Targeting eNOS in Pancreatic Cancer

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Abstract

Mortality from pancreatic ductal adenocarcinoma cancer (PDAC) is among the highest of any cancer and frontline therapy has changed little in years. Activation of endothelial nitric oxide synthase (eNOS, NOS3, or NOS III) has been implicated recently in the pathogenesis of PDACs. In this study, we used genetically engineered mouse and human xenograft models to evaluate the consequences of targeting eNOS in PDACs. Genetic deficiency in eNOS limited the development of preinvasive pancreatic lesions and trended toward an extended lifespan in mice with advanced pancreatic cancer. These effects were also observed upon oral administration of the clinically evaluated NOS small molecule inhibitor N\(^{-}\)-nitro-L-arginine methyl ester (L-NAME). Similarly, other transgenic models of oncogenic KRas-driven tumors responded to L-NAME treatment. Finally, these results were recapitulated in xenograft models of human pancreatic cancer, in which L-NAME was found to broadly inhibit tumorigenic growth. Taken together, our findings offer preclinical proof-of-principle to repurpose L-NAME for clinical investigations in treatment of PDACs and possibly other KRas-driven human cancers. Cancer Res; 72(17): 4472–82. ©2012 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has 5-year survival rates less than 5% (1). Current frontline treatment for advanced PDAC, gemcitabine, was introduced 13 years ago with only incremental progress made thereafter (2, 3). Thus, there is a need to develop new and less toxic therapeutic approaches that can be rapidly introduced into the clinic. In this regard, 90% of PDACs possess oncogenic mutations within the gene KRAS, which renders the encoded small GTPase constitutively GTP-bound and active (4). Oncogenic KRas can convert normal human ductal pancreatic epithelial cells to a tumorigenic state (5), and pancreatic expression of the activated oncogene leads to pancreatic intraepithelial neoplasm (PanIN) lesions that progress to PDAC in mice (6). Conversely, silencing oncogenic Ras in established tumors causes spontaneous regression (7). KRas is thus the initiating oncogene in PDAC, and like chronic myelogenous leukemia is addicted to BCR-Abl and hence sensitive to imatinib (8). pancreatic cancers required oncogenic KRas for continued growth.

Despite the pivotal role of oncogenic KRas in PDACs, it has proved challenging to inhibit (9). KRas exerts its tumorigenic functions by activating primarily 3 effector proteins, Raf kinases, phosphoinositide 3-kinases (PI3K), and Ral guanine nucleotide exchange factors. Pharmacologic inhibitors of the first 2 pathways reduce tumor growth in some cancers, with numerous clinical trials underway (4). As such, targeting druggable components of oncogenic KRas signaling is one potential strategy to treat PDAC.

Of the 3 KRas effectors, only active PI3K, or its principal target AKT kinases, maintains xenograft tumor growth upon silencing oncogenic Ras (10), suggesting that pancreatic cancer cells become addicted to PI3K/AKT signaling. Consequently, components of this pathway represent attractive targets. While the families of PI3K and AKT proteins are druggable, they are composed of highly related proteins involved in a large number of normal physiologic processes, and general inhibitors of these kinases can be toxic (11). However, the AKT substrate endothelial nitric oxide synthase (eNOS, NOS3, or NOS III; ref. 12) has been detected in the active state in human PDAC tissues (13). eNOS is a member of the NOS family additionally composed of neuronal NOS (nNOS, NOS1, or NOS I) and inducible NOS (iNOS, NOS2, or NOS II) that generate nitric oxide (12). Unlike AKT, eNOS plays a limited role in normal physiology, mainly in vasorelaxation (14), and eNOS\(^{-/-}\) mice are viable (15). Moreover, mounting evidence suggests that inhibition of eNOS has antitumor effects. Specifically, eNOS\(^{-/-}\) mice are resistant to 7,12-dimethylbenz(a)anthracene...
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Xenograft assays
– days of age or at moribundity endpoints), according to a Duke approach using a small-molecule NOS inhibitor. We thus whether this could be translated into a clinically relevant evaluated the impact of genetically and pharmacologically LSL-KrasG12D wild-type or null. KC and KPC mice were randomly assigned mice were generated in a similar manner except the drug L-NAME to treat PDACs. However, nitric oxide can the possibility that eNOS could be targeted by repurposing genetic ablation of eNOS (13). Thus, inhibiting eNOS may be a way to indirectly exploit the reliance of pancreatic cancer cells on oncogenic KRas for tumorigenesis. In this regard, the NOS inhibitor N\textsuperscript{\textbeta}-nitro-L-arginine methyl ester (\textbeta-NAME), which is moderately selective for eNOS and nNOS over iNOS (12), was developed and clinically evaluated in phase II trials for cardiogenic shock, and in numerous other clinical trials, including those involving normal healthy subjects (22). This drug is relatively benign when compared with conventional cytotoxic chemotherapy; the major side effect of chronic administration is hypertension (23). These findings support the possibility that eNOS could be targeted by repurposing the drug \textbeta-NAME to treat PDACs. However, nitric oxide can both inhibit and enhance tumorigenesis (24), and the effect of ablating the eNOS gene on PDAC was unknown. Even if genetic ablation of eNOS inhibited PDACs, it was unknown whether this could be translated into a clinically relevant approach using a small-molecule NOS inhibitor. We thus evaluated the impact of genetically and pharmacologically inhibiting eNOS on PDAC.

Materials and Methods

Cell lines
AsPC-1, CAPAN-1, CF Pac-1, HPAC, HPAF-II, MiaPaCa-2, PAN C-1, and SW-1990 (purchased from American Type Culture Collection) were not independently authenticated. Tumor tissue from KPC mice was minced in collagenase V (Sigma-Aldrich) for 30 minutes at 37°C, after which cells were cultured in Dulbecco’s Modified Eagle’s Media (DMEM) + 10%FBS for 4 passages.

Mouse pancreatic cancer models
Pdx-1-Cretg\textsuperscript{+/+} (6); eNOS\textsuperscript{+/+} (15) mice were interbred with LSL-KrasG12D\textsuperscript{+/-} (25); eNOS\textsuperscript{+/-} to generate eNOS\textsuperscript{+/+} and eNOS\textsuperscript{-/-} KC (LSL-Kras\textsuperscript{G12D/+}; Pdx-1-Cre\textsuperscript{+/+}) littermates. KPC [LSL-Kras\textsuperscript{G12D/+}; Pdx-1-Cre\textsuperscript{+/+}; LSL-Tp53\textsuperscript{R172H/+}; ref. (26)] mice were generated in a similar manner except the final step consisted of crossing littermates that were either eNOS wild-type or null. KC and KPC mice were randomly assigned to received water untreated or treated with 1 g/L \textbeta-NAME (Sigma-Aldrich; ref. 27) after weaning until endpoints (330 ± 7 days of age or at moribundity endpoints), according to a Duke Institutional Animal Care and Use Committee-approved protocol.

Xenograft assays
Cells (\texttimes 10^7) suspended in 100 µL of Matrigel (BD Biosciences) were injected subcutaneously into flanks of severe combined immunodeficient (SCID)/beige mice (Charles River) and resultant tumors measured 3 times weekly (28). Mice were treated with 1 g/L \textbeta-NAME (see above) beginning on the day of xenograft injection, or once tumors reached a size of 0.75 cm\(^2\), with 120 mg/kg (intraperitoneally) gemcitabine (Eli Lilly) twice weekly for 2 weeks once tumors reached a size of 0.75 cm\(^2\), or with 10 mg/kg (intraperitoneally) amloidine (Sigma) 5 times weekly as previously described (29), beginning on the day of xenograft injection. Equal volumes of PBS were injected in control mice.

Human PDAC tissue samples
Resected archived primary PDAC specimens were provided for eNOS immunohistochemical analysis devoid of all identifying information, in accordance with Institutional Review Board protocols.

Grading of ductal lesions
Hematoxylin and eosin (H&E)-stained histologic sections were reviewed by 2 pathologists (M.J. Shealy and D.M. Cardona) blinded to the experimental groups. Examined slides consisted of a single longitudinal section of pancreas (head to tail) with adjacent small intestine and spleen from each mouse. Quantification of mouse PanIN lesions was accomplished by first determining the total number of anatomic pancreatic lobules per specimen. Lobules were counted and subsequently evaluated if they contained at least a single identifiable duct and surrounding circumscribed pancreatic acini and/or fibrosis. Within each lobule, the highest grade mouse PanIN lesion (normal, 1A, 1B, 2, or 3) was identified (30). Quantification of normal acinar area was determined in a blinded fashion from typically 5 randomly identified high-power fields from 15 or more pancreatic sections and expressed as a percentage of the total area.

Mouse squamous papilloma and carcinoma analysis
Vulvar papillomas were excised from KC mice at time of necropsy and weighed. Total number of new facial papillomas arising until endpoints were determined for each mouse.

eNOS immunohistochemistry
Heat-induced epitope retrieval was conducted on H&E-stained sections followed by staining with an \textalpha-eNOS (1:70, Assay Designs 905-386) antibody (Vectastain Elite ABC kits, Vector Labs, were used for peroxidase-based detection). Photographs taken from the areas of the strongest immunoreactivity were qualitatively assessed within the vessels (internal control), duct epithelium, stroma, pancreatic acini, and adenocarcinoma using a 4-tier scale (0–3+) in a blinded fashion by a pathologist (D.M. Cardona).

CD31 immunohistochemistry
Heat-induced epitope retrieval was conducted on H&E-stained sections followed by staining with an \textalpha-CD31 (1:100 Abcam ab2864 or 1:50 BD Pharmingen 350274) antibody (Vectastain Elite ABC kits, Vector Labs, were used for peroxidase-based detection). Usually 5 random high-power field images of pancreatic sections from 6 eNOS\textsuperscript{+/+} and 5 eNOS\textsuperscript{-/-} KPC mice each at moribundity endpoints were analyzed using ImageJ.
software to quantitate pixels of CD31 reactivity per field and an average calculated.

**Ki67 immunohistochemistry**
Heat-induced epitope retrieval was conducted on H&E-stained sections followed by staining with an αKi67 (1:50 Dako M7249) antibody (Vectorstain Elite ABC kits, Vector Labs, were used for peroxidase-based detection). Ki67 immunoreactive cells were counted from 4 to 5 random high-power fields of CFPac-1 xenograft tumor sections from 7 tumors each from mice treated or untreated with 1 g/L t-NAME and an average calculated.

**PCR of KRas alleles**
DNA, purified from pancreatic, facial papilloma, or vulvar tumor tissue, was PCR amplified to detect wild-type and recombinant KRas alleles, as described previously (31).

**Reverse transcription PCR of eNOS mRNA**
RNA was purified from pancreatic tissue or tumor cell lines established from pancreatic tumors of KC or KPC mice in the absence or presence of the eNOS gene using the RNeasy reagent (Qiagen) and PCR amplified with the primers 5′-TCTTCCATCAGAGATGGTCAA-3′ and 5′-CTCATCTCATCATGCACAGG-3′ to detect eNOS and 5′-GCACAGTCAGGCGGAGAT-3′ and 5′-GCCTTCTCCATGTCGGTTGA-3′ to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Blood pressure measurements**
The average daily cohort blood pressure was determined by averaging the average of the last 10 of 15 blood pressure measurements from each conscious mouse in the cohort using a computerized tail cuff monitor (Hatteras Industries) as previously described (32). Average daily blood pressure was calculated from measurements taken on 26 days over an 8-week span.

**eNOS and HRas activation status**
CFPac-1 cells transfected with 6 μg pcMV-neo-HA-eNOS using Fugene 6 (Roche) were treated for 2 hours with dimethyl sulfoxide or 20 μmol/L LY294002 (Cell Signaling Technologies). These cells, KPC cell lines grown overnight in 0.5% fetal calf serum, or subcutaneous tumors derived from CFPac-1 cells in cohorts of mice untreated or treated with t-NAME were lysed in radioimmunoprecipitation assay (RIPA) buffer, and resolved by SDS-PAGE and probed using αAKT, αSer1177 Phospho-eNOS or αSer473 Phospho-akt (Cell Signaling Technology) or αHRas (Santa Cruz) antibodies. GTP-bound HRas was detected as previously described (13). Quantitation of phospho-eNOS and HRas-GTP levels was done using ImageJ software. Results were normalized to total AKT and total HRas levels, respectively.

**t-NAME dose titration**
Twenty-four hours after HPAF-II, PANC-1, or CAPAN-2 cells were plated (4,000 cells per well), cells were treated with 2 μL t-NAME in an 11-point dilution standard curve with concentrations ranging from 10 μmol/L diluted 1:3 down to 0.2 nmol/L then 24 hours later assayed with the Cell Titer-Glo Luminescent Cell Viability Assay (Promega). Luminescence was detected with an Analyst HT Luminometer and graphed in a dose–response curve to calculate pIC50 values.

**Statistical analyses**
For 2 cohort xenograft studies, tumor volumes of untreated and treated mice on the final day of the experiment were compared using a 2-sided unpaired t test. For xenograft studies with multiple arms, 1-way ANOVA was used with a Tukey multiple comparison test. For comparison of individual arms in blood pressure studies, for comparison of individual arms in studies analyzing the area of normal acinar tissue remaining in KC pancreata, for studies comparing relative CD31 and Ki67 immunoreactivity in tumors, and for comparison of facial papilloma appearances in the mice, a 2-sided unpaired t test was again used. For comparison of vulvar papillomas, the Mann–Whitney U test was conducted. For analysis of normal ducts and PanIN-1A lesions, χ2 analysis was conducted to compare each arm with control untreated mice. Ducts were grouped into 2 groups (e.g., normal vs. abnormal or PanIN-1A vs. all other) and χ2 analysis was conducted. Kaplan–Meier survival curves were generated for each of the KPC mice cohorts, and P values were calculated using the log-rank (Mantel–Cox) test. All statistical analyses were performed using GraphPad Prism v5 (GraphPad Software).

**Results**

**eNOS expression during PDAC development**

- **LSL-KrasG12D/+**; **Pdx-1-CreGec/+ (KC) mice conditionally express endogenous mutant KrasG12D in the pancreas** develop PanInNs, presumed precursors to invasive disease that at low frequency progress to PDACs (6). **LSL-KrasG12D/+**; **LSL-Tp53R172H/+; Pdx-1-CreGec/+** (KPC) mice conditionally express endogenous mutant KrasG12D and p53R172H in the pancreas, which leads to lethal PDACs similar to human PDAC (33, 34). Capitalizing on the ability to model different stages of pancreatic cancer in mice, we measured eNOS levels by immunohistochemistry in the pancreas of 2 wild-type, KC, and KPC animals. As previously reported (35), eNOS was confined to endothelial cells in the normal pancreas (+2 to +3 staining in a scale of 0 – +3), with no visible staining in ducts or acini. Variable eNOS reactivity (0 – +2) was observed in PanIN lesions in KC mice. Patchy (+2) to a widespread blush (+1) with focal eNOS staining was detected in adenocarcinomas of KPC mice, which was lost in eNOS−/− KPC mice (Fig. 1A). eNOS was detected at variable levels by reverse transcription PCR in 3 normal pancreatic tissue samples, presumably from the endothelial cells, in all 4 pancreatic samples from KC mice, and 11 of 13 pancreatic samples from KPC mice at the time these tissues developed PanIN or adenocarcinomas. Eight of the 13 tumor cell lines devoid of stromal tissue that were derived from pancreata of KPC mice also expressed eNOS. As a negative control, eNOS was not detected in the pancreata analyzed from 1 normal, 4 KC, or 1 KPC eNOS−/− mice.
In humans, eNOS was previously shown to be elevated, particularly in the vasculature, in pancreatic cancer cell lines and specimens (36), and a number of human PDAC cell lines exhibit activated eNOS (13). In agreement, 4 of 9 human pancreatic cancer specimens had regions of eNOS positivity (e.g., Supplementary Fig. S1).

Genetic ablation of eNOS decreases PanIN development

To test whether ablation of the eNOS gene disrupts the onset of pancreatic tumorigenesis, KC and eNOS−/− KC mice were aged to approximately 330 days to allow a spectrum of PanINs to develop (6). Pancreata were removed, stained, and the area of remaining normal acinar tissue quantified from usually 5 high-power fields per pancreas from cohorts of 12 to 16 mice (amounting to 80 random high-power fields analyzed) as well as the highest grade ductal PanIN lesion per lobule scored from more than 2,600 lobules per group.

As previously reported (6), most of the pancreata in KC animals were replaced with abnormal tissue consisting of varying grades of PanIN lesions with abundant surrounding fibrotic stroma and associated chronic inflammatory cells (Fig. 2A). Quantification revealed that the average area of normal acinar tissue remaining was reduced to 17% (Fig. 2B), with 94.5% of lobules having abnormal ducts, mainly consisting of low-grade PanIN-1A lesions (Fig. 2C). eNOS−/− KC mice exhibited over twice as much normal pancreatic tissue area (P < 0.0001) doubling in the number of lobules with normal ducts (Fig. 2C).

Genetic ablation of eNOS decreases development of other oncogenic KRas–driven tumors

KC mice develop vulvar and facial papillomas (Fig. 3A and B) presumably because of a recombined oncogenic KrasG12D allele (Fig. 3C) arising from Pdx-1–restricted Cre expression (6, 37, 38). eNOS−/− KC mice exhibited a significant (P < 0.05) 50% decrease in the number of facial papillomas and a significant (P < 0.05) 85% decrease in the weight of vulvar papillomas per mouse at the time of sacrifice (~330 days) compared with KC mice (Fig. 3D).

Genetic ablation of eNOS trends toward an increase in the lifespan of mice with lethal PDAC

To test whether genetic ablation of eNOS provides a survival benefit, the most clinically relevant endpoint, eNOS-null alleles were crossed into the KPC background, and littermates generated over the course of a year were used to populate cohorts of 35 KPC and 32 eNOS−/− KPC mice. Mice were euthanized at moribundity endpoints known to immediately precede death (33). Consistent with previous studies, KPC mice had a median survival of 142 days (33), whereas eNOS−/− KPC mice exhibited a median survival of 142 days (33), whereas eNOS−/− KPC mice exhibited a median survival of 176 days, a trend that although did not reach significance [P = 0.093; HR, 0.709; 95% confidence interval (CI), = 0.431–1.167], corresponded to an increase of 34 days or nearly 25% of the control lifespan (Fig. 4A).
**L-NAME treatment decreases development of preinvasive pancreatic lesions**

To test whether pharmacologic inhibition of eNOS impacts pancreatic cancer, KC mice were treated with L-NAME. This drug was chosen because it has a 10-fold preference for eNOS and nNOS over iNOS (12), can be orally dosed, a quality that would facilitate administration in the clinic, is one of only 2 NOS inhibitors to have been brought to a phase II clinical trial for the treatment of shock (18–21), and appears to be a relatively benign drug, with the main side effect of chronic administration being hypertension and resultant end-organ damage, such as left ventricular hypertrophy and glomerulosclerosis (23), all of which can be suppressed by co-administration of antihypertensives (39). A cohort of 16 KC mice was provided with L-NAME-treated water at a dose established to increase blood pressure, indicating effective eNOS inhibition (see below and ref. 15) at weaning until the termination of the experiment (230 days of age). The L-NAME-treated group trended, although did not reach significance, toward retaining more normal tissue architecture than untreated controls, with the mean percentage of remaining normal acinar tissue increasing to 20.5% in 16 mice analyzed (Fig. 2A and B), exhibited a significant ($P < 0.001$) decrease in lobules with PanIN-1A lesions and a nearly 2-fold significant ($P < 0.0001$) increase in lobules with normal ducts in 12 mice analyzed (Fig. 2C). Anecdotally, mice progressing to invasive PDAC were also reduced, with 3 untreated, 2 eNOS$^{+/−}$, and 1 L-NAME–treated KC mice developing PDACs.

**L-NAME treatment can inhibit other oncogenic KRas–driven tumors**

L-NAME treatment significantly ($P < 0.05$) halved the number of facial papillomas and trended, but did not reach significance, toward an almost three-quarters reduction in the weight of vulvar papillomas in KC mice (Fig. 3D).

**L-NAME treatment trends toward an increase the lifespan of mice with lethal PDAC**

To address whether L-NAME increases survival in a PDAC setting, 32 KPC mice treated as above with L-NAME were monitored for moribundity endpoints. All-cause mortality was assessed to include any deaths that may be because of adverse effects from the drug itself. Median survival for the
L-NAME–treated mice was 161 days, a trend that although did not reach significance (P = 0.208; HR, 0.725; 95% CI, 0.439–1.196), reflected a 19-day survival advantage over untreated KPC mice (Fig. 4A). Whereas this effect could be ascribed to an initial decrease in PanIN lesions, as observed in KC mice treated with L-NAME (Fig. 2), L-NAME could similarly inhibit the adenocarcinomas directly. Indeed, the final tumor volume of a PDAC cell line established from a KPC mouse was significantly (P < 0.05) reduced in mice treated with L-NAME (Fig. 4B).

**L-NAME treatment decreases tumorigenic growth of human PDAC cell lines**

To evaluate the potential of targeting NOS in a human cell setting, an important consideration given that oncogenic Ras signaling can exhibit species differences (28, 40), 8 KRAS mutation–positive human PDAC cell lines were each injected into 8 or more immunocompromised mice, of which half were untreated and half were treated with l-NAME. Five cell lines showed a significant response (P < 0.05), 2 lines trended toward a response, and 1 did not respond to L-NAME, typically halving the tumor size by the termination of the experiment (Fig. 5A and Supplementary Fig. S2A–S2G). Anecdotally, the one cell line not affected by L-NAME, PANC-1, was the only line with undetectable levels of activated eNOS (13). In agreement, N-nitro-L-arginine (L-NNA), an insoluble active metabolite of L-NAME, halved both subcutaneous and orthotopic xenograft tumor growth of the human PDAC cell line L3.6pl (41) and aminoguanidine, a broad NOS inhibitor with some specificity for iNOS, decreased tumor growth when given at high doses (42).

To evaluate the effect of treating established human tumors, once tumors from CFPac-1 cells reached a size of 0.75 cm³, animals were left untreated or dosed with l-NAME. Within days of beginning l-NAME treatments, tumor size was reduced and at the termination of the experiment tumor size was...
which 10 blood pressure measurements were taken a total of 26 days over the course of 8 weeks, and tumor volume was measured. Amlodipine significantly ($P < 0.05$) reduced systolic blood pressure of L-NAME–treated animals, as previously reported (39), from 141 $\pm$ 2.2 mm Hg to the reference level of 109 $\pm$ 2.7 mm Hg (Fig. 5C) but had no effect on the antitumor activity of L-NAME, as tumors in the L-NAME and L-NAME + amlodipine cohorts were identical in size, but nevertheless were significantly ($P < 0.05$) smaller than the untreated or amlodipine-treated mice at the termination of the experiment (Fig. 5D).

eNOS promotes tumorigenesis through effects in the tumor and stroma

KRas signaling can stimulate AKT (4), and AKT can phosphorylate S1177 and activate eNOS (14), which in turn can elevate the level of activated GTP-bound wild-type HRas and NRas to promote tumor growth (13). In agreement, the PI3K inhibitor LY294002 reduced the level of activated eS1177 phosphorylated eNOS in CFPac-1 cells (Fig. 6A). Similarly, the level of GTP-bound HRas, as detected by affinity capture with the Raf1 RBD followed by immunoblot with an $\alpha$Hras antibody, was diminished in tumor cell lines devoid of stromal tissue derived from the pancreas of 3 eNOS$^{+/–}$ KPC mice compared with tumor cell lines from 3 control KPC mice (Fig. 6B) and in 2 CFPac-1 derived tumors from mice treated with L-NAME relative to tumors from 2 untreated mice (Fig. 6C).

Interestingly, L-NAME did not inhibit the in vitro proliferation of 3 different human PDAC cell lines (Supplementary Fig. S3), suggesting a role for eNOS in the tumor stroma as well. In this regard, oncogenic Ras activates eNOS to maintain tumor growth (13), and the first effect observed upon silencing Ras oncogene expression was an increase in apoptotic CD31+ cells (43). Activation of Ras also leads to secretion of pro-angiogenic cytokines (44, 45). Similarly, eNOS is known to play a role in angiogenesis (46, 47). In this regard, CD31 staining from typically 5 randomly chosen fields of PDAC tumors from 5 eNOS$^{+/–}$ KPC mice compared with tumors from 6 control KPC mice was significantly ($P < 0.05$) reduced by one third (Fig. 6D), but Ki67 immunoreactivity from 4 to 5 randomly chosen fields from tumors arising in 7 mice injected with the CFPac-1 human pancreatic cancer cell line and treated with L-NAME compared with tumors from 7 control untreated mice was significantly ($P < 0.05$) reduced by half (Fig. 6E).

Discussion

Loss of eNOS or L-NAME treatment reduced the development of pancreatic lesions, facial papillomas, and vulvar papillomas in the KC mouse model of preinvasive pancreatic cancer. eNOS ablation and L-NAME treatment also trended, but did not reach significance, toward a 34- and 19-day increase in survival in KPC mice, corresponding to a 25% and 13% increase in lifespan compared with that of control KPC mice, respectively. In terms of human cell settings, eNOS was detected in human PDAC samples, although admittedly this was a small sample set, and L-NAME broadly inhibited the tumorigenic growth of human PDAC cell lines. Given the variability in eNOS levels detected in PDAC samples, we cannot rule out that the
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Figure 5. Tumorigenic growth of human pancreatic cancer cells treated with L-NAME, amlodipine, or L-NAME and gemcitabine in cohorts (n = 5) of mice untreated or L-NAME-treated.

A, mean size ± SEM of subcutaneous tumors derived from CFPac-1 cells in animals without L-NAME treatment. B, mean size ± SEM of subcutaneous tumors derived from CFPac-1 tumors in cohorts of mice (n = 4–5) that, upon reaching an established size of 0.75 cm² (arrow), were untreated or treated with L-NAME, gemcitabine, or L-NAME + gemcitabine. C, mean size ± SEM of CFPac-1 xenografts in animals corresponding to treatment groups described in C. D, mean size ± SEM of CFPac-1 xenografts in animals corresponding to treatment groups described in C. 

more general inhibition of pancreatic tumorigenesis observed in multiple animal model systems may be more limited in a clinical setting. Similarly, given that L-NAME tended but did not reach significance toward a 19-day increase in survival in the metastatic KPC mouse model, we also cannot rule out the possibility that the drug may not be effective in the highly aggressive late stage of the disease in humans. With these caveats in mind, these data nevertheless suggest that eNOS is a potential therapeutic target that could be inhibited with the available drug L-NAME for the treatment of PDACs.

KC mice treated daily with L-NAME for roughly 10 months had no overt adverse effects, and analysis of all-cause mortality in KC mice, which would take into account deaths because of adverse drug events, was actually decreased in the L-NAME treatment group compared with untreated controls. In fact, the undesirable effects of L-NAME on blood pressure were ameliorated with a commonly used antihypertensive with no change to the antitumor activity of L-NAME. Indeed, L-NAME has been used in many clinical trials, including those involving normal human subjects (22), suggesting that this drug may have low toxicities in a cancer setting.

Because eNOS knockout and L-NAME treatments exhibited similar effects on tumorigenesis in KC and KPC mice, and because L-NAME inhibits eNOS in vitro, as measured by an increase in blood pressure, the antitumor effects of L-NAME can be attributed, at least in part, to eNOS inhibition. Nevertheless, we cannot rule out the possibility that this may also be a consequence of inhibiting other NOS isoforms. Similarly, the cell type sensitive to eNOS inhibition during the development of pancreatic cancer remains to be determined. NO can be produced both by the tumor and stromal cells (24). In regard to the tumor, eNOS is detected in PDAC tumor cells (Fig. 1A), loss of the gene reduced the level of GTP-bound wild-type HRas (Fig. 6B), and knockdown of eNOS in 2 pancreatic cancer cell lines reduced their tumorigenic potential (13). In regard to the stroma, L-NAME did not decrease the viability of pancreatic cancer cell lines in vitro (Supplementary Fig. S3). Moreover, both Ras and eNOS promote angiogenesis (44–47), eNOS−/− animals are deficient in endothelial progenitor cell mobilization and neovascularization (48), and CD31 and Ki67 staining were reduced upon inhibiting eNOS (Fig. 6D and E). Thus, activation of stromal eNOS, perhaps by paracrine signaling from the tumor or other sources, may also promote tumorigenesis, for example, through effects on angiogenesis. An orthotopic model of pancreatic cancer would help resolve the issue of which tissue eNOS functions, and additionally, in the context of human cells. Regardless of the source of eNOS, the net result of genetic ablation and/or pharmacologic inhibition of eNOS in the tested pancreatic cancer models was a reduction in tumorigenesis.

The inhibition of eNOS may have therapeutic use in malignancies beyond pancreatic cancer as oncogenic KRAs–driven vulvar and facial papillomas responded to L-NAME treatment and exhibited reduced tumor growth in an eNOS−/− background. This effect may extend even beyond KRAS mutation-positive cancers. Specifically, eNOS−/− mice are also resistant to DMBA/TPA chemical carcinogenesis (13) and were recently reported to have prolonged survival in a PDGF-induced glioma mouse model (16). Similarly, peptide-mediated inhibition of eNOS decreases tumor vascular permeability and tumor growth in hepatic carcinoma and lung carcinoma xenograft models (17). Finally, eNOS-generated NO can regulate recruitment of pericytes and stabilization of angiogenic vessels in a xenograft model of murine melanoma (49). Thus, we suggest...
that eNOS is an attractive target that can be inhibited with the available drug L-NAME, which could potentially be exploited for the treatment of PDAC and perhaps other oncogenic Ras-driven cancers.

Disclosure of Potential Conflicts of Interest
B.B. Ancrile, D.F. Kashatus, and C.M. Counter submitted a patent application to inhibit eNOS for the treatment of Ras mutation-positive cancers. K.S. Barrientos and C.M. Counter had a collaboration with GlaxoSmithKline to develop eNOS-specific inhibitors. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: B.L. Lampson, S.D. Kendall, B.B. Ancrile, M.J. Shealy, K.S. Barrientos, C.M. Counter Development of methodology: B.L. Lampson, S.D. Kendall, B.B. Ancrile, K.S. Barrientos, S.B. Gurley Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.L. Lampson, S.D. Kendall, B.B. Ancrile, K.S. Barrientos, M.S. Crowe, D.F. Kashatus, R.R. White, S.B. Gurley Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.L. Lampson, S.D. Kendall, B.B. Ancrile, M.J. Shealy, K.S. Barrientos, M.S. Crowe, D.M. Cardona, C.M. Counter Writing, review, and/or revision of the manuscript: B.L. Lampson, S.D. Kendall, B.B. Ancrile, M.M. Morrison, M.J. Shealy, K.S. Barrientos, R.R. White, D.M. Cardona, C.M. Counter Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.L. Lampson, M.M. Morrison Animal experiments (related to blood pressure data) and data analysis: M.M. Morrison Study supervision: M.J. Shealy, C.M. Counter

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References


