg/ml canstatin). All wells received polymyxin B to eliminate potential effects of endotoxin. Cells were lysed at 30, 60, and 180 min with passive lysis mix (Promega, Madison, WI) plus leupeptin, phenylmethylsulfonyl fluoride, NaF, Na3VO4, β-glycerophosphate, and sodium pyrophosphate, and the lysates were analyzed for FLIP levels by Western blot with anti-FLIP antibody (17).

Annexin V-FITC Binding—0.5 × 106 CAE cells were added to each well of a 6-well tissue culture plate in 10% FBS-supplemented DMEM (BioWhittaker, Walkersville, MD) overnight. The next day the fresh medium was added to all wells together with 15 μg/ml canstatin or 40 ng/ml tumor necrosis factor-α. Control cells received an equal volume of PBS. After 24 h of treatment medium containing detached cells was collected; and attached cells were trypsinized and added together with detached cells and centrifuged at 3,000 × g. Cells were then washed and phosphatidylserine externalization (an early apoptotic indicator) was measured by labeling with FITC-labeled annexin V (CLONTECH, Palo Alto, CA) according to manufacturer's instructions. Annexin-V-FITC-labeled cells were counted using a Becton-Dickenson FACScan Plus flow cytometer. For each treatment 15,000 cells were counted and stored in listmode. The data were then analyzed using standard Cell Quest software.

In Vivo Tumor Studies—Human prostate adenocarcinoma cells (PC-3) were harvested from culture, and 2 × 106 cells were subcutaneously implanted into each flank. Subsequently, mice were treated with either canstatin, BSA, sterile PBS, or α5-NC1 domain in increasing concentrations. All assays were performed in triplicate. Cells were incubated for 24–48 h at 37 °C and viewed using a CK2 Olympus microscope (3.3 ocular, 10× objective). The cells were then photographed using 400 DK-coated TMAX film (Eastman Kodak Co.). Cells were stained with diff-quick fixative (Sigma) and photographed again (15). Ten fields were viewed, and tubes were counted and averaged.

Migration Assay—The inhibitory effect of canstatin on VEGF-induced chemotaxis was tested on human umbilical endothelial cells (HUVECs) using the Boyden chamber assay (Neuroprobe, MD). HUVECs were grown in M199 containing 0.5% FBS, 5 mg/ml DiIC18(3) living fluorescent stain (Molecular Probes, Eugene, OR) overnight. After trypanosmizing, washing, and diluting cells in M199 containing 0.5% FBS, 60,000 cells were seeded on the upper chamber wells, together with or without canstatin (0.01 or 1 μg/ml). M199 medium containing 1% FBS plus 10 ng/ml VEGF (R & D Systems, Minneapolis, MN) was placed in the lower chamber as a chemotactant. The migrating cells were counted using a fluorescent microscope and a motorized stage.
nude mice, and tumors grew for approximately 2 weeks after which animals were divided into groups of 4 mice. Experimental groups were injected daily intraperitoneally with canstatin at a dosage of 3 mg/kg in a total volume of 0.2 ml of PBS or recombinant mouse endostatin at a dosage of 8 mg/kg in the same volume of PBS. The control group received equal volumes of PBS each day. Tumor length and width were measured using a Vernier caliper, and the tumor volume was calculated using the standard formula length × width squared × 0.52 (18). Vol-

RESULTS AND DISCUSSION

Human canstatin was produced in E. coli as a fusion protein with a C-terminal six histidine tag using pET-22b(+), a bacterial expression plasmid, and also produced as a secreted soluble protein in 293 embryonic kidney cells using the pcDNA 3.1 eukaryotic vector. The E. coli-expressed protein was isolated predominantly as a soluble protein, and SDS-PAGE analysis revealed a monomeric band at 27 and 3 kDa that arises from polylinker and histidine tag sequences. Un cleaved signal peptide may account for additional molecular weight seen in some preparations (Fig. 1, A and B). Canstatin protein was analyzed by SDS-PAGE and visualized using the standard formula length × width squared × 0.52 (18). Vol-

Canstatin and Angiogenesis

Fig. 1. Recombinant production of canstatin in E. coli. A, the sequence encoding canstatin was amplified by PCR from the a2 NCI (IV)pD vector (12) using the given primers. The resulting cDNA fragment was digested with BamHI and HindIII and ligated into pre-
digested pET22b(+) (Novagen, Madison, WI). B, Coomassie-stained 12% SDS-
PAGE showing different stages of His-
tagged canstatin nickel-nitrilotriacetic acid-agarose column (Qiagen) purification as follows: lane 1, undigested bacterial ly-
sate; lane 2, isopropyl-1-thio-β-D-galacto-
pyranoside-induced bacterial lysate; lane 3, 10 mM imidazole wash; lane 4, 25 mM imidazole wash; lane 5, 50 mM imidazole wash; lane M, prestained low range molecular weight standards (Bio-Rad); lane 6, 125 mM elution; lane 7, 250 mM elution. 125 and 250 mM elutions were combined and dialyzed against PBS for use in as-
asays. Sizes of visible molecular mass markers (kDa) are given left of the figure. C, anti-polyhistidine tag antibody Western blot of purification fractions as fol-

lows: lanes 1–7 are the same as B. D, anti-polyhistidine tag antibody Western blot of dialyzed protein as follows: lane 1, soluble fraction of dialyzed purified can-

statin, non-reducing conditions; lane 2, insoluble fraction of dialyzed purified can-

statin, non-reducing conditions; lane 3, soluble canstatin, reducing conditions; lane 4, insoluble canstatin, reducing con-
ditions. E, affinity chromatogram of 293 embryonic kidney cell-expressed cansta-
tin. Conditioned medium containing se-
creted canstatin was subjected to affinity chromatography using canstatin-specific antibodies (13), and fractions were collected. A major peak was identified, con-
taining a monomer of about 24 kDa. The arrow indicates the point where canstatin fraction collection began. F, anti-a2 NC1 antibody (1:200 dilution), Western blot of 6-fold concentrated 293 human embryonic kidney cell-conditioned medium. Lane M, molecular mass marker; lane 1, cansta-
tin-transfected 293 cells; lane 2, untrans-
fected 293 cells; lane 3, empty vector transfection 293 cells; lane 4, a3 NC1 transfection 293 cells; lane 5, affinity pu-
rified canstatin.

Canstatin Antibodies—Canstatin antibodies were detected by Western blotting with an anti-polyhistidine tag antibody (Fig. 1, C and D). Canstatin antibodies also detected
bacterially expressed recombinant canstatin protein (data not shown). The 293 cell-produced recombinant protein (without any purification or detection tags) was isolated using affinity chromatography, and a pure monomeric form was detected in the major peak by SDS-PAGE and immunoblot analyses (Fig. 1, E and F). In addition, human canstatin was isolated from human placenta by gel filtration, high pressure liquid chromatography, and affinity chromatography techniques; a 24-kDa molecule was detected by SDS-PAGE and immunoblot analyses (data not shown).

We tested the ability of canstatin to disrupt endothelial cell tube formation in matrigel, a solid gel of mouse basement membrane proteins derived from sarcomas. When mouse aortic endothelial cells are cultured on matrigel, they rapidly align and form hollow tube-like structures (15). Canstatin, produced in 293 cells, selectively inhibited endothelial tube formation in a dose-dependent manner, with a near-complete inhibition of tube formation seen with the addition of 1 μg of canstatin protein (Fig. 2, A and C). Neither a control protein, bovine serum albumin (BSA), nor the NC1 domain of type IV collagen α5 chain had an effect on endothelial tube formation (Fig. 2, A and D).

During the process of angiogenesis, endothelial cells proliferate and migrate to form new blood vessels. Therefore, we assessed the effect of canstatin on endothelial cell migration. HUVECs were cultured in a Boyden chamber and stimulated to migrate toward a 10 ng/ml VEGF gradient (16). Canstatin, produced in 293 cells, inhibited the migration of HUVECs with a significant effect observed at 10 ng/ml (Fig. 2, E and F). The ability of canstatin to inhibit both proliferation and migration of endothelial cells may indicate that it works at more than one step in the process of angiogenesis or that the effect is common to both migratory and proliferative processes.

In thymidine incorporation assays of endothelial cell proliferation, using E. coli produced soluble protein, a dose-dependent inhibition of 10% serum-stimulated CPAE cells was detected with an ED50 value of 0.5 μg/ml (Fig. 3A). Furthermore, we found that HUVECs were also inhibited by canstatin with a similar ED50 value (Fig. 3B). These results support earlier observations that α1 and α2 type IV collagen chains isolated from the Engelbreth-Holm-Swarm sarcoma tumor may be inhibitory to capillary endothelial cell proliferation (19). No significant effect was observed on the proliferation of renal carcinoma cells (786-O), prostate cancer cells (PC-3), or human embryonic kidney cells (HEK 293) at canstatin doses up to 40 μg/ml (Fig. 3C). Methylene blue colorimetric proliferation assay confirmed the results of the thymidine assay for the CPAE, PC-3, and HEK 293 cells (data not shown).
In order to understand further the molecular mechanisms involved in the anti-proliferative and anti-migratory activities of canstatin, we assessed the effect of canstatin on ERK activation induced by 20% fetal bovine serum and endothelial mitogens. Fig. 3D shows that ERK phosphorylation in HUVECs was evident within 5 min after growth factor stimulation, and treatment with 20 μg/ml canstatin did not alter early activation of ERK. A decrease in ERK phosphorylation was observed at later time points, a profile that is consistent with responses observed with several mitogens (20, 21).

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Observations indicate that canstatin does not primarily work by inhibiting proximal events activated by VEGF or basic fibroblast growth factor receptors.

In order to establish apoptosis of endothelial cells as a potential mode of action for canstatin, we used annexin V-FITC labeling of externalized phosphatidylserine to assess for apoptotic cells. Our results show that canstatin specifically induces apoptosis of endothelial cells (Fig. 3E) with no significant effect observed on PC-3, 786-0, or HEK 293 cell lines (data not shown). It is probable that canstatin acts by predisposing cells to apoptosis, as evidenced by a steady decrease in FLIP protein levels during treatment with canstatin in the presence of both canstatin and Angiogenesis
serum and growth factor (basic fibroblast growth factor and VEGF) stimulation (Fig. 3F). Canstatin did not effect FLIP levels in the absence of growth factors. A transient increase in FLIP levels was seen with the addition of serum only medium, probably due to the removal of growth factors. In contrast, the addition of growth factor-containing medium induced a transient decrease in FLIP levels that returned to base line or increased in the absence of canstatin. Since endothelial cells express both Fas and FasL constitutively (17), it is likely that this decrease in FLIP sensitizes endothelial cells to the apoptotic signal. Furthermore, the lack of effect of canstatin on endothelial cells in the absence of growth factors indicates it may target angiogenic and not pre-formed endothelium.

For in vivo experiments, established xenograft tumor models in mice were used to test the effectiveness of canstatin as an inhibitor of angiogenesis-dependent tumor growth. Canstatin, produced as soluble protein in E. coli, inhibited the growth of small (Fig. 4A) and large (Fig. 4B) renal cell carcinoma (786-0) tumors by 4- and 3-fold with respect to placebo-treated mice. Established human prostate (PC-3) tumors in severe combined immunodeficient (SCID) mice or athymic (nu/nu) mice exhibited fractional tumor volumes of 1.8–2.4-fold less than placebo-treated mice when treated with 10 (Fig. 4C) or 3 mg/kg (Fig. 4D) canstatin, respectively. This decrease in tumor size was consistent with a decrease in CD31-positive vasculature (Fig. 4, E and F). A similar suppressive effect was observed with 8 mg/kg of endostatin (Fig. 4D). Further studies will be required to determine the minimal effective doses of these proteins, but results in our laboratory indicate that a 5 mg/kg dose of endostatin is not able to suppress tumor growth (data not shown), suggesting that human canstatin may be more potent than endostatin. In all of the in vivo studies, mice appeared healthy with no signs of wasting, and none of the mice died during treatment.

The ability of canstatin to inhibit specifically endothelial cell proliferation, migration, and tube formation strongly suggests that it is an anti-angiogenic agent and that it may function via a cell surface protein/receptor. Integrins are potential candidate molecules for binding to canstatin based on their extracellular matrix binding capacity and ability to modulate cell behavior such as migration and proliferation (22). In particular, α5β3 integrin is a possible canstatin receptor, due to its induction during angiogenesis and its promiscuous binding capacity (23). Also, integrins α1β1, and α2β1 have been implicated as cell adhesion receptors that bind to type IV collagen (24), but whether canstatin is the collagen IV domain that is mediating this interaction or any other integrin binding has yet to be determined. The recent identification of other collagen-derived inhibitors of angiogenesis, such as endostatin and resitin, C-terminal fragments of collagen XVIII and XV, respectively (18, 25, 26), lends credence to the theory that collagen matrix is critical to angiogenesis, although no significant sequence (<14% at the amino acid level) or structural homology is evident between canstatin and these other inhibitors. Furthermore, canstatin, endostatin, and resitin are non-collagenous fragments of collagen molecules underscoring the importance of this portion of the protein in mediating cell behavior (2, 18, 25). Future comparative studies with these inhibitors may also give insight into their unique mechanism of action.

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