Drug Redeployment to Kill Leukemia and Lymphoma Cells by Disrupting SCD1-Mediated Synthesis of Monounsaturated Fatty Acids

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Abstract

The redeployed drug combination of bezafibrate and medroxyprogesterone acetate (designated BaP) has potent in vivo anticancer activity in acute myelogenous leukemia (AML) and endemic Burkitt lymphoma (eBL) patients; however, its mechanism-of-action is unclear. Given that elevated fatty acid biosynthesis is a hallmark of many cancers and that these drugs can affect lipid metabolism, we hypothesized that BaP exerts anticancer effects by disrupting lipogenesis. We applied mass spectrometry–based lipidomics and gene and protein expression measurements of key lipogenic enzymes [acetyl CoA carboxylase 1 (ACC1), fatty acid synthase (FASN)], and stearoyl CoA desaturase 1 (SCD1)] to AML and eBL cell lines treated with BaP. BaP treatment decreased fatty acid and phospholipid biosynthesis from 13C-n-glucose. The proportion of phospholipid species with saturated and monounsaturated acyl chains was also decreased after treatment, whereas those with polyunsaturated chains increased. BaP decreased SCD1 protein levels in each cell line (0.46- to 0.62-fold; P < 0.023) and decreased FASN protein levels across all cell lines (0.87-fold decrease; P = 1.7 × 10−5). Changes to ACC1 protein levels were mostly insignificant. Supplementation with the SCD1 enzymatic product, oleate, rescued AML and e-BL cells from BaP cell killing and decreased levels of BaP-induced reactive oxygen species, whereas supplementation with the SCD1 substrate (and FASN product), palmitate, did not rescue cells. In conclusion, these data suggest that the critical anticancer actions of BaP are decreases in SCD1 levels and monounsaturated fatty acid synthesis. To our knowledge, this is the first time that clinically available antileukemic and antilymphoma drugs targeting SCD1 have been reported. Cancer Res; 75(12); 2530–40. ©2015 AACR.

Introduction

Acute myelogenous leukemia (AML) and endemic Burkitt lymphoma (eBL) are two aggressive and biologically very distinct blood cancers that kill patients quickly if untreated. AML mainly affects elderly patients, who often cannot tolerate routine high-grade cytotoxic chemotherapy treatment (1, 2). eBL is a B-cell non-Hodgkin lymphoma predominantly affecting children in equatorial Africa. It responds well to high-grade chemotherapy; however, the high costs of treatment and supportive care often preclude their use in the endemic setting (3). Thus, in elderly AML patients in high-income countries and eBL patients in low-income countries, there is an urgent need for inexpensive treatments that selectively kill tumor cells with low systemic toxicity. One approach is drug redeployment where existing drugs with low toxicity are screened for efficacy against cancers (4). This approach has already been successful in reducing mortality in hematologic malignancies (5, 6). Using this method, we previously identified that the combination of the lipid lowering drug, bezafibrate, and the contraceptive, medroxyprogesterone acetate (MPA), has potent and cancer cell selective in vitro anticancer effects against AML, eBL, chronic lymphocytic leukemia, and B-cell non-Hodgkin lymphoma cells (7–10). The potency of this combination (denoted BaP) extended to the clinic demonstrating efficacy with low toxicity in clinical trials in AML (trial registration number ISRCTN50635541; ref. 11) and eBL patients (ISRCTN34303497; ref. 12). In vitro, BaP induces intracellular reactive oxygen species (ROS) in tumor cells, which is thought to be important for cell killing (8–10); however, as yet, we do not have a complete mechanism that fully explains the action of BaP against these diverse hematologic malignancies.

Both bezafibrate and MPA individually alter lipid metabolism (13, 14); however, nothing is known about their combinational effect on lipogenesis. We hypothesized that regulation of lipid metabolism could be an important component of BaP anticancer activity given that abnormally rapid lipogenesis is considered to be a hallmark of most cancers (15, 16). Elevated lipogenesis in tumor cells is at least partly caused by high rates of fatty acid biosynthesis (15–18), a phenomenon also observed in hematologic malignancies (19, 20). Enzymes that synthesize saturated fatty acids [acetyl CoA carboxylase 1 (ACC1) and fatty acid synthase (FASN)] and monounsaturated...
fatty acids [stearoyl CoA desaturase 1 (SCD1)] are often over-expressed in malignant cells, and selective inhibition of these enzymes exerts an anticancer effect in vitro (18, 20–24). Accelerated fatty acid synthesis may permit faster growth of tumor cells. Furthermore, synthesis of monounsaturated fatty acids may have additional benefits as it has been shown that oleate can protect cells against saturated fatty acid toxicity and cellular stress (25, 26). FASN, ACC1, and SCD1 have therefore been identified as plausible targets for cancer therapy, but agents shown to decrease their activities in the laboratory have yet to be translated to the clinic.

Here, we have investigated the effect of BaP on lipid metabolism in AML- and eBL-derived cell lines by measuring changes to the lipidome and lipid synthesis from 13C-labeled D-glucose using direct-infusion Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry. We also measured mRNA and protein levels of key lipogenic enzymes. We show that BaP slows fatty acid and phospholipid biosynthesis, which appears to be achieved by downregulation of lipogenic enzymes, particularly SCD1, at the gene and protein levels. External provision of SCD1 product oleate rescued both AML and eBL cell lines from killing by BaP. This suggests that the dysregulation of lipogenesis is a key component of the anticancer action of BaP.

Materials and Methods

Cell culture

AML (K562 and HL60; European Collection of Cell Cultures) and Burkitt lymphoma (BL2 and Blor; gift from A.B. Richardson, University of Birmingham, Birmingham, United Kingdom) cell lines were maintained in exponential growth (between 0.25 and 1.5 × 104 cells/mL) at 37°C with 5% CO2 in RPMI-1640 medium (containing 2 g/L D-glucose) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL of streptomycin ( Gibco-Invitrogen).

Drug treatments

Unless specified, cells were seeded at 2.5 × 103 cells/mL (AML) or 5 × 103 cells/mL (BL) in flasks (Falcon) and treated for 24 hours with either: BaP (0.5 mmol/L bezafibrate and 5 μmol/L MPA; Sigma-Aldrich); 0.5 mmol/L bezafibrate; 5 μmol/L MPA; or solvent control (ethanol and DMSO). DMSO and ethanol was at a final concentration of 1 in 1,000 in all classes. The 13C 0-glucose tracer study was set in glucose-free RPMI-1640 (Gibco-Invitrogen) supplemented with 2 g/L 1-13C 0-glucose (Sigma-Aldrich). Where indicated, HL60 cells were treated with a commercial SCD1 inhibitor (4-(2-Chlorophenoxy)-N-[3-[(methylamino)carboxyl]phenyl]-1-piperidinene carbamimide; ab142089; Abcam; 10 nmol/L).

Metabolomics/lipidomics cell quenching and extraction

Cell quenching (to rapidly halt metabolic activity) was done as described (27) with minor modifications. Cell suspensions were centrifuged (2,500 × g, 5 minutes, 20°C) and supernatant was discarded. Cell pellets were resuspended in residual media, of which 200 μL was added to 1.6-mL 60% methanol on dry ice (−40°C) and then centrifuged (2,500 × g, 5 minutes, −9°C). Supernatant was discarded and cell pellet weighed and transferred to a −80°C freezer until extraction. Polar metabolites and lipids were extracted from cell pellets using a modified Bligh-Dyer procedure (28, 29) using 8-μL methanol/mg biomass, 8-μL chloroform/mg biomass, and 7.2-μL water/mg biomass. The polar phase (100 μL) was dried in a centrifugal concentrator and the nonpolar phase (100 μL) was dried under nitrogen. Both were kept at −80°C until analysis.

Mass spectrometry analysis and data processing

Dried samples were resuspended in either: 50 μL 80:20 methanol:water with 20 mmol/L ammonium acetate (polar extracts), or 200 μL 2:1 methanol:chloroform with 5 mmol/L ammonium acetate (nonpolar extracts). All analyses were conducted by direct infusion mass spectrometry (DIMS), using a Triversa nanoelectrospray system (Advion Biosciences; +1.7 kV/−1.7 kV—positive/negative ion mode, 0.3-psi backing pressure) and a hybrid linear ion trap FT-ICR mass spectrometer (LTQ FT Ultra; Thermo Fisher Scientific) using resolutions of 100,000 to 400,000 (at 400 m/z). Each sample was collected as transient data in triplicate using the selected ion monitoring (SIM) stitch approach (Supplementary Data; ref. 30). To measure phosphatidylcholine and phosphatidylserine phospholipids, a novel DISMs approach, termed tandem mass spectrometry (MSMS)-stitch, was used comprising collision-induced dissociation (CID; 80-eV collision energy) fragmentation of phospholipid ions, resulting in characteristic neutral losses from phosphatidylcholine and phosphatidylserine lipids and thereby aiding their identification (see Supplementary Data and Supplementary Fig. S1). SIM stitch transient data were averaged, Fourier transformed, and internally calibrated (Supplementary Tables S1–S4) in MATLAB (Mathworks; ref. 30). For each sample, peaks were retained if they were present in at least two of the triplicated analyses. When collating samples into a data matrix, peaks were retained if they were present in at least 50% of all samples (31). Data matrices were probabilistic quotient normalized (32). Prior to principal components analysis (PCA), a generalized log transformation (λ = 7.3 × 10−11) was applied (33).

Mass spectral peak annotation

Peaks were putatively annotated using KEGG (http://www.genome.jp/kegg/) and LIPID MAPS (http://www.lipidmaps.org) databases in conjunction with Mi-Pack software (34). Annotation m/z tolerance was ±1 ppm (for peaks < 500 m/z) and ±3 ppm (300–1,000 m/z). For the DISMs approach, fatty acids, phospholipids, and lysophospholipids were identified as [M−H]−, except phosphatidylcholine and lysophosphatidylcholine that ionized as an acetate adduct ([M+OAc]−). Diacylglycerol (DAG) and triacylglycerol (TAG) were identified as [M+NH4]++. Polar metabolites were identified as [M−H]−, [M+OAc]−, or [M+NaOAc−H]−. For the MSMS-stitch DISMs approach, phosphatidylcholine was identified as [M−CH3]− and phosphatidylserine was identified as [M−3C5H5N3O5H−]. The identities of phospholipids, lysophospholipids, and glycerol 3-phosphate (G3P) were confirmed by CID MS3 on the LTQ FT Ultra (35 eV; Supplementary Fig. S2).

13C 0-glucose tracer study data analysis

The 13C isotopes of fatty acids and phospholipids were assigned by looking for multiple additions of 1.003355 abundance to the 12C parent peak. To establish the amount of each compound that came from 13C 0-glucose, the intensity of all the 13C isotope peaks was summed minus the naturally occurring 13C intensity from the 12C peak. Naturally occurring 13C (1.1% abundance) was calculated: (13C intensity × 1 × number of
carbon atoms present in $^{12}C$ identification)/100. This was calculated for $^{13}C_1$, $^{13}C_2$, … $^{13}C_n$ peaks.

cDNA preparation and quantitative real-time PCR

RNA was extracted from 4 × 10^5 cells using the RNeasy Mini Kit (Qiagen) and quantified on a NanoDrop (Thermo Scientific). RNA quality was assessed using A260/280 nm and A260/230 nm ratios measured by the NanoDrop. cDNA was synthesized using SuperScript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). Quantitative real-time PCR (qRT-PCR) was carried out using preoptimized QuantiTect Primer Assays (Qiagen) and a SensiMix SYBR Low-ROX Kit (Bioline) on an Agilent Mx3005P quantitative PCR cycler. 18S rRNA was used as an internal standard for cDNA. The qPCR cycle protocol included an initial heating of 95°C for 10 minutes, then 44 cycles of 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 20 seconds, then a final cycle of 95°C for 1 minutes, 55°C for 30 seconds and 95°C for 30 seconds. Delta cycle threshold ($\Delta Ct$) values were calculated by subtracting 18S Ct from test gene Ct. Relative mRNA levels were determined by subtraction of control $\Delta Ct$, values from treatment $\Delta Ct$, values to give a $\Delta \Delta Ct$ value. This was inverse logged ($2^{-\Delta \Delta Ct}$) to generate relative fold changes.

Western blotting

Cells treated with drugs for 24 hours were lysed in radio-immunoprecipitation assay (RIPA) buffer. Thirty micrograms of protein was combined with Laemmli 4× loading dye (Bio-Rad) and 10% β-mercaptoethanol (Sigma) and heated at 70°C (5 minutes), and then separated on SDS-PAGE precast gradient gels (4%–15%; Bio-Rad). Proteins were transferred to Immobilon-P membrane (Millipore Corp.) and probed with 1/1,000 dilution of anti-SCD1 (ab19862; Abcam), anti-ACC1 (4190; New England Biolabs), anti-phospho-ACC Ser79 (11818; New England Biolabs), and anti-FASN (ab22759; Abcam). Detection was by horse-radish peroxidase (HRP)–conjugated anti-rabbit/mouse diluted at 1/1,000 and ECL using Supersignal West Pico Chemiluminescent substrate (Pierce). Loading controls used anti-β-actin antibody (Sigma) diluted at 1/25,000 and anti-mouse-HRP secondary at 1/2,500. Densitometry was performed using the ImageJ software (http://rsb.info.nih.gov/ij/) and protein expression normalized to β-actin.

Palmitate and oleate supplementation and measurement of cell viability

To generate soluble fatty acid stocks, palmitic acid (7.5 mmol/L) was complexed to bovine serum albumin (BSA; 1.5 mmol/L; Sigma) as described (35) and oleic acid (3 mmol/L) was purchased precomplexed to BSA (1.5 mmol/L; Sigma). Vehicle control stock of 1.5-mmol/L BSA was created. Cells were set in 100-µL cultures in 96-well flat-bottomed plates, with starting cell counts of 5,000 (AML) or 10,000 (Burkit lymphoma) cells per well. Cells were treated with BaP and escalating concentrations of palmitic or oleate. After 48 hours, a further 100 µL of media was added, including reaplication of BaP and the fatty acids. After 96 hours, the 200-µL volume of cells was removed into a flow cytometry tube, centrifuged (250 × g) and supernatant discarded. FACS fix (200 µL; 1% formaldehyde, 2% FBS) and 20-µL (20,000 beads) CytoCount beads (Dako) were added, the sample vortexed (10 seconds), and analyzed by flow cytometry (FACSCalibur flow cytometer; Becton Dickenson). A minimum of 1,000 bead events were collected and ratio of the beads to the number viable cells in the forward/side scatter plots was proportional to the cell density. Cell death (after 96 hours) was measured by FACSCalibur flow cytometry (Becton Dickenson) using the Annexin V/propidium iodide kit (Becton Dickenson) according to the manufacturer’s instructions.

Measurement of ROS

After drug treatment, 200 µL of cell suspension (approximately 1 × 10^5 cells) was incubated at 37°C with 2',7'-dichlorofluorescein diacetate (DCFDA; Sigma) for 1 hour. The oxidized product 2',7'-dichlorofluorescein (DCF) was measured according to the manufacturer’s instructions using a FACSCalibur flow cytometer and CellQuest software (Becton Dickenson). Relative ROS levels were calculated as DCF fluorescence intensity relative to untreated control cells.

Statistical analysis

PCA was conducted using PLS_Toolbox (Eigenvector) in MATLAB. Statistical outliers were defined as samples that fell outside the 95% confidence limit on the PCA Q residuals and/or Hotelling axes. Univariate statistics composed of two-tailed t tests or one-way ANOVA with Dunnett post hoc testing where more than two sample classes were tested (in Minutah 17). Where multiple tests were applied in the metabolomics/lipidomics datasets, a false discovery rate (FDR) of <10% was used to correct for multiple hypothesis testing (36). Intensity changes between treatments are expressed as average fold changes. All bar charts represent the mean ± the standard error of the mean (SEM) unless indicated.

Results

BaP treatment of AML cell lines perturbs the phospholipidome

Negative ion DIMS ([-]DIMS) analysis of lipid extracts from HL60 and K562 AML cells revealed that the majority of the abundant peaks were between 600 and 1000 m/z and attributable to intact phospholipids (Fig. 1A and Supplementary Fig. S2). Lipid extracts from HL60 and K562 cells treated for 24 hours with either BaP (combination of 0.5-mmol/L bezafibrate and 5-µmol/L MPA) or solvent control were analyzed by [-]DIMS. As reported previously, at 24 hours, BaP had little effect on cell viability (9), so any changes observed in the lipidome reflect changes upstream of cell differentiation or death. The PCA scores plot of the [-]DIMS data demonstrated the major segregation to be between HL60 and K562 cells along principal component 1 (Fig. 1B). This is not surprising, given that the cell lines were derived from different individuals with distinct forms of AML (HL60 are de novo AML M2 cells and K562 are AML cells derived from a chronic myeloid leukemia patient undergoing blast crisis; ref. 37, 38). Despite these baseline differences, both cell lines displayed a uniform response to BaP along principal component 2 (Fig. 1B). The PCA scores plot of 24-hour treatment of HL60 cells with 0.5-mmol/L bezafibrate, 5-µmol/L MPA, or the combination demonstrated that bezafibrate contributed most to the BaP effect (Fig. 1C). However, MPA-treated samples were significantly different to control samples along principal component 1 ($P = 0.004$), as were bezafibrate and BaP samples ($P = 0.013$), indicating that MPA is required to generate the lipid perturbation profile observed with BaP treatment (Fig. 1C).
The quantities of the four major diacyl-phospholipid classes (phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, and phosphatidylserine) were relatively unchanged after 24-hour BaP treatment of HL60 or K562 cells (Fig. 1D and Supplementary Table S5). Both cell lines were significantly depleted of lysophosphatidylethanolamine and lysophosphatidylcholine after BaP treatment (Fig. 1D and Supplementary Table S5). Lysophosphatidylethanolamine and lysophosphatidylcholine are major breakdown products of phosphatidylethanolamine and phosphatidylcholine, respectively, and a decrease in their steady-state concentrations might indicate a decrease in the rate of phospholipid turnover.

In addition, BaP treatment induced a consistent perturbation of the phospholipid acyl chain composition in HL60 and K562 cells (Fig. 1E and Supplementary Table S5). This is illustrated in Fig. 1E (and Supplementary Table S5) that shows the relative fold changes of individual phosphatidylcholine species in BaP-treated cells compared with control cells. The relative proportions of phosphatidylcholine species with saturated and monounsaturated chains were decreased and those with polyunsaturated chains were increased (Fig. 1E and Supplementary Table S5). This suggested that in the presence of BaP, HL60 and K562 cells had lower de novo synthesis of saturated and monounsaturated fatty acids.

BaP treatment decreases fatty acid and phospholipid synthesis and increases triacylglycerol levels

Glycerol 3-phosphate (G3P) and diacylglycerol (DAG) are intermediates in the de novo phospholipid biosynthetic pathway: two fatty acyl chains are added to G3P-generating phosphatidate, which can then be dephosphorylated to generate DAG. [-]DIMS analysis of polar extracts of HL60 and K562 cells treated with BaP for 24 hours showed elevated steady-state levels of G3P and positive ion DIMS analysis of lipid extracts identified depleted DAG levels (Fig. 2A and Supplementary Table S6). This suggested phospholipid synthesis may be disrupted at the acyl chain addition stage. In contrast, the concentrations of triacylglycerol (TAG) were substantially increased in BaP-treated cells (Fig. 2A and Supplementary Table S6).

C18:1 was the most abundant of the four observed free fatty acids in HL60 cells (Fig. 2B), and BaP treatment led to an approximately 15% decrease in its steady-state concentration (P = 0.005), whereas C16:0, C16:1, and C18:0 levels were unchanged (Fig. 2B). To determine the effects of BaP treatment on fatty acid synthesis,
and phospholipid biosynthesis from glucose, HL60 cells were grown in the presence of $^{13}$C D-glucose for 24 hours with and without BaP, and $^{13}$C incorporation into lipids was measured by [1-$^{13}$C]DIMS. BaP treatment decreased the incorporation of labeled carbon from $^{13}$C D-glucose into some free fatty acids (C16:1 0.66-fold, $P = 0.008$; C18:0 0.68-fold, $P < 0.001$; C18:1 0.25-fold, $P < 0.001$) and phosphatidylcholine (0.71-fold; $P = 0.001$), phosphatidylethanolamine (0.6-fold; $P < 0.001$), and phosphatidylglycerol (0.59-fold; $P = 0.025$) phospholipids but did not significantly alter $^{13}$C D-glucose incorporation into C16:0 fatty acid ($P = 0.63$) or phosphatidylserine ($P = 0.22$). Bezaflibrate was the major contributor to the effects of BaP; however, given that the changes associated with BaP were more substantial and/or more significant than bezafibrate treatment alone, MPA also appears to play a role (Fig. 2C and D and Supplementary Table S7).

The absolute intensities of each newly synthesized $^{13}$C phosphatidylcholine (PC) species from $^{13}$C D-glucose in HL60 cells are shown in Supplementary Table S8 and Supplementary Fig. S3A. The five most highly produced species [PC(32:1), PC(34:1), PC(36:2), PC(36:1), and PC(32:3)] accounted for >80% of all synthesized phosphatidylcholine contained at least one monounsaturated fatty acyl chain and C18:1 was present in all but one of the species (Supplementary Table S8). The incorporation of $^{13}$C from $^{13}$C D-glucose into these five phosphatidylcholine species was significantly decreased after BaP treatment (0.65- to 0.73-fold decrease; $P < 0.01$; Supplementary Table S8), which is consistent with the observed BaP-induced decrease in de novo C18:1 synthesis, as described above. Phosphatidylcholine species that had not been newly synthesized from $^{13}$C D-glucose ($^{12}$C isotope peaks; Supplementary Fig. S3B) were generally increased after BaP treatment, particularly the long-chain polyunsaturated species, suggesting that cells were obtaining these from sources other than glucose, including exogenous import. Phosphatidylcholine species distribution heatmaps for each of the $^{12}$C and $^{13}$C datasets (Supplementary Fig. S4) showed that BaP decreased the de novo synthesis of phosphatidylcholine species with 0 to 2 double bonds and increased the abundance of polyunsaturated species mainly via a non-glucose, and most likely exogenous source. This is consistent with the earlier findings and gives a rationale for the observed pattern seen in Fig. 1E.

BaP treatment modifies the expression of key enzymes involved in lipogenesis.

The decreases in fatty acid and phospholipid synthesis led us to ask whether 24-hour BaP treatment was altering expression levels of the enzymes responsible for fatty acid synthesis and desaturation. These were investigated in the two AML cell lines (HL60 and K562) plus two Burkitt lymphoma cell lines (BL31 and Glor).
Synthesis of malonyl-CoA by ACC1 is the first committed step in de novo fatty acid biosynthesis. There were decreases in ACC1 mRNA (HL60: 0.73-fold, $P = 0.016$; BL31: 0.78-fold, $P = 0.008$; Glor: 0.8-fold, $P = 0.029$) following 24-hour BaP treatment in HL60, BL31, and Glor cells, but not in K562 cells (Fig. 3A), whereas ACC1 protein levels remained relatively unchanged across the four cell lines (Fig. 3B). ACC1 can be posttranslationally inhibited by phosphorylation at serine 79 (denoted pACC). Immunoassay of pACC showed increased ACC1 phosphorylation in K562 cells (2.8-fold; $P = 0.0001$), but no change in the other three cell lines (Fig. 3B).

FASN catalyzes the synthesis of palmitate (C16:0) from acetyl-CoA and malonyl-CoA. BaP treatment decreased mRNA expression in three of the four cell lines (HL60: 0.41-fold, $P < 0.001$; BL31: 0.62-fold, $P = 0.006$; Glor: 0.67-fold, $P = 0.036$), but not in K562 cells (Fig. 3C), and FASN protein levels were decreased in BaP-treated cells across all cell lines (0.87-fold, $P = 0.009$; Fig. 3D).

The clearest effects of BaP treatment were on SCD1, which mainly converts stearate (C18:0) to oleate (C18:1) and is the rate-limiting step in the synthesis of monounsaturated fatty acids. SCD1 mRNA levels were decreased in HL60, BL31, and Glor cells (HL60: 0.63-fold, $P = 0.005$; BL31: 0.8-fold, $P = 0.007$; Glor: 0.83-fold, $P = 0.042$), though not in K562 cells (Fig. 4A). However, BaP treatment caused a significant decrease in SCD1 protein concentration in all four cell lines (HL60: 0.6-fold,
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P = 0.016; K562: 0.62-fold, P = 0.017; BL31: 0.52-fold, P = 0.023; Glor: 0.46-fold, P < 0.001; Fig. 4B), with both bezafibrate and MPA both contributing to this effect.

BaP-treated cells can be rescued by olate (C18:1) but not palmitate (C16:0)

The BaP-induced depletion of C18:1 free fatty acid levels and decrease in SCD1 concentration led us to consider whether provision of exogenous olate (C18:1) might rescue the leukemia and lymphoma cell lines from killing by BaP during 96-hour culture. Supplementation with olate, which was added as a complex with BSA, increased the viable cell count of BaP-treated cells in a dose-dependent manner in all four cell lines (between 1.4- and 2.1-fold increase with 300-μmol/L olate compared with no olate supplementation, P < 1 × 10⁻⁶; Fig. 5A). Analysis of the effect on bezafibrate and MPA as single treatments showed that 300-μmol/L olate rescued bezafibrate-treated cells almost back to untreated control levels in all four cell lines, whereas there was only a small rescue effect in MPA-treated K562 and BL31 cells (Fig. 5B). In contrast, supplementation of BaP-treated cells with palmitate-BSA complex (C16:0), the product of FASN, did not rescue the cells from the effects of BaP, and concentrations of palmitate above 100-μmol/L reduced viable cell counts in both BaP-treated and untreated cells (Fig. 5C).

Our earlier observations demonstrated that AML cell killing by BaP requires a >96-hour exposure (9), whereas killing of eBL cells is more rapid (7). Consistent with this, olate induced a strong antiapoptotic effect in the Burkitt cell lines, as measured by decreased Annexin V staining, which was not observed in AML cells (Fig. 5D).

A commercial SCD1 inhibitor can partially recapitulate the effect of BaP on lipogenesis.

As SCD1 appeared to be a target of BaP, we treated cells with a commercial SCD1 inhibitor (4-[(2-Chlorophenoxy)-N-[3-((methylamino)carboxyl)-phenyl]-1-piperidinocarboxamide [ab142089]) in the presence of ¹³C-D-glucose to identify if it could generate a similar lipid profile to that seen in BaP-treated cells. With respect to fatty acid synthesis from ¹³C-D-glucose, ab142089 decreased C18:1 (0.70-fold; P = 6 × 10⁻⁴) and C16:1 (0.83-fold; P = 0.003) production, increased C18:0 production (1.2-fold; P = 0.03) and had little effect on C16:0 production (P = 0.13; Supplementary Fig. S5A). The levels of synthesis of C16:0, C16:1, and C18:1 after ab142089 treatment were consistent with those observed after BaP treatment (Fig. 2C); however, the effect on C18:0 synthesis was inconsistent: this was decreased by BaP treatment and increased by ab142089 treatment (Fig. 2C and Supplementary Fig. S5A). When looking at individual classes of fatty acid, ab142089 decreased synthesis of monounsaturated fatty acids from ¹³C-D-glucose (0.73-fold; P = 4 × 10⁻⁴), but did not significantly alter saturated fatty acid synthesis (P = 0.07; Supplementary Fig. S5B). Considering phosphatidylethanolamine synthesis from ¹³C-D-glucose, ab142089 decreased production of PC (36:2) (0.77-fold; P = 0.005) and increased production of PC (36:1) (1.37-fold; P = 0.008; Supplementary Fig. S6A), whereas BaP decreased the synthesis of many phosphatidylcholine species, including PC (36:1) and PC (36:2) (Supplementary Fig. S3A). These findings suggest that BaP decreases SCD1 activity but also negatively regulates other lipogenic processes, including FASN (see above).

Olate decreases the levels of BaP-induced ROS

BaP induces ROS in all cell types that are sensitive to this drug combination (6–10). We therefore tested whether the rescue of cells mediated by olate was associated with diminished ROS production. As shown in Fig. 6, olate (300 μmol/L) decreased ROS levels in both AML and eBL control cells (between 0.51- and 0.83-fold) and in BaP-treated cells (between 0.57- and 0.82-fold; Fig. 6). In BaP-treated K562, BL31, and Glor cells, olate treatment brought ROS down to levels similar to those found in the untreated control cells. Given that ROS production is indicated as a major reason for the killing action of BaP, the ROS decrease is noteworthy and suggests that olate is cytoprotective to BaP-treated cells by lowering ROS to tolerable levels. The rescue of AML and Burkitt lymphoma cells by olate was associated with the accumulation of cytoplasmic lipid droplets in both BaP-treated and untreated cells (Supplementary Fig. S7). These are likely to represent the safe storage of fatty acids as TAG (25, 26).

Discussion

There is a need for new low-toxicity therapies that selectively target cancer cells. Enhanced lipogenesis, arising from increased activities of fatty acid biosynthetic enzymes (including ACC1, FASN, and stearoyl CoA desaturase (SCD1)—see the pathways map in Fig. 7), is a metabolic hallmark of many cancer cells (15–17). Inhibition of these enzymes has been demonstrated to be selectively toxic to some cancer cells in vitro (18, 20–24), suggesting that this pathway might represent a good target for antitumor drugs. In particular, cancer cells appear to have a high dependence on monounsaturated fatty acids (39), which are key components of phospholipids. Monounsaturated fatty acids can also protect cells against saturated fatty acid toxicity by enhancing safe storage of saturates into TAGs (25, 26). In cancer cells, SCD1 inhibition leads to endoplasmic reticulum stress and apoptosis (40), symptoms that are similar to palmitate-induced lipotoxicity (41), and in cardiac myocytes, SCD1 activity protects cells against fatty acid-induced apoptosis (42). Given their rapid saturated fatty acid synthesis by ACC1 and FASN (15), cancer cells may be heavily reliant on SCD1 activity, making SCD1 a potential target for anticancer therapy. However, there are currently no routine therapeutics targeting SCD1 in the clinic.

Here, we describe an investigation, at the lipid, gene, and protein levels, of the effects of the antileukemia and antilymphoma therapy BaP (combined 0.5-mmol/L bezafibrate and 5-μmol/L MPA) on lipogenesis in AML and eBL cells. Our clearest observations on the effects of BaP treatment on lipogenesis in these two disease settings are the following: (i) phospholipid species with saturated and monounsaturated acyl chains were decreased, whereas phospholipids with polyunsaturated chains were increased, (ii) BaP treatment decreased the incorporation of ¹³C from ¹³C-D-glucose into a variety of cellular lipids, especially into monounsaturated free fatty acids and phospholipids with monounsaturated acyl chains, (iii) BaP caused a decrease in the concentration, and probably the activity, of SCD1, and (iv) supplementation of BaP-treated cells with olate (the product of SCD1 activity), but not with palmitate, protected AML and eBL cells from killing by BaP. Both bezafibrate and MPA appear to have antilipogenic action individually but the effect against AML and eBL cell lines was enhanced when the two drugs were combined.

At first sight, two of these observations appear contradictory: the rate of introduction of new double bonds into fatty acids
was decreased, but phospholipid acyl chains became more unsaturated. However, de novo fatty acid biosynthesis in human cells is only capable of producing saturated or omega-7/omega-9 monounsaturated fatty acids (via SCD1). Fatty acids with other double bond structures are classed as essential and have to be imported from exogenous sources (43). We demonstrate that phosphatidylcholine species with saturated and monounsaturated acyl chains represent the overwhelming majority of

Figure 5.
Oleate rescues AML and Burkitt lymphoma cells from killing by BaP drug treatment. The effect of fatty acid supplementation on survival of AML (K562 and HL60) and Burkitt lymphoma (BL31 and Glor) cell lines treated with BaP (0.5 mmol/L bezafibrate (BEZ) and 5 μmol/L MPA), bezafibrate (0.5 mmol/L), and MPA (5 μmol/L) for 96 hours. Viable cell counts (measured by flow cytometry) of drug-treated cells in the presence of escalating amounts of oleate (A) or 300 μmol/L oleate (B). C, viable cell counts of drug-treated cells in the presence of escalating amounts of palmitate. D, apoptosis was measured by Annexin V staining and flow cytometry after cells were treated with drug in the presence of 300 μmol/L oleate. Data, n ≥ 4 ± SEM.
**de novo** synthesized PCs in cells used in this study, and the synthesis of these species from $^{13}$C D-glucose was strongly decreased by BaP. Conversely, phosphatidylcholine species that were not synthesized **de novo** from $^{13}$C D-glucose increased after BaP treatment, especially those with polyunsaturated acyl chains, which suggested that the cells were taking up exogenous lipids to compensate for the reduction in **de novo** synthesis. This phenomenon has been observed in prostate cancer cells after ACC1 inhibition that led to a doubling of the quantity of essential polyunsaturated fatty acids being incorporated into phospholipids (44). The increase in unsaturation of phospholipids in ACC1-inhibited prostate cancer cells caused them to become more sensitive to ROS-induced lipid peroxidation and apoptosis and this could be a useful therapeutic consequence of the treatment (44). Therefore, our observation of the change to phospholipid saturation status could be particularly relevant in the case of BaP, which is a strong inducer of intracellular ROS and also lipid peroxidation (9).

The changes to phospholipid synthesis and acyl chain composition can be explained by lowered fatty acid biosynthesis due to a BaP-induced decrease in FASN and SCD1 protein levels. SCD1 disruption appeared to be the more dominant factor as its protein levels were more strongly decreased in all four cell lines compared with FASN. Also the synthesis of the SCD1 products (C18:1 and C16:1 fatty acids) from $^{13}$C D-glucose was strongly decreased by BaP, whereas the synthesis of the FASN product (16:0 fatty acid) was unchanged, which was consistent with the actions of a commercial SCD1 inhibitor (ab142089). BaP had a broader negative effect on lipid synthesis than ab142089, including more widespread decrease in the synthesis of phosphatidylcholine species, which can be explained to some extent by the BaP-induced decreases of FASN protein levels and FASN and ACC1 gene expression. However, the critical anticancer component of BaP appeared to be the decrease in SCD1 protein levels because oleate (C18:1, SCD1 product), but not palmitate (C16:0, ACC1 and FASN product), supplementation could rescue both AML and eBL cells from BaP treatment.

Monounsaturated fatty acids have been shown to be important in cancer (39, 45), which, in part, is due to their requirement for cellular growth and proliferation. SCD1 is suggested
be a major driver of fatty acid synthesis from glucose (46) and has also been shown to help negotiate cellular stress, with SCD1 inhibition leading to saturated fatty acid accumulation, toxicity, and endoplasmic reticulum stress (40, 47, 48). This is likely to be of particular importance in the cancer phenotype where levels of saturated fatty acid synthesis are often high (15, 16). BaP treatment led to increases in ROS and TAG levels, which are consistent with elevated saturated fatty acid levels as ROS is a symptom of endoplasmic reticulum stress and saturated fatty acid lipotoxicity (49, 50), whereas TAG represent a safe storage of excess saturated fatty acids in the cell (25, 26).

In support of the BaP-induced ROS being related to saturated fatty acid build-up, supplementation of BaP-treated cells with oleate, which can rescue cells from palmitate toxicity (25), decreased ROS levels. This was accompanied with an increase in cytoplasmic lipid droplets, a similar phenomenon to that seen during oleate rescue of cells from saturated fatty acid–induced lipotoxicity (25).

To conclude, we show that the redeployed drug combination of bezafibrate and MPA kills AML and eBL cells, in part, by decreasing SCD1 protein levels and slowing monounsaturated fatty acid synthesis. This study is relevant to the wider context of cancer therapeutics where significant effort is being invested into developing therapeutics that can disrupt fatty acid biosynthesis by interfering with SCD1 due to its apparent anticancer activity (21–23). To our knowledge, this is the first study to describe clinically available drugs with demonstrable antileukemic and antilymphoma activity in clinical trials (11, 12) that target this pathway.

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**Disclosure of Potential Conflicts of Interest**

C.M. Bunce is a Research Director at Leukaemia & Lymphoma Research. No potential conflicts of interest were disclosed by the other authors.

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