Preclinical Development of a Nontoxic Oral Formulation of Monoethanolamine, a Lipid Precursor, for Prostate Cancer Treatment

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Abstract

**Purpose:** Most currently available chemotherapeutic agents target rampant cell division in cancer cells, thereby affecting rapidly dividing normal cells resulting in toxic side-effects. This nonspecificity necessitates identification of novel cellular pathways that are reprogrammed selectively in cancer cells and can be exploited to develop pharmacologically superior and less toxic therapeutics. Despite growing awareness on dysregulation of lipid metabolism in cancer cells, targeting lipid biosynthesis is still largely uncharted territory. Herein, we report development of a novel nontoxic orally deliverable anticancer formulation of monoethanolamine (Etn) for prostate cancer by targeting the Kennedy pathway of phosphatidylethanolamine (PE) lipid biosynthesis.

**Experimental Design:** We first evaluated gastrointestinal tract stability, drug–drug interaction liability, pharmacokinetic, and toxicokinetic properties of Etn to evaluate its suitability as a nontoxic orally deliverable agent. We next performed in vitro and in vivo experiments to investigate efficacy and mechanism of action.

**Results:** Our data demonstrate that Etn exhibits excellent bioavailability, gastrointestinal tract stability, and no drug–drug interaction liability. Remarkably, orally fed Etn inhibited tumor growth in four weeks by approximately 67% in mice bearing human prostate cancer PC-3 xenografts without any apparent toxicity. Mechanistically, Etn exploits selective overexpression of choline kinase in cancer cells, resulting in accumulation of phosphoethanolamine (PhosE), accompanied by downregulation of HIF-1α that induces metabolic stress culminating into cell death.

**Conclusions:** Our study provides first evidence for the superior anticancer activity of Etn, a simple lipid precursor formulation, whose nontoxicity conforms to FDA-approved standards, compelling its clinical development for prostate cancer management.

Clin Cancer Res; 1–13. ©2017 AACR.

Introduction

Malignant transformation alters several physiologic processes including reprogramming of biochemical and metabolic pathways, which can be exploited to develop superiorly efficacious and less toxic cancer therapies (1, 2). Altered lipid metabolism is one of the hallmarks of cancer. Thus, cellular lipids and lipid biosynthesis enzymes may serve as promising anticancer targets (3, 4). Literature reports that lipids and their precursor-based formulations are attractive anticancer drug candidates. For example, alkylphospholipids (ALP) exert cytotoxic effects by targeting cell membranes instead of conventional targets like DNA or microtubules (5). Lipid precursors such as omega-3 polyunsaturated fatty acids (ω-3 PUFA) exhibit antiproliferative activity against multiple cancers (6).

Another lipid precursor, phosphoethanolamine (PhosE), has recently garnered a great deal of attention in Brazil (7, 8) and is a subject of intense anticancer research (9–13). PhosE is a biosynthetic precursor of phosphatidylethanolamine (PE) lipids, which constitute the second-most abundant lipid class in cells, (16) and is synthesized in the first step of Kennedy pathway of PE lipid biosynthesis through ATP-dependent phosphorylation of monoethanolamine (Etn; ref. 17). PhosE exhibits antitumor activity in various in vitro and in vivo models by affecting multiple signaling pathways (9–15). Interestingly, vitamin K2–induced apoptosis in Jurkat cells is ascribed to intracellular PhosE accumulation (18).

Herein, we evaluated Etn and PhosE, biosynthetic precursors of PE lipids, as anticancer agents to ultimately develop a nontoxic orally deliverable formulation for prostate cancer. We found that Etn, first precursor in Kennedy pathway, exhibits remarkable anticancer activity in vitro and in vivo models. The Lipinski’s rule for molecular properties endorsed Etn’s appropriateness for oral administration in humans. Mechanistically, Etn induces cell death by downregulation of HIF-1α, accompanied by depletion of cellular glucose and glutamine levels; this results in metabolic stress and triggers apoptosis. Etn spares normal cells by exploiting
Translational Relevance

Severe toxicity of currently available chemotherapeutic agents and their intravenous infusion necessitating hospital visits limit their usefulness in cancer management. In this work, we have identified Etn (a precursor of phosphatidyl-ethanolamine lipids) as an orally deliverable anticancer agent whose nontoxicity conforms to FDA-approved standards. We demonstrated that Etn exhibits excellent pharmacokinetic and toxicokinetic profiles and anticancer activity, attributes that are most sought after in orally deliverable anticancer drugs. Mechanistically, Etn targets energy and metabolite source (glucose and glutamine) of cancer cells which are unconventional targets in cancer therapy. These exciting results set the stage to systematically develop Etn as an IND (investigational new drug) candidate for cancer therapy. We are hopeful that clinical translation of Etn would bring an "effective, safer, and kinder" oral therapy to cancer patients, and eliminate inconveniences associated with intravenous infusion of chemotherapeutic drugs that necessitates multiple hospital visits and admission.

Materials and Methods

Information on cell lines

PC-3, DU145, MDA-MB-468, OVCAR-3, CFPAC-1, and HCT116 cancer cell lines were purchased from ATCC and used as such as authentication of the cell lines was provided with their purchase from ATCC.

C4-2B cell line is a derivative of the LNCaP parental cell line. C4-2B was developed by Leland Chung's group (Cedars-Sinai Medical Center, Los Angeles, CA) and was obtained from his lab as a generous gift. C4-2B cells were tested for mycoplasma contamination using MycoAler Mycoplasma Detection Kit from Lonza.

PC-3-luc cells were purchased from Perkin Elmer and periodically tested for mycoplasma contamination using MycoAler Mycoplasma Detection Kit from Lonza.

All experiments were performed with cells between 10–15 passages.

Cell lines, media, antibody, and reagents

Prostate (PC-3, PC-3-luc, DU145, C4-2B), breast (MDA-MB-468), ovarian (OVCAR-3), pancreatic (CFPAC-1), colon (HCT116) cancer cell lines and near-normal prostate RWPE-1 cell lines were used. PC-3-luc cells were from Perkin Elmer and all other cell lines were from ATCC except C4-2B which was obtained from Leland Chung's lab as a generous gift. Primary antibodies against Cdk4, Cdk2, p-Rb, p21, Bim, Bid, Bcl-2, pBcl-2, cleaved PARP, and β-actin were from Cell Signaling Technology, Ki67, and HIF-1α, were from BD Biosciences, and CK was from Proteintech. Bax, GAPDH, and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology. Phosphoethanolamine, monoethanolamine, lucif-erin and dimethylxylglycine, N-(Methoxysuccinyl)-glycine methyl ester (DMOG) were from Sigma. CK-α inhibitor was from Calbiochem. siRNA against CK was from GE Dharmacon.

Stability of Etn and PhosE in simulated gastric and intestinal fluid

Simulated gastric fluid (SGF) and intestinal fluid (SIF) were prepared following US Pharmacopeia methods. PhosE and Etn were incubated in SGF and SIF for varying times followed by their quantification using LC/MS-MS analysis.

Pharmacokinetic and toxicologic studies

Pharmacokinetic studies (oral and intravenous) were performed in male BALB/c mice (Harlan Laboratories). Plasma was extracted from blood samples collected from animals at different time points by centrifugation (8,000 × g/10 minutes) and stored below –80°C until analysis. Pharmacokinetic parameters were calculated using noncompartmental analysis tool of Phoenix WinNonlin software (V6.3, Pharsight). Toxicity studies of Etn were performed in male and female Sprague-Dawley (SD) rats (Harlan Laboratories).

Cell proliferation and colony survival assay

Proliferation of PC-3, DU145, C4-2B, RWPE-1, MDA-MB-468, OVCAR-3, and CFPAC-1 cells treated with Etn/PhosE was evaluated employing MTT assay as described earlier (19). Clonogenic assay was performed as described previously (19).

In vivo tumor growth and bioluminescent imaging

In vivo prostate tumor growth in athymic male BALB/c mice was measured using vernier calipers and bioluminescence imaging as described previously (19). Animal experiments were in compliance with GSU IACUC guidelines.

Immunoblotting and IHC

Immunoblotting was performed as described earlier (20). Five-micron prostate tumor sections were stained for Ki67 and c-PARP and prostate cancer TMA (US Biomax) was immunostained for CK. All stained slides were examined by a pathologist in a blinded manner.

In situ analysis of CK expression

Expression level of CK-A in prostate cancer was analyzed using Oncomine (https://www.oncomine.org/resource/login.html). Reporter ID and platform for datasets used were as follows: gene rank 229 (21), 839 (22), 2059 (23), 1589 (24), 8514 (25), 1206 (26), 546 (27) analyzed on Human Genome U133 Plus 2.0 Array.

RNA, DNA preparation, and real-time PCR

RNA extracted from control and 2 mg/mL Etn-treated PC-3 cells using RNeasy kit from Qiagen, and quantified using NanoDrop. Reverse transcription of RNA was performed for the first strand cDNA synthesis using First-Strand Synthesis Kit (GoScript Reverse Transcription System-A5000) from Promega. The quality of cDNA was checked on agarose gel. RT-PCR of cDNA samples (in duplicates) was performed using iQ SYBR Green Supermix from Bio-Rad as per the manufacturer's instructions. The RT-PCR primers were designed manually, checked with the primer3 software, and ordered from Sigma.

Measurement of oxygen consumption rate

Oxygen consumption rate (OCR) was measured using a computer-interfaced oxygen electrode (Hansatech Instruments Inc.) by monitoring initial rate of oxygen consumption at 37°C and atmospheric oxygen concentration (230 μmol/L O₂).
Electron microscopy and lipidomics

Tumor tissue was processed for electron microscopy as described previously (19). Lipidomic analysis of control and Etn-treated tumors was performed by Lipidomics Core Facility, Wayne State University (Detroit, MI).

Statistical analysis

Results are expressed as mean values ± SE values of at least three independent experiments. P-values (Student t test) were calculated using Microsoft Excel software.

Results

PE lipid precursors as anticancer candidates: gastrointestinal tract stability, drug–drug interactions, and pharmacokinetics

Given that development of oral drugs is impeded by limited solubility, poor gastrointestinal tract stability, low permeability, and extensive first-pass metabolism (28), we first asked whether PE lipid precursors are good candidates for oral delivery. Interestingly, Etn and PhosE both satisfy Lipinski’s and Veber’s rule that examine molecular properties and druggability of a compound, suggesting that these PE lipid precursors (Supplementary Fig. S1) can induce pharmacologic effects in humans upon oral consumption, thus qualifying them as viable candidates for further evaluation.

Many drugs are degraded in the gastrointestinal tract when confronted with extreme pH and harsh digestive enzymes, thereby explaining their decreased bioavailability or inability to reach the target at optimal concentrations. SGF (pH 1.2) and SIF (pH 6.8) mimic gastrointestinal tract environment and are amenable in vitro systems to evaluate compound degradation in gastrointestinal tract (US Pharmacopoeia). We found that both Etn and PhosE were stable in SGF over 1 hour (Fig. 1A1). While Etn remained unchanged in SIF over time (Fig. 1A, ii), PhosE exhibited an approximately 35% decrease in its concentration after 2 hours in SIF suggesting its degradation (Fig. 1A, ii). These results demonstrate enhanced stability of Etn compared with PhosE in the gastrointestinal tract.

Polypharmacy increases the risk of drug–drug interaction (DDI) and has led to drug marketing with black box warnings (29). Thus, we evaluated the potential of Etn and PhosE to inhibit major drug metabolizing cytochrome P450 enzymes. For all the nine tested CYPs (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) Etn and PhosE showed IC₅₀ values of more than 100 μmol/L (equivalent to 6.1 μg/mL Etn and 14.1 μg/mL PhosE) suggesting no CYP related drug–drug interaction liabilities (Supplementary Table S1).

Next, we developed a bioanalytic method to quantify Etn and PhosE using LC/MS-MS [representative chromatograms for Etn (retention time (RT): 6.3 minutes) and PhosE (RT: 5.3 minutes) in Supplementary Fig. S2A]. To select a suitable matrix for

Figure 1.

Gastrointestinal stability and pharmacokinetic parameters of Etn and PhosE. A, Stability of Etn and PhosE in (i) SGF (pH 1.2) and (ii) SIF (pH 6.8). Etn (10 μmol/L) and PhosE (10 μmol/L) were spiked into preincubated SGF and SIF. Samples (100 μL) were drawn at 0, 15, 30, and 60 minutes from SGF reaction-vials and at 0, 30, 60, and 120 minutes from SIF reaction-vials, quenched with acetonitrile, vortex-mixed, and centrifuged before supernatant analysis by LC/MS-MS analysis. B, Cmax (i) and (ii) AUCₑ₅₀ for Etn and PhosE upon oral administration of Etn and PhosE. C, CL (clearance; i) and Vss (volume of distribution; ii) for Etn and PhosE upon intravenous administration of Etn and PhosE. While PhosE and Etn were orally fed at 60 and 40 mg/kg, respectively, intravenous administration were 3 and 2 mg/kg, respectively. For pharmacokinetic studies, a sparse sampling design with 3 mice per time point was used to collect blood samples at 5, 10, 15, 30 minutes, 1, 2, 3, 4, 5, and 6 hours in K₂EDTA-coated tubes. The pharmacokinetic parameters (AUCₑ₅₀, Cmax, CL, and Vss) were calculated using noncompartmental analysis tool of Phoenix WinNonlin software (version 6.3).
pharmacokinetic studies and to rule out any red blood cell (RBC) accumulation of Etn and PhosE, blood-to-plasma concentration ratio (BPR) was determined in BALB/c mice. Our data confirmed lack of preferential partitioning into RBCs; hence, plasma was selected as matrix for pharmacokinetic studies (Supplementary Fig. S2B).

To evaluate bioavailability, we performed pharmacokinetic studies in BALB/c mice following oral (Etn: 40 mg/kg; PhosE: 60 mg/kg) and intravenous (Etn: 2 mg/kg; PhosE: 3 mg/kg) dose (Supplementary Fig. S2). Irrespective of the compound dosed, we analyzed both Etn and PhosE in all samples. The time to reach peak plasma concentration was 10 minutes for both Etn and PhosE upon oral Etn administration. Maximum concentration (C_{max}) achieved following oral administration of 40 mg/kg Etn was 32-fold higher at 17.37 μg/mL compared with 0.55 μg/mL of PhosE. Similarly, AUC_{last} of Etn was 13-fold higher at 9.10 μg h/mL compared with 0.72 μg h/mL of PhosE (Fig. 1B, i and ii; Supplementary Table S2). However, oral administration of PhosE gave similar exposure (C_{max} and AUC_{last}) for both PhosE (1.14 μg/mL and 1.89 μg h/mL) and Etn (4.32 μg/mL and 2.02 μg h/mL) suggesting that PhosE gets converted into Etn in vivo (Fig. 1B, i and ii). A similar trend was observed following intravenous administration of PhosE (Supplementary Table S2). Intravenous Etn led to a moderate clearance at 57.23 mL/min/kg compared with normal liver blood flow of 90 mL/min/kg (Fig. 1C, i). The volume of distribution (V_{d}) of Etn was 4-fold higher compared with normal body water of 0.7 L/kg confirming its extensive distribution into various tissues (Fig. 1C, ii). PhosE showed high clearance of 101.92 mL/min/kg equivalent to normal liver blood flow with a high V_{d} at 4.82 L/kg (Fig. 1C, i and ii). Both Etn and PhosE showed a plasma half-life of less than 1 hour. While oral bioavailability of Etn was excellent at 78%, PhosE was poorly bioavailable at 19% (Supplementary Table S2), perhaps due to its conversion to Etn by alkaline phosphatases present in intestine and liver. Summarizing, our pharmacokinetic data strongly suggest Etn’s superiority over PhosE for an orally deliverable agent.

To evaluate concentration–time profile of Etn and to understand its accumulation following repeated oral dosing for 28 days, we used Phoenix WinNonlin software with single-dose administration data. Etn showed T_{max} of less than 30 minutes following daily repeated dose confirming rapid absorption (Supplementary Table S3). No Etn accumulation was predicted on dosing over 28 days. Interestingly, a dose proportional increase in exposure (AUC_{last}) was observed from 40 mg/kg to 200 mg/kg. A 2-fold increase in dose (40 to 80 mg/kg) elicited a 2-fold increase in exposure. Similarly, a 5-fold increase (from 40 to 200 mg/kg) led to a 5-fold increase in exposure on day 1 and day 28. The simulated data profile suggests twice-a-day dosing regimen for toxicologic studies.

We next asked whether Etn is nontoxic, well-tolerated, and safe for oral consumption. As per OECD guidelines, we first tested acute toxicity of Etn in male and female Sprague–Dawley (SD) rats. Five male and five female rats were orally administered with single dose of 5 g/kg Etn and monitored for 1 week for any sign of distress/sickness. After one week, all rats were alive and did not display any sign of distress or toxicity suggesting that Etn does not induce any acute toxicity even at the limit dose. We further investigated organ-related toxicity by histopathologic evaluation of organs obtained from control and Etn-fed mice. Comparative analysis of various blood components and serum chemistry parameters indicative of liver, kidney, and cardiac function and muscle integrity in control and Etn-fed groups was performed. Immunotoxicity of Etn was evaluated by estimating percentages of CD4+T, CD8+T, CD19-B, NK cells, and macrophages in spleens from control and Etn-fed mice. Our data show that Etn feeding did not induce any observable organ-related or immune toxicity and was safe for oral consumption over an extended period (Supplementary Figs. S3–S5).

**Antiproliferative activity of PE lipid precursors**

Next, we evaluated the antiproliferative activity of various Etn and PhosE concentrations on human prostate PC-3 cancer cells. Quantitation of cell survival showed that Etn was more effective in inhibiting cell proliferation compared with PhosE as shown in Fig. 2A, i. The half-maximal concentration (IC_{50}) of Etn was approximately 0.88 mg/mL. Interestingly, PhosE only showed limited inhibition of PC-3 cell proliferation up to 0.5 mg/mL concentrations greater than 0.5 mg/mL were mostly ineffective (Fig. 2A, i). In a clonogenic assay to assess the reproductive capacity of cells upon drug removal, we found that while 2 mg/mL Etn decreased colony numbers by approximately 97%, 2 mg/mL PhosE was ineffective in decreasing colony numbers compared with control cells (Fig. 2A, ii). We further tested antiproliferative activity of Etn on other prostate cancer lines (DU145 and C4-2B) and near-normal prostate cell line, RWPE-1. We found that 0.5 and 1 mg/mL Etn were more effective in reducing viability of prostate cancer lines (PC-3, DU145, and C4-2B) compared with normal prostate cells (RWPE-1; Fig. 2B, i). While 0.5 mg/mL Etn reduced survival of PC-3, DU145, and C4-2B cells by approximately 30%–52%, RWPE-1 cells remained unaffected. To test the generality of Etn’s antiproliferative activity on representative cancer cell lines from different tissues [breast (MDA-MB-468), ovary (OVCAR-3), and pancreas (CPCAC-1)], we performed MTT assay to obtain dose–response curves upon Etn treatment. The IC_{50} of Etn was 0.55, 0.29, and 1.9 mg/mL in MDA-MB-468, OVCAR-3, and CPCAC-1, respectively (Fig. 2B, ii), suggesting its broad applicability in inhibiting proliferation of various cancer cell types. Furthermore, PhosE was ineffective in inhibiting proliferation and colony formation of cancer cells of varying tissue origin (Supplementary Fig. S6). To understand why Etn was more effective than PhosE in inhibiting proliferation, we quantified changes in intracellular levels of PhosE and Etn upon treatment with Etn or PhosE in PC-3 cells. Given that Etn and PhosE are interconvertible, we were curious to pinpoint the species responsible for inhibiting proliferation. Intriguingly, both Etn and PhosE remarkably increased PhosE intracellularly in Etn-treated cells (Fig. 2Ci). While Etn elevated PhosE levels by approximately 40-fold compared with control cells, PhosE increased intracellular PhosE levels by only approximately 15-fold relative to control cells (Fig. 2C, ii). Thus, higher intracellular PhosE levels clearly correlated with reduced cell survival. These data confirmed that Etn serves as an easily absorbable produg, which upon entry into cells, gets converted into cytotoxic intracellular PhosE.

**In vivo efficacy of Etn in prostate cancer xenografts**

Given its superior absorption, gastrointestinal tract stability, nontoxicity, and antiproliferative activity, Etn is clearly a better candidate than PhosE for development of an oral anticancer formulation. However, as PhosE gets converted into Etn upon oral administration, we reasoned that formulations containing both Etn and PhosE may provide a 2-fold advantage and could be potentially developed for cancer treatment. First, PhosE converts...
into Etn in vivo, thereby increasing Etn exposure more than Etn alone. Second, the acidic nature of PhosE can attenuate the basicity of Etn making it suitable for oral administration. To this end, we tested the in vivo efficacy of a repertoire of formulations containing Etn and PhosE in various molar ratios with pH adjusted to 5 or 7.4 using phosphoric acid. Unfortunately, formulations with pH 7.4 and/or containing PhosE were either not as effective in inhibiting tumor growth as Etn at pH 5 or in some cases even accelerated tumor growth (data not shown). Moreover, Etn absorption was independent of pH (5/7.4) or acid (H₃PO₄/HCl/H₂SO₄) employed to adjust formulation pH (Supplementary Table S4). These data generated compelling grounds to exclusively pursue the Etn-containing formulation at pH 5 adjusted using phosphoric acid.

We next examined the in vivo anticancer efficacy of Etn formulation in prostate (PC-3-luc and DU145) and colon (HCT116) cancer xenografts. Tumor-bearing control and treated mice received vehicle (water) and 40 mg/kg Etn, respectively, by oral gavage for 2 (HCT116) or 4 (PC-3-luc and DU145) weeks. Tumor growth was measured by both vernier calipers (twice/week) and bioluminescence imaging (only PC-3-luc; Fig. 3A, i and ii; once/week) for 2/4 weeks. We observed approximately 67% reduction in tumor volume (Fig. 3A, iii) and approximately 55% reduction in tumor weight (Fig. 3A, iv) after 4 weeks of Etn treatment in PC-3-luc xenograft model. In DU145 xenografts, we observed an approximately 42% reduction in tumor volume (Fig. 3D, i) and approximately 29% reduction in tumor weight (Fig. 3D, ii) after 4 weeks of Etn treatment. Furthermore, we observed approximately 44% decrease in tumor volume in HCT116 xenograft model after 2 weeks of Etn treatment (Supplementary Fig. S7A). Importantly, no apparent change in body weight of control and Etn-treated mice over the treatment course in both models (Fig. 3B and E; Supplementary Fig. S7B) implied that Etn is nontoxic.

Quantiﬁcation of intratumoral levels of Etn and PhosE after 4 weeks of Etn treatment showed that PhosE level in Etn-treated PC-3-luc tumor-bearing mice was approximately 38% higher than control mice (Fig. 3C). However, we did not observe any signiﬁcant change in intratumoral Etn levels between control and Etn-treated mice. These data are in consonance with our in vitro findings and reconﬁrm that the intracellular conversion of Etn into PhosE results in intratumoral accumulation of cytotoxic PhosE, which perhaps is crucial for tumor growth inhibition.

Inhibition of CK activity attenuates Etn’s antiproliferative activity

Having conﬁrmed that Etn converts into PhosE intracellularly, we next sought to identify the enzyme responsible for this conversion. Two enzymes, namely, ethanolamine kinase and CK are known to catalyze conversion of Etn into PhosE. However, CK has been reported to be overexpressed in multiple cancers including lung, prostate, and breast (30). To examine whether CK catalyzes the conversion of Etn into PhosE, we determined survival of PC-3...
cells upon Etn treatment in the presence/absence of CK function. While Etn alone reduced cell proliferation by approximately 33% (Fig. 4A), pharmacologic CK inhibition significantly attenuated Etn-induced reduction in cell proliferation from approximately 33% to approximately 17% (Fig. 4A). CK inhibitor itself did not significantly affect PC-3 proliferation at the employed concentration. We also observed that CK inhibition reduced conversion of Etn into PhosE by approximately 19% (Fig. 4B). We further employed siRNA approach to confirm the role of CK in Etn-induced cell death. Knockdown (KD) of CK using siRNA significantly abated Etn-mediated reduction in viability of PC-3 cells (Fig. 4C). While 0.5 mg/mL Etn reduced cell survival of PC-3 cells by approximately 38%, cell viability in CK KD PC-3 cells was decreased by only approximately 11% (Fig. 4C). These results underscore CK's role in the conversion of Etn to PhosE in PC-3 cells. Furthermore, we found that CK expression is low in normal prostate cell line (RWPE-1) compared with prostate cancer cell lines (PC-3, DU145, and C4-2B; Fig. 4D), which may underlie differential sensitivity of normal versus cancer cells to Etn (Fig. 2B, i). Next, we asked why Etn selectively affected cancer cells and spared normal ones. To this end, we explored publicly available datasets for CK gene expression in prostate cancer patients as well as evaluated CK protein expression immunohistochemically in tumor versus adjacent normal tissue from prostate cancer patients. We found that CK is highly overexpressed in prostate cancer tissue compared with adjacent normal (Fig. 4E and F, i and ii). Our in silico analysis showed that prostate cancer exhibits 2.1-fold higher CK expression compared with normal prostate tissue (Fig. 4E). Quantification of CK immunostaining showed that CK expression was 1.5-fold higher in prostate cancer tissue compared with normal prostate tissue (Fig. 4F).
with adjacent normal (Fig. 4F, ii). This differential CK expression (both at gene and protein level) perhaps explains increased sensitivity of cancer cells to Etn.

Etn activates mitochondrially mediated death pathways and affects cellular respiration and metabolism

To gain mechanistic insights, we evaluated protein expression and transcript levels of cell-cycle and apoptosis-regulatory molecules in PC-3 cells using immunoblotting (Fig. 5A and B). While Etn caused downregulation of protein expression of pRb, Cdk4, and Cdk2, upregulation of p21 suggested that Etn stalls cell-cycle progression. Furthermore, an increase in protein expression of proapoptotic markers (c-PARP and Bim), and a decrease in antiapoptotic molecules (Bcl-2) upon Etn treatment implicates a mitochondrially mediated death pathway (Fig. 5A). This was confirmed at transcriptional level where Etn upregulated p21, PARP1, Bax and Bid, and downregulated Bcl-2 (Fig. 5B). The binding of Annexin-V to phosphatidylserine (PS) lipids followed by flipping to the outer plasma membrane leaflet in apoptotic cells (31) serves as an indicator of apoptosis. We observed flow cytometrically that Annexin-V–positive cells increased from approximately 8% to approximately 25% upon Etn treatment, suggesting that Etn induces apoptosis in PC-3 cells (Supplementary Fig. S6C). To uncover signaling pathways responsible for in vivo tumor inhibition, we examined expression levels of molecular regulators of cell-cycle and apoptosis in tumor lysates prepared from control and 40 mg/kg Etn-treated mice. Etn caused upregulation of p21, Bax and pBcl-2, c-PARP, Bim, and Bid (Fig. 5C). Upregulation of protein/transcript levels of Bim and Bid in Etn-treated PC-3 cells/prostate tumors suggests activation of cell death pathway involving BH3-only proteins of Bcl-2 family. Immunohistochemical staining of paraffin-embedded samples for Ki67 (cell proliferation) and c-PARP (apoptotic) showed decrease in Ki67 and increase in c-PARP in treated tumors compared with control ones (Fig. 5D, i and ii). While approximately 62% cells were Ki67-positive in control tumors, Ki67 positivity was reduced to approximately 40% in Etn-treated tumors (Fig. 5D, ii).
To further address the mechanism of action of Etn, we extrapolated the in vitro efficacy dose (IC50: 0.88 mg/mL) to the corresponding in vivo dose (140.57 mg/kg; therapeutic index = 5) using NIH guidelines (32). Intriguingly, we realized that supraphysiologic Etn concentrations are required for in vitro activity. However, Etn displays remarkable in vivo efficacy with 40 mg/kg Etn which is approximately 3.5 times lower than the extrapolated dose for its in vivo efficacy. This indicated that Etn takes advantage of an unknown aspect of the in vivo physiology of cancer to evoke its anticancer activity, which cannot be mimicked in a cell culture system.

It is well recognized that HIF-1α plays a pivotal role in cancer progression by regulating several survival pathways in cancer cells (33, 34). For instance, HIF-1α regulates glucose metabolism under hypoxia by inducing expression of glucose transporters to increase glucose uptake to fulfill energy demands of rapidly proliferating cancer cells through glycolysis (35). Recently, HIF-1α has been shown to regulate glutamine metabolism (36). Reports indicate that BH3-only proteins of Bcl-2 family (members include Bim, Bid, Noxa etc) play an important role in inducing apoptosis in response to energy/metabolic stress in cells through both p53-dependent and -independent pathways (37, 38). However, mechanisms linking cellular stress and activation of BH3-only proteins are not well understood (39). Since PC-3 cells are p53 null, it is likely that p53-independent pathway involving BH3-only proteins are activated in response to cellular stress in these cells upon Etn treatment. Literature also indicates that cellular PhosE accumulation affects respiration and that both Etn and PhosE impair mitochondrial respiration by altering OCR in isolated mitochondria (40, 41). This led us to hypothesize that PhosE accumulation alters HIF-1α function that impairs glucose/glutamine metabolism leading to bioenergetic/metabolic stress in cells, which induces cell death by involving BH3-only proteins and its downstream effectors. To test this hypothesis, we examined HIF-1α protein and gene expression in Etn-treated tumors and PC-3 cells, respectively. We found that Etn treatment resulted in 50-fold reduction in HIF-1α transcripts levels in PC-3 cells compared with control cells (Fig. 5B). We found downregulation of HIF-1α protein expression in Etn-treated tumors compared with control tumors (Fig. 5E). Using DMOG, we tested the effect of HIF-1α stabilization [active HIF-1α signaling] on Etn-mediated cell death in PC-3 cells. Etn was more effective in reducing survival in PC-3 cells with active HIF-1α signaling owing to HIF-1α stabilization by DMOG compared with controls (Fig. 5F), suggesting the possible role of HIF-1α signaling axis in Etn-mediated cell death. These data lend plausible explanation for the discrepancy between in vitro and in vivo efficacy doses. It is likely that Etn is more effective in vivo due to active HIF-1α signaling in hypoxic tumor tissues compared with cultured cells under normoxic condition. Furthermore, we measured OCR in control and Etn-treated cells as a function of cell number as well as evaluated glucose and glutamine content in cultured cells and tumors from control and Etn-treated mice. We observed that Etn affected OCR in PC-3 cells and the extent of reduction varied with cell number such that OCR in treated cells was reduced by approximately 26% compared with controls at a concentration of 1 million cells/mL. (Fig. 5G). Both glucose and glutamine content were also significantly reduced in Etn-treated tumors (Fig. 5H, 1 and ii) and cells (Fig. 5I, 1 and ii) in comparison with control tumors and cultured cells, although the effect was more pronounced in tumors than cultured cells. It is likely that HIF-1α-dependent pathways are not so active in cultured PC-3 cells with adequate supply of oxygen and nutrients leading to discrepancy in pharmacologic effects of Etn observed under in vitro and in vivo setting. This may partly explain the inconsistency in our immunoblotting data from cell and tissue lysates. Our data underscore the likelihood that Etn employs different cell-cycle and apoptosis regulators to mediate in vitro and in vivo effects due to differential extent of HIF-1α signaling under the two conditions. Furthermore, as alluded earlier, Etn possibly exploits an unknown aspect of the in vivo cancer environment which may drive disparate mechanisms in cultured cells versus physiologic systems. Furthermore, we found that CK inhibition abrogated Etn-mediated decrease in cellular glucose and glutamine content (Fig. 5I, 1 and ii). Taken together, these data suggest that Etn altered intracellular glucose and glutamine levels in tumors and cultured prostate cancer cells.

Having identified the effects of Etn on intracellular glucose and glutamine that impact glycolysis and other metabolic pathways, we performed 2D gel electrophoresis of tumor lysates from control and Etn-treated animals to identify and characterize differentially expressed proteins using LC/MS-MS analysis. We found two enzymes of glycolysis (GAPDH and phosphoglycerate kinase-1) and one enzyme of glutamine metabolism (delta-1-pyrroline-5-carboxylate synthase) down-regulated upon Etn treatment, which could exacerbate the metabolic crisis in Etn-treated tumor cells (Supplementary Fig. S8; Supplementary Table S5).

**Figure 5.** Etn activates mitochondrially mediated death pathways and affects oxygen consumption rate (OCR) and cellular metabolism in cancer cells. A, Immunoblots of control and Etn-treated (2 mg/mL for 48 hours) PC-3 cell lysates for molecular regulators of cell cycle (pRb, Cdk4, Cdk2, p21) and apoptosis (c-PARP, Bim, and Bcl-2). B-actin was used as a loading control. B, Relative transcripts of p21, PARP1, Bcl-2, HIF-1α, Bax, and Bid in control and Etn-treated (2 mg/mL for 48 hours) PC-3 cells. RNA samples were run on MOPS agarose gel to check integrity and two clear bands were observed for each sample. C, Immunoblots of control and 40 mg/kg Etn-treated PC-3-luc tumors lysates for p21, Bax, pBcl-2, c-PARP, Bim, and Bid. B-Actin was used as a loading control. D, Micrographs showing immunohistochemical staining of K67 and c-PARP in control and Etn-treated PC-3-luc prostate xenografts. E, Quantification of K67 staining in control and Etn-treated prostate xenografts. F, Effect of Etn treatment on HIF-1α expression level in PC-3-luc prostate xenografts. G, Effect of HIF-1α stabilization on Etn-induced cell death in PC-3 cells. PC-3 cells were pretreated with 35 µg/mL DMOG (HIF-1α activator) for 4 hours followed by treatment with 1 and 2 mg/mL Etn and DMOG together for 48 hours and estimation of cell survival by MTT assay. H, Effect of Etn treatment on oxygen consumption rate in PC-3 cells. PC-3 cells were treated with 2 mg/mL Etn for 48 hours at pH 7.4 and cells were suspended at a concentration of 0.5, 0.75, and 1 x 10^5/mL. OCR was measured by using oxygen electrode. Measurements were initiated by adding 500 µL of control and 2 mg/mL Etn-treated cell suspension at various concentrations into electrode chamber preequilibrated with 500 µL fresh media. The plot shows representative OCR as a function of cell number for control and Etn-treated cells. I, Intracellular glucose (i) and glutamine (ii) levels in control and 40 mg/kg Etn-treated PC-3-luc tumors. Glucose and glutamine levels in control and Etn-treated tumors were estimated by LC/MS-MS method. J, Effect of CK inhibition on intracellular levels of glucose and glutamine in Etn-treated cells. Treatment of PC-3 cells with 2 mg/mL Etn for 48 hours reduced intracellular level of glucose (i) and glutamine (ii) and this reduction in glucose and glutamine level was abrogated upon inhibition of CK enzyme. Values and error bars shown in the figure represent mean and SE, respectively, from three independent experiments (*, P < 0.05 compared with control; ***, P < 0.0001 compared with Etn treatment).
Figure 6.
Effect of Etn treatment on mitochondrial integrity and cellular lipids. A, Representative transmission electron micrographs of control and 40 mg/kg Etn-treated tumors showing changes in mitochondrial morphology and accumulation of lipids upon Etn treatment. Ultra-thin sections were cut on Boeckeler MTx ultramicrotome, counterstained with lead citrate, and examined on a LEO 906e transmission electron microscope. Mitochondria and accumulated lipid granules are highlighted by arrows in the panel. Treated tumors showed elongated mitochondria with degrading mitochondrial matrix (ii) and abundant lipid rich granules (iv) in comparison with control tumors (i and iii). Left panels, scale bar = 2 μm; right panels, scale bar = 5 μm. B, Etn treatment increases lipid levels in Etn-treated tumors. Levels of PE (i), PS (ii), PC (iii), and SM (iv) lipids in control and Etn-treated tumors. In the abbreviation of lipid first and second numbers denote the number of carbon atoms and unsaturated bonds present in the lipid, respectively. Lipid amounts were quantified by LC/MS-MS analysis. Values and error bars shown in the figure represent mean and SE, respectively. C, Schematic diagram depicting proposed model for anticancer activity of Etn in prostate cancer cells. We propose that accumulation of PhosE and phospholipid downregulates HIF-1α which precipitates a bioenergetics/metabolic crisis leading to activation of BH3-only proteins-mediated signaling cascade culminating into cell death.
Etn alters cellular lipids and impairs mitochondrial integrity in vivo

As Etn is a precursor of lipid constituents of membrane-bound cellular structures, we next asked whether Etn altered structural integrity of membrane-bound organelles or adversely affected membrane fission–fusion events. Transmission electron microscopy (TEM) micrographs of tumors showed structural differences in mitochondria from control and Etn-treated groups. Specifically, mitochondria were elongated along with degraded matrix in treated tumors (Fig. 6A, ii) compared with controls (Fig. 6A, i). We observed more osmiophilic granules in treated versus control tumors (Fig. 6A, iii and iv) indicating lipid accumulation in treated tumor cells. These results suggest that Etn treatment leads to lipid accumulation in cells, causes alteration of mitochondrial structure, and induces lipid-mediated activation of cell death pathways. As TEM micrographs of Etn-treated samples showed many lipid granules, we next examined which lipids are specifically upregulated upon Etn treatment by a lipidomic analyses of tumors from control and Etn-treated groups.

We quantified a total of 402 lipids from various lipid classes such as phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), lyso phospholipids, ceramides, and sphingomyelin (SM). Levels of 21 lipids, out of 402, were increased in tumors from Etn-treated group (Fig. 6B). While these lipids mostly belonged to PE class (Fig. 6B, i), other lipids from the PS (Fig. 6B, ii), PC (Fig. 6B, iii), and SM (Fig. 6B, iv) lipid classes were also increased. In summary, we infer that altered lipid levels can perturb lipid homeostasis upon Etn treatment resulting in changes in membrane properties that could initiate a cascade of events detrimental to cell survival.

Discussion

We have reported the preclinical development of Etn, including in vitro and in vivo efficacy, mechanism of action, pharmacokinetic evaluation, and toxicity measurements of its oral formulation for prostate cancer management. Taken together, our results demonstrate that Etn possesses desirable molecular traits and anticancer attributes with favorable ADMET properties for its development as an orally deliverable broad-spectrum cancer therapeutic. Etn exploits intrinsic overexpression of CK in cancer cells to convert into cytotoxic PhosE. Furthermore, Etn treatment through downregulation of HIF-1α precipitates a bioenergetics/metabolic crisis, which activates signaling cascade culminating into cell death (Fig. 6C).

Essentially, malignant transformation involves extensive rewiring of metabolic circuitries to fulfill metabolic needs of rampant proliferating cancer cells. HIF-1α plays a significant role in metabolic adaptation (shifting toward anaerobic glycolysis) of cancer cells by regulating glucose metabolism (33). HIF-1α controls expression level of glycolysis and gluconeogenesis enzymes including GAPDH and phosphoglycerate kinase-1, which were found to be downregulated upon Etn treatment (33, 42). In cancer cells, glycolysis also provides intermediates for the synthesis of cellular building blocks (43). Glycolytic intermediates are used for the synthesis of amino acids such as alanine, serine, and glycine. Recent reports demonstrate that HIF-1α regulates metabolism of glutamine, a key nutrient that maintains redox homeostasis and contributes to energy production and lipid biosynthesis (44, 45). Etn-mediated HIF-1α downregulation results in reduced intracellular levels of glucose and glutamine uncoupling several metabolic pathways and leading to metabolic stress in cells. Downregulation of glycolysis and glutamine metabolism enzymes further exacerbates metabolic stress, which triggers cell death pathway involving BH-3 only proteins.

However, the question remains how Etn treatment down-regulates HIF-1α resulting in a metabolic catastrophe in cancer cells that destines them to death. As Etn treatment increases both PhosE and other lipids from various lipid classes, it is difficult to pinpoint the actual mediator of deleterious metabolic imbalance in cells. This is further complicated by lack of knowledge of biological implications of higher intracellular levels of PhosE. Phospholipids are one of the most abundant macromolecules present in cells and maintenance of cellular lipid homeostasis is crucial for cell survival. Furthermore, cell membranes exhibit a tightly regulated asymmetric distribution of various lipids that is necessary for their proper functioning in various cellular processes such as endocytosis, cell signaling, membrane protein activation, etc (46). Although lipid-mediated regulation of HIF-1α represents an untapped research area, there exists one report which suggests that changes in membrane properties can modulate HIF-1α expression by affecting EGFR function (47). This raises the possibility that accumulation of lipids may hamper the function of membrane proteins that are positive regulators of HIF-1α expression. Because of limited knowledge of the biological ramifications of high intracellular PhosE levels, further detailed investigations are indeed warranted to systematically delineate the molecular mechanism of HIF-1α downregulation upon Etn treatment.

We believe our study uncovers the previously unrecognized molecular link between the Kennedy pathway of lipid biosynthesis and cellular respiration/metabolism in cancer cells. On the basis of our results, Etn represents an extremely promising candidate for the development of an orally deliverable nontoxic formulation for prostate cancer treatment by targeting glucose metabolism, a driver of cancer progression. Indeed we are excited about the prospects of our study in the context of recent articles spotlighting the agony and demand of cancer patients to access a compound (essentially, a variant of PhosE) that has been deemed as miracle cure for cancer (7, 8).

Disclosure of Potential Conflicts of Interest

P.C.G. Rida is an employee of Novozoi Theranostics. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors would like to thank Dr. Charlie Benson and Samantha Simon from GSU for help with the acquisition of immunotoxicity data.
Grant Support
This work was supported by institutional grants to the R. Aneja (principal investigator).

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Received July 11, 2016; revised January 10, 2017; accepted January 22, 2017; published OnlineFirst February 6, 2017.

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Clin Cancer Res  Published OnlineFirst February 6, 2017.

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Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-16-1716

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