Impact of APE1/Ref-1 Redox Inhibition on Pancreatic Tumor Growth

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Abstract

Pancreatic cancer is especially a deadly form of cancer with a survival rate less than 2%. Pancreatic cancers respond poorly to existing chemotherapeutic agents and radiation, and progress for the treatment of pancreatic cancer remains elusive. To address this unmet medical need, a better understanding of critical pathways and molecular mechanisms involved in pancreatic tumor development, progression, and resistance to traditional therapy is therefore critical. Reduction–oxidation (redox) signaling systems are emerging as important targets in pancreatic cancer. AP endonuclease1/Redox effector factor 1 (APE1/Ref-1) is upregulated in human pancreatic cancer cells and modulation of its redox activity blocks the proliferation and migration of pancreatic cancer cells and pancreatic cancer-associated endothelial cells in vitro. Modulation of APE1/Ref-1 using a specific inhibitor of APE1/Ref-1’s redox function, E3330, leads to a decrease in transcription factor activity for NFκB, AP-1, and HIF1α in vitro. This study aims to further establish the redox signaling protein APE1/Ref-1 as a molecular target in pancreatic cancer. Here, we show that inhibition of APE1/Ref-1 via E3330 results in tumor growth inhibition in cell lines and pancreatic cancer xenograft models in mice. Pharmacokinetic studies also show that E3330 attains more than 10 μmol/L blood concentrations and is detectable in tumor xenografts. Through inhibition of APE1/Ref-1, the activity of NFκB, AP-1, and HIF1α that are key transcriptional regulators involved in survival, invasion, and metastasis is blocked. These data indicate that E3330, inhibitor of APE1/Ref-1, has potential in pancreatic cancer and clinical investigation of APE1/Ref-1 molecular target is warranted. Mol Cancer Ther; 10(9); 1698–708. ©2011 AACR.

Introduction

Pancreatic cancer is a particularly insidious form of cancer with the worst 5-year survival rate of any cancer at less than 2% (1). There is no early detection method for pancreatic cancer, which often displays only nonspecific symptoms such as abdominal pain, weight loss, and vomiting, until the cancer is well advanced (2). Pancreatic cancers are hypoxic tumors that respond poorly to existing chemotherapeutic agents and radiation (3). NFκB and HIF-1α have been identified as leading drivers of cell growth in pancreatic cancer; both are under APE1/Ref-1 redox signaling control, which is the focus of our studies (4–6).

Cellular response to base DNA damage is a highly regulated and complex biological process. APE1/Ref-1 is a vital protein in this process and acts as a master regulator of the DNA damage response by contributing to the maintenance of the genome. APE1/Ref-1 is a dual function protein involved in base excision repair pathways of DNA lesions, as the major apurinic/apyrimidinic endonuclease, and in eukaryotic transcriptional regulation of gene expression as a reduction–oxidation (redox) factor. APE1/Ref-1 can stimulate DNA-binding activity of numerous transcription factors that are involved in cancer promotion and progression such as HIF-1α, NFκB, AP-1, p53, and others (6–13). The functional regions of APE1/Ref-1, redox, and DNA repair are completely independent in their function; that is, mutations of the cysteine at position 65 removes the redox function but does not affect the DNA repair function and vice versa (14). Although the DNA repair active site of APE1/Ref-1 is delineated (15), the redox region is less obvious. Recently, the mechanism of APE1/Ref-1 redox structure...
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function has been elucidated (16–18). APE1/Ref-1 redox inhibitor E3330 recognizes an alternate, redox active conformation of APE1/Ref-1 and potentially inhibits its redox activity by inducing disulfide bond formation within APE1/Ref-1 (16).

To investigate the role of redox regulation by APE1/Ref-1 in pancreatic cancer, we used E3330, a highly selective inhibitor of APE1/Ref-1 redox function. Originally discovered in a search for NFκB inhibitors, E3330 was used in liver inflammation and hepatitis, but was never investigated for its therapeutic potential in cancer (19, 20). We previously showed that APE1/Ref-1 is upregulated in human pancreatic cancer cells and modulation of its redox activity blocks the proliferation and migration of pancreatic cancer cells (21, 22) and pancreatic cancer-associated endothelial cells (PCEC) in vitro (23). Here, we study the effects of redox signaling through APE1/Ref-1 in animal models of pancreatic cancer. The effectiveness of E3330 in vivo is shown with good pharmacokinetic (PK) and pharmacodynamic properties as well as tumor growth reduction. Our in vitro data support the in vivo results showing that blocking the redox activity of APE1/Ref-1 inhibits the proliferation and adhesion of pancreatic cancer cell lines, arrests cell cycle progression, and decreases the transcriptional activation of 3 major transcription factors known to be important in pancreatic cancer progression, survival, and metastasis (NFκB, HIF-1α, and AP-1; ref. 24). In conclusion, this is the first specific APE1/Ref-1 redox inhibitor that shows in vivo effectiveness and further work on this and other related compounds will allow the development of first in class and first in human agents for the treatment of pancreatic cancer.

Materials and Methods

Cell lines

Panc-1 and PaCa-2 were purchased from and authenticated by American Type Culture Collection (ATCC). ATCC authenticates the cell lines by short tandem repeat analysis and cytochrome C oxidase I assay and testing for bacterial, fungal, and mycoplasma contamination. Both were maintained at 37°C in 5% CO2 and grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) with 10% cosmic calf serum (Hyclone; Logan).

Proliferation of PaCa-2 and Panc-1 cells

The proliferative capacity of PaCa-2 and Panc-1 cells was assessed using trypan blue (0.4%; Invitrogen) and the xCELLigence system (25, 26), and E3330 was synthesized as previously described (16, 26). For trypan blue assays, PaCa-2 and Panc-1 cells were treated with E3330 for 72 and 48 hours, respectively. Effects of E3330 on the growth were determined using the xCELLigence DP System (Roche Applied Science ref. 25). Following background reading, cells (PaCa-2: 3,000 and Panc-1: 4,000 cells/well) were plated in 16-well plates in 100 μL volume. After adding cells to the wells, plates were kept at room temperature for 30 minutes after which they were inserted into the cradles. Cells were allowed to grow for 18 to 22 hours before E3330 was added. Continuous impedance measurements were then monitored more than 72 hours. Assays were done in triplicate.

Adhesion assay

For the adhesion assays (27), serum-free media containing increasing concentrations of E3330 was added to each well in triplicate. A background reading was taken and then PaCa-2 or Panc-1 cells were added in complete media. Plates were kept at room temperature for 15 minutes and inserted into the cradle. Continuous impedance measurements were taken every 15 to 30 minutes more than 30 hours.

Western blot analysis

Cells were harvested, lysed in RIPA buffer (Santa Cruz Biotechnology), and protein was quantified and electrophoresed on a 12% SDS-PAGE. The following antibodies were used: APE1/Ref-1 (Novus Biologicals), PARP-1 (Cell Signaling Technology Inc.), tubulin (Sigma Aldrich), p21 and p27 (Santa Cruz Biotechnology; ref. 22).

Apoptosis assays via Alexa Fluor 488-conjugated Annexin V/propridium iodide staining

Apoptosis was assayed 48 hours after E3330 treatment. Cells were trypsinized, pelleted, washed in ice-cold PBS, and resuspended in 1× binding buffer. Apoptosis was analyzed using the Alexa Fluor 488 Annexin V Vybrant Apoptosis Assay Kit in combination with propidium iodide (PI; Molecular Probes) as previously described (9).

Cell cycle staining via BrdU

To stain the cells for DNA content and analyze the movement of cells through G0–G1, S, and G2–M, cells were plated, allowed to attach overnight and treated with E3330 in serum-free media for 24 hours. Following treatment, cells were processed according to the manufacturer’s directions (BD Pharmingen ref. 9).

Transient luciferase reporter assays

Panc-1 and PaCa-2 cells were cotransfected with constructs containing luciferase driven by NFκB, AP-1, or HIF (pLuc-MCS with the NFκB, AP-1, or HIF1α responsive promoter; PathDetect cis-Reporting Systems, Stratagene; ref. 5). The following HIF1 consensus sequence was cloned into the pLuc-MCS vector: 5’- AGCGGCGGTACGACGCGGAATTCCTCTTCTAC- GTGACCACATGCACCTCCTGATCGAGCCACACT- CACCTGTCACTGACGCGGAAATTCCTACCTCTTCTC-3’. Renilla control vector pRL-TK (Promega Corp.) was used in a 20:1 ratio using Lipofectamine 2000 (Invitrogen Life Technologies). After transfection for 16 hours, cells were treated with E3330 in serum-free media for 24 hours (for HIF1 drug added immediately before placement in hypoxic chamber). Firefly and Renilla luciferase activities were assayed by using the Dual Luciferase Reporter

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Assay System (Promega Corp.) with Renilla luciferase activity for normalization in a 96-well microtiter plate luminometer (Thermolabs Systems). Each time, a media only control was included, and the final data is expressed as the fold change of the relative luciferase units (RLU) compared with the vehicle control, dimethyl sulfoxide (DMSO). DMSO concentration was equivalent in all samples and the final concentration was less than 0.25%. All of the transfection experiments were done in triplicate and repeated at least 3 times in independent experiments.

Stable cell lines for reporter assays
Using lentiviral transcriptional reporter vectors, pGreenFire-NFkB, pGreenFire-HIF1, and pGreenFire-mCMV (negative control), from System Biosciences Inc., we transfected HEK293-T cells (Lonza, Inc.) with the constructs and collected the media containing the viral supernatants. To generate stably expressing reporter cell lines, we infected pancreatic cancer cells with the viral supernatant and selected the cells with puromycin (5 ng/mL) for 5 days. For the experiments inducing NFkB activity via TNFα, Panc-1 and PaCa-2 were treated with E3330 for 24 hours in serum-free media and then treated with TNFα for 6 hours. Cells were then assayed for luciferase activity as described above except the normalization was done using MTS-based assay. For the MTS assay (11, 22), 7.5 × 10^4 cells per 96-well plate were plated in parallel with the cells for luciferase assay, allowed to adhere overnight, and then treated with E3330 and TNFα as above. Alongside the luciferase assay, MTS reagent was added to the 96-well plates for 2 hours. Values were standardized to wells containing media alone and then used to account for effects of drug treatment on the cells.

Quantitative reverse transcriptase-PCR reactions
Quantitative reverse transcriptase PCR (qRT-PCR) was used to measure the mRNA expression levels of NFkB downstream target, carbonic anhydrase-IX (CA-IX) gene. PaCa-2 and Panc-1 were treated with increasing amounts of E3330 in the presence or absence of hypoxia (1% O2) for 24 hours, then total RNA was extracted from cells using the Qiagen RNeasy Mini Kit according to the manufacturer’s instructions (28). The extracted RNA was quantified by a Qubit fluorometer (Invitrogen Corp.). First-strand cDNA was obtained from RNA using random hexamers and MultiScribe reverse transcriptase (Applied Biosystems). Quantitative PCR was conducted by using Taqman Gene Expression Assays and Universal PCR Master Mix (Applied Biosystems) in a 7900HT Sequence Detection System (Applied Biosystems). The relative quantitative mRNA level was determined using the comparative C_{t} method using 18S rRNA as the reference gene (28-30). The primers for CA-IX and 18S are commercially available (Applied Biosystems). Experiments were done in triplicate for each sample.

PK studies in NOD/SCID mice
Nonobese diabetic/severe combined immunodeficient mice (NOD/SCID) mice were administered 25 mg/kg E3330 intraperitoneal (i.p.) in DMSO (9%)/sterile saline for initial PK studies. To determine PK parameters, following administration of E3330, blood was collected via tail vein at multiple time points between 0.5 to 36 hours following administration. We collected 2 samples (~25 μL) per mouse with each sample collected at least 6 hours apart. Following half-life determination, a dosing regimen for the tumor studies was established in mice that consisted of 2 doses of E3330 each day, approximately 8 hours apart for 14 days.

E3330 was quantified in plasma and solid tissue using an internal standard (bromomethylcoumarin), liquid–liquid extraction with ethyl acetate and high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS-MS; Agilent HPLC, Applied Biosystems, API 4000). The HPLC was run in isocratic mode using acetonitrile: 5 mmol/L ammonium acetate (60:40, v/v). The API 4000 was run in negative mode for E3330 (Q1/Q3: 377/333) and positive mode for bromomethylcoumarin (Q1/Q3, 301/220). The lower limit of quantification was 1 ng/mL using 10 μL of blood or plasma. For E3330 in solid tissue, tissue was weighed, reconstituted with phosphate buffer (100 mmol/L NaPO4, pH = 7.4) to up to 2 mL of total volume, and homogenized by a handheld homogenizer. An aliquot of the homogenate was used for the extraction. Tissue concentration was expressed in ng/g tissue.

PK parameters for E3330 including area under the curve (AUC), area under the moment curve (AUMC), and t1/2 were estimated using noncompartmental methods with Excel. The maximum plasma concentration (C_{max}) and time of C_{max} (t_{max}) were obtained from the data. The AUC from 0 to infinity (AUC_{0–∞}) was estimated from the AUC_{0–t} (time 0 to the last quantifiable concentration C_{last}) and the AUC from C_{last} to infinity, C_{last}/k_{el}, where k_{el} is the rate constant of elimination. The AUMC_{0–∞} was estimated by an analogous manner. The systemic clearance (Cl/F, where F = bioavailability) of E3330 was calculated from the dose and AUC_{0–∞}. The apparent volume of distribution was estimated by the following equation: (dosage/AUC_{0–∞}) × (AUMC_{0–∞}/AUC_{0–∞}).

Animals
All animal studies were conducted under the guidelines of the NIH and were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine and Johns Hopkins School of Medicine. Animals were maintained under pathogen-free conditions and a 12-hour light-dark cycle.

For the patient-derived xenografts, 6-week-old female nu/nu athymic mice (Harlan) were used, whereas PaCa-2 cells were grown as ectopic xenografts in NOD/SCID mice. NOD/SCID (NOD.CB17-Prkdc<sup>scid</sup>/J) mice were obtained from the In Vitro Therapeutics Core of the Indiana University Simon Cancer Center.
Establishment of ectopic xenografts from PaCa-2 cells

PaCa-2 cells (2.5 × 10^6) in 0.2 mL of DMEM media were implanted s.c. into the right flanks of NOD/SCID mice. E3330 was dissolved in 4% CremophorEL:EtOH (1:1)/saline solution or methylcellulose (0.5%, Sigma). When tumor volumes were greater than 100 mm^3, E3330 was administered orally twice daily, 8 hours apart, at 25 mg/kg for 10 to 12 days (5 days on 2 days off schedule). Tumors were measured biewise and followed for approximately 6 weeks. Tumor volumes were monitored by caliper measurement [tumor volume = length × (perpendicular width)^2 × 0.5] and the average tumor volume in mm^3 for each treatment group was plotted. Treatment corresponds to days 1 to 15. Average tumor volume ± SE for the vehicle and E3330-treated PaCa-2 xenografts (n = 7) was analyzed by statistical analysis.

Ectopic xenograft established from patient-derived cells

Fresh pancreatic cancer specimens resected from patients at the time of surgery, with informed written patient consent, were implanted s.c. into the flanks of 6-week-old female nude athymic mice (Harlan). The patients had not undergone chemotherapy or radiation therapy before surgery. Grafted tumors were subsequently transplanted from mouse to mouse and maintained as a live PancXenoBank according to an Institutional Review Board approved protocol. Panc253 xenograft was used for the study. When tumors reached a volume of approximately 200 mm^3, mice were individually identified and randomly assigned to treatment groups, with 5 to 6 mice (8–10 evaluable tumors) in each group: (i) control; (ii) E3330, administered twice daily, 8 hours apart, at 25 mg/kg for a total of 10 doses (5 days on 2 days off, 5 days on and 2 days off schedule).

Statistics

For the PaCa-2 xenograft data, all data points for vehicle and E3330-treated mice were analyzed. Continuous variables were summarized by typical parameters such as mean, SD and range and compared using 2-sample t test (if the normality assumption holds) or Wilcoxon rank-sum test (if the normality assumption does not hold). Normality of distribution was determined using the Kolmogorov–Smirnov goodness-of-fit test. Categorical data were summarized by frequency and percentage and analyzed using the χ² or Fisher’s exact test, as appropriate. A linear mixed-effects model was fit to the repeatedly collected tumor size data. The fixed effects include experimental group and distinct linear, quadratic, and cubic effects of time for each of the 2 groups. The random effects include intercept and linear effect of time (31). The model was estimated using SAS 9.2 (SAS Institute, Inc.). A P value less than 0.05 was considered statistically significant.

Results

APE1/Ref-1 redox inhibitor, E3330 inhibits the proliferation and ability of pancreatic cancer cell lines to adhere but does not induce apoptosis

We tested the effect of APE1/Ref-1 redox inhibition on the growth of pancreatic cancer cells using a trypan blue assay. E3330 was found to effectively slow the growth rate of cells in a dose-dependent manner (Fig. 1A), with an ED_{50} of 135 and 87 μmol/L for PaCa-2 and Panc-1, respectively. The ability of E3330 to inhibit proliferation and/or reduce survival of PaCa-2 and Panc-1 cells was further characterized using the xCELLigence DP system (Roche). This system measures a dimensionless parameter called cell index (CI), which integrates information on cell viability, number, morphology, and adhesion (27). In this assay, E3330 dramatically reduced CI of PaCa-2 and Panc-1 cells efficiently and in a dose-dependent manner, suggesting that E3330 inhibits growth and proliferation of pancreatic cancer cells as well as affects morphology and adhesion (Fig. 1B). The ED_{50} of E3330 was reduced by 2-fold using the xCELLigence system (43 and 54 μmol/L for PaCa-2 and Panc-1 cells), therefore we used the xCELLigence system to monitor the adhesion of PaCa-2 and Panc-1 cells. Adhesion of the cells was blocked at doses more than 33.75 μmol/L (Fig. 1D). The CI value was reduced by 50% in both cell lines at 67.5 μmol/L between 8 to 12 hours. Doses that blocked proliferation also had a dramatic effect on the cells’ ability to adhere to the plate. The sensitivity of the xCELLigence system shows the effects of APE1/Ref-1 redox inhibition on proliferation, adhesion, and morphology.

To investigate whether the cells were undergoing apoptosis following treatment with E3330, we used 2 methods: Annexin/PI staining and PARP-1 cleavage (Fig. 1C). Both assays showed similar results showing that although we had a reduction in proliferation, there was not a corresponding increase in apoptosis. These data are consistent with the idea that inhibition of the redox function of APE1/Ref-1 affects the cells’ proliferative capacity whereas the DNA repair function is critical for cell survival (4).

Inhibition of the redox function of APE1/Ref-1 via E3330 arrests cell cycle progression

Using bromodeoxyuridine (BrdU) incorporation assays, the percentage of PaCa-2 and Panc-1 cells in S phase is significantly decreased following treatment with E3330 (Fig. 2A). DMSO, the vehicle control, does not differ significantly from the media (data not shown). Furthermore, we synchronized PaCa-2 cells and monitored reentry into cell cycle following treatment with E3330. At 8 hours after adding serum-containing media, 60% less cells have entered S phase (Supplementary Fig. S1), showing that progression out of G1 is slower in the PaCa-2 cells when the redox function of APE1/Ref-1 is inhibited.

We quantitated the levels of endogenous cell cycle inhibitors, p21 and p27 (Fig. 2B and C). Although we
observe no change in p27 levels, the levels of p21 increase 2 to 2.5-fold in cells that have been treated with E3330. The effects on cell cycle progression and levels of p21 and p27 are very similar to our recent report of the effects of APE1/Ref-1 siRNA on PaCa-2 and Panc-1 cells (22). However, the increase in apoptosis that we observe when APE1/Ref-1 protein levels are decreased by siRNA is not observed when we inhibit the redox function of APE1/Ref-1. These data suggest that the redox function of APE1/Ref-1 affects both cell cycle progression and the time the cells take to traverse the cell cycle in pancreatic cancer cells.

**Inhibition of APE1/Ref-1 via E3330 results in a decrease in transcriptional activity for 3 known targets of APE1/Ref-1 redox activity**

To further characterize the redox function of APE1/Ref-1 on pancreatic cancer cell signaling, we used transient luciferase assays. In these experiments, the luciferase gene expression was driven by NFκB, HIF1, or AP-1 and normalized to Renilla gene expression for transfection efficiency. Inhibition of APE1/Ref-1 via E3330 results in a dose-dependent reduction in NFκB, HIF1, and AP-1 activity in vitro (Fig. 3A–C). HIF1 activity was induced by exposure to hypoxia for 24 hours before luciferase assay. The basal levels of AP-1 activity in PaCa-2 are approximately 20-fold lower than the basal activity in Panc-1 cells, and we were unable to detect a significant decrease in activity with the low levels of basal activity in PaCa-2 cells (data not shown). The doses of E3330 required to inhibit 50% of promoter activity were approximately 67.5 μmol/L for NFκB and HIF1 and approximately 15 μmol/L for AP-1.

To further show that inhibition of APE1/Ref-1 blocks downstream signaling of HIF1, we treated Panc-1 and PaCa-2 cells with E3330 for 24 hours. We then analyzed HIF1 target CA-IX (32) mRNA levels using qRT-PCR assay. As predicted, there was a significant...
dose-dependent decrease in HIF1 target CA-IX mRNA in pancreatic cancer cells following APE1/Ref-1 inhibition (Fig. 3D). Because inhibition of mRNA for CA-IX serves as a biomarker for HIF1 activity (32), we hypothesize that inhibition of APE1/Ref-1’s redox function by E3330 could disable the tumors’ ability to respond to hypoxic conditions that are known to contribute to the chemotherapeutic resistance of these tumors.

**Blockade of APE1/Ref-1 redox activity diminishes the pancreatic cancer cells ability to induce HIF1α and NFκB**

We generated PaCa-2 and Panc-1 lines that stably express NFκB-driven and HIF1α/C0-driven luciferase/green fluorescent protein (GFP) using lentiviral constructs, pGreenFire (pGF) from System Biosciences Inc. As a negative control, stable cell lines were generated that contain the luciferase/GFP cassette but do not have the NFκB or HIF1 response element (pGF-mCMV). Inhibition of the redox function of APE1/Ref-1 blocks the ability of PaCa-2 and Panc-1 cells to induce NFκB through TNFα (Fig. 4A, B) and HIF1 activity (Fig. 4C). PaCa-2 and Panc-1 cells that express pGF-mCMV did not show detectable luciferase activity above baseline, and treatment with TNFα or hypoxia did not induce expression of luciferase activity (data not shown).

**Inhibition of APE1/Ref-1 redox activity delays the growth of pancreatic cancer xenografts in vivo and exhibits favorable PK parameters**

The estimated half-life (t_1/2) of E3330 was 3.7 hours (Fig. 5A). From this, a dosing regimen was established in mice that consisted of 2 doses of E3330 each day, approximately 8 hours apart. As shown in Fig. 5B, 2 dosages of E3330 maintained the concentrations of E3330 1 or more...
μmol/L for more than 24 hours. In formulation studies, E3330 was dissolved in 4% CremophorEL: EtOH (1:1) in saline or 0.5% methylcellulose. Both vehicles were similar in their PK levels in plasma and tumor tissue (Supplementary Fig. S2) and replaced the less desirable vehicle, DMSO. Concentrations of E3330 attained in the blood of mice were within the required range for target inhibition from in vitro studies, suggesting that E3330 has favorable properties in vivo.

Next we evaluated the antitumor efficacy of E3330 using both patient-derived tumors and xenografts from established cell line, PaCa-2. In contrast to vehicle-control tumors, both patient-derived and PaCa-2 xenografts showed a significant tumor growth delay (Fig. 5C). All animals treated with E3330 had detectable drug in the tumor tissue (40–7,500 ng/g tissue). E3330 was detectable in pancreas, liver, lung, kidney, heart, and brain, but was not overly toxic to the animals as measured by weight loss and bone marrow cellularity (data not shown and Supplementary Fig. S2A and B). Furthermore, Ki67 staining is reduced in E3330-treated tumors at day 7 and day 11, supporting the notion that APE1/Ref-1 redox activity is blocking proliferation of the tumor cells (Supplementary Fig. S2C). Another patient-derived xenograft was used and did not show the dramatic growth delay that we observed in Fig. 5C. However, tumor tissue analysis indicated that levels of E3330 in the unresponsive tumors were 10-fold less than in the Panc 253 responsive tumors: 2,500 ng/g (~6.6 μmol/L) versus 250 ng/g (~0.7 μmol/L), data not shown. These results indicate that if we can efficiently deliver APE1/Ref-1 redox inhibitor, tumor growth is strongly inhibited.

Discussion

Although today’s standard of care for pancreatic cancer strives for a cure, debulking surgery along with chemotherapy and/or radiation is almost always palliative rather than curative, with few cases of long-term regression (33). Researchers agree that pancreatic cancer defies most of what we have come to know about other types of cancer; therefore, a different therapeutic approach is needed (1, 33, 34). Blocking a single step...
in a pathway or a single pathway has very limited clinical utility in the face of the tumors’ cumulative defects. Jones and colleagues found that pancreatic cancers contain a core set of 12 cellular signaling pathways and processes, each of which was altered in 67% to 100% of the tumors analyzed (24). Novel targets that modulate multiple pathways may offer the most promise for clinical utility against this dreaded disease. Transcription factors including NFκB, AP-1, and HIF1α are key in the regulation of multiple signals in pancreatic cancer, which provides strong evidence for investigating the effects of targeting APE1/Ref-1 to kill pancreatic cancer cells. In this report, we show that inhibition of APE1/Ref-1 reduces the proliferation of ectopic pancreatic tumors in mice using both established pancreatic cancer cell lines and primary human pancreatic tumors. Inhibition of APE1/Ref-1 redox activity led to cell cycle arrest and a reduction in NFκB, AP-1, and HIF1 activity, key regulators of pathways that are involved in the progression, maintenance, invasive, and metastatic potential of pancreatic cancer (5, 35–37). Furthermore, we show downregulation of HIF1 target, CA-IX, and surmise that APE1/Ref-1 inhibition may be able to sensitize these tumors to therapy by disabling their response to the hypoxic environment in which they are growing.

We recently showed that human adenocarcinoma and peri-pancreatic metastases have a significant increase in APE1/Ref-1 expression in adenocarcinoma as compared with normal pancreas tissue (22). The experiments described here and our previous work showing both in vitro and in vivo data support APE1/Ref-1 as a viable target in this deadly disease (21, 22). We used the redox-specific APE1/Ref-1 inhibitor, E3330, which recognizes an alternate, redox active conformation of APE1/Ref-1, and potentially inhibits its redox activity by inducing disulfide bond formation (16, 17). Biochemical studies using radiolabeled E3330 and proteins renatured on membrane blots showed that 14C-labeled E3330 very selectively bound to both recombinant APE1/Ref-1 and purified APE1/Ref-1 from cell nuclear extracts (20). Although E3330 blocks Ref-1’s redox function, it has no effect on APE1/Ref-1 endonuclease activity or base excision repair activity of an AP site (5). These studies formally show the specificity of E3330 for APE1/Ref-1’s redox activity, without impacting on its DNA repair function. The PK/pharmacodynamic studies (Fig. 5) show that tumors with significant levels of E3330 have their rate of growth significantly reduced. We are currently investigating additional delivery methodology such as nanoparticle technology to ensure efficient delivery of APE1/Ref-1.
inhibitor and novel compounds with submicromolar inhibition of APE1/Ref-1 redox activity. Another potential mechanism for the different amount of E3330 in these 2 patient-derived tumors is that the drug is pumped out of the cells. For example, there are several ATP-binding cassette transporters in the MDR/MRP gene family. Given the chemical structure of E3330, we predict that it is not a substrate for P-gp (MDR1, ABCB1) since this efflux transporter typically binds cationic, lipophilic structures. However, E3330 could be a substrate for MRP (ABCC1) or BCRP (ABCG2), as these transporters are known to bind anionic substrates including glutathione conjugates (38). However, it should be noted that MRP is not always associated with extracellular transport but may concentrate substrates into intracellular vesicles, thus protecting potential targets from drug. This is one possibility for the tumors with low levels of E3330 detected. However, further research is warranted.

The mechanism of action of E3330 is through the blockage of the transcriptional regulation of APE1/Ref-1 therefore, it blocks activity of NFκB, AP-1, and HIF1 as shown in Figs. 3 and 4. These data provide fundamental evidence that E3330 inhibits 3 known transcriptional targets of APE1/Ref-1 in pancreatic cancer cells. Pancreatic tumors are characterized as one of the most hypoxic tumors that clinicians encounter (3, 35). Our previous work and the data here show that blockade of APE1/Ref-1 redox signaling does affect the activity of HIF1 in pancreatic cancer cells (21, 23). This supports our hypothesis that inhibition of APE1/Ref-1 could disable the tumors’ ability to respond to hypoxic conditions, which contributes to chemotherapeutic resistance. Using in vitro luciferase-based assays, E3330 can inhibit the activity of these 3 important transcription factors in pancreatic cancer. Using a lentiviral system, we created stable cell lines with a construct containing an NFκB or HIF1 response element that drives GFP and luciferase. In PaCa-2 and Panc-1 cells that express luciferase driven by the NFκB or HIF1 promoter, E3330 treatment results in a reduction in activity as shown by reduction of GFP positivity and luciferase activity (Fig. 4 and data not shown). Inhibition of NFκB (with and without induction by TNFα) and HIF1 activity is observed both in transient and stable luciferase assays. Decreases in the activity of downstream transcription factors lead to a reduction in tumor growth and delay in tumor growth as seen in Figs. 5A and 5C.
factors provide important support for APE1/Ref-1 as a target in pancreatic cancer. We can now use these stable cell lines in vitro as markers of NFκB, AP-1, and HIF1 and dissect which of these transcription factors are critical for the survival and spread of pancreatic cancer.

Inhibition of APE1/Ref-1 via E3330 inhibits the growth of pancreatic tumor xenografts, both the cell line and xenograft models, and we plan to extend our studies to orthotopic models. In addition to evaluating E3330 as a single agent, combination of E3330 with gemcitabine will also be tested to know whether E3330 potentiates gemcitabine sensitivity. A reduction in APE1/Ref-1 protein expression does indeed sensitize pancreatic cells in vitro to gemcitabine (39) suggesting that combination therapy might be useful in treating this disease. Due to the decrease in proliferative capacity and the decrease in cells in S phase (Figs. 1 and 2), careful selection of the agent(s) that are chosen for combination therapy and the dosing schedules should be chosen carefully. The studies here provide preclinical validation for a novel therapeutic strategy for this largely incurable cancer.

Disclosure of Potential Conflicts of Interest

Mark R. Kelley is also a consultant for Apex Therapeutics, a company that has licensed IP from his work.

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