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# Functional CRISPR and shRNA screens identify involvement of mitochondrial electron transport in the activation of evofosfamide

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**Running Title:** Mitochondrial involvement in evofosfamide activation

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**Abbreviations:** Br-IPM, bromo-*iso*-phosphoramidate mustard; Cl-IPM, chloro-*iso*-phosphoramidate mustard; *cyt a*, cytochrome *a*; *cyt b*, cytochrome *b*; *cyt c*, cytochrome *c*; DMF, dose-modifying factor; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GO, gene ontology; PC1, first principal component; POR, P450 oxidoreductase; RNAseq, RNA sequencing; SF, surviving fraction; sgRNA, single guide RNA.

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## Abstract

Evofosfamide (TH-302) is a hypoxia-activated DNA-crosslinking prodrug currently in clinical development for cancer therapy. Oxygen-sensitive activation of evofosfamide depends on one-electron reduction, yet the reductases that catalyse this process in tumours are unknown. We used RNA sequencing, whole-genome CRISPR knockout and reductase-focused shRNA screens to interrogate modifiers of evofosfamide activation in cancer cell lines. Involvement of mitochondrial electron transport in the activation of evofosfamide and the related nitroaromatic compounds EF5 and FSL-61 was investigated using 143B  $\rho^0$  (rho zero) cells devoid of mitochondrial DNA and biochemical assays in UT-SCC-74B cells. The potency of evofosfamide in 30 genetically diverse cancer cell lines correlated with the expression of genes involved in mitochondrial electron transfer. A whole-genome CRISPR screen in KBM-7 cells identified the DNA damage response factors *SLX4IP*, *C10orf90* (*FATS*) and *SLFN11*, in addition to the key regulator of mitochondrial function *YME1L1* and several complex I constituents as modifiers of evofosfamide sensitivity. A reductase-focused shRNA screen in UT-SCC-74B cells similarly identified mitochondrial respiratory chain factors. Surprisingly, 143B  $\rho^0$  cells showed enhanced evofosfamide activation and sensitivity but had global transcriptional changes including increased expression of non-mitochondrial flavoreductases. In UT-SCC-74B cells, evofosfamide oxidised cytochromes *a*, *b* and *c* and inhibited respiration at complexes I, II and IV without quenching ROS production. Our results suggest that the mitochondrial electron transport chain contributes to evofosfamide activation and that predicting evofosfamide sensitivity in patients by measuring the expression of canonical bioreductive enzymes such as P450 oxidoreductase (POR) is likely to be futile.

## Introduction

Hypoxia has been pursued as an oncology target due to its severity in tumours and its roles in cancer progression and therapy resistance (Wilson and Hay, 2011). The latter reflects the central role of O<sub>2</sub> in the conversion of radiation-induced DNA radicals to strand breaks, in addition to extensive evidence that hypoxic regions are refractory to systemic cytotoxic and immune therapies (Trédan *et al.*, 2007; Chouaib *et al.*, 2017). One strategy for targeting hypoxia involves the use of prodrugs that undergo reductive activation that is suppressed by molecular oxygen. The leading example of this class, evofosfamide (TH-302), is a *bis*-alkylating bromo-*iso*-phosphoramidate mustard (Br-IPM) deactivated by a bioreductive 2-nitroimidazole trigger (Duan *et al.*, 2008). One-electron reduction of evofosfamide produces a radical anion that rapidly fragments to release Br-IPM (Meng *et al.*, 2012), which in turn undergoes halide exchange to produce a second DNA crosslinking agent, Cl-IPM (Hong *et al.*, 2018). In the presence of O<sub>2</sub>, the prodrug radical is oxidised, thus conferring hypoxia selectivity (Fig. 1A).

Evofosfamide has been extensively tested in preclinical models, where it shows hypoxia-dependent monotherapy activity (Sun *et al.*, 2012) and augments the efficacy of approved therapies including radiation (Peeters *et al.*, 2015; Lohse *et al.*, 2016; Jamieson *et al.*, 2018; Takakusagi *et al.*, 2018), immune checkpoint blockade (Jamieson *et al.*, 2018; Jayaprakash *et al.*, 2018), bortezomib (Hu *et al.*, 2013), mTOR inhibitors (Sun *et al.*, 2015), transarterial chemoembolisation (Duran *et al.*, 2017) and various cytotoxic agents (Liu *et al.*, 2012; Zhang *et al.*, 2016; Haynes *et al.*, 2018). Clinically, evofosfamide is well-tolerated (Weiss *et al.*, 2011) and was active in combination with gemcitabine for advanced pancreatic cancer (Borad *et al.*, 2015) and doxorubicin for soft-tissue sarcoma (Chawla *et al.*, 2014) in phase II randomised and single-arm studies, respectively. Disappointingly, definitive trials in both indications were

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negative for overall survival benefit in patients not selected for tumour hypoxia (Cutsem *et al.*, 2016; Tap *et al.*, 2017). While the negative SARCO21 result may in part reflect chemical antagonism between evofosfamide and doxorubicin (Anderson *et al.*, 2017), the narrow shortfall in primary endpoint in the MAESTRO pancreatic cancer study has been attributed to diminished prodrug exposures caused by a formulation change (Higgins *et al.*, 2018) and lack of predictive biomarkers (Domenyuk *et al.*, 2018). Reflecting the key role of hypoxia in immune suppression, evofosfamide is currently being evaluated in combination with ipilimumab for solid tumours in a phase IB study (NCT03098160).

Since reductive activation of evofosfamide is required for anti-tumour activity, profiling the enzymes that catalyse this reaction is potentially an important aspect of predictive biomarker strategies. As for other prodrugs that depend on initial one-electron reduction, the activation of evofosfamide is inhibited by diphenyleneiodonium, indicating catalysis by FMN- and FAD-dependent flavoproteins that mediate one-electron transfer from NAD(P)H to substrates (Meng *et al.*, 2012). While P450 oxidoreductase (POR) (Hunter *et al.*, 2012; Meng *et al.*, 2012) and FAD-dependent oxidoreductase domain containing 2 (FOXRED2) (Hunter, Jaiswal, *et al.*, 2014) have been shown to activate evofosfamide when expressed at supra-physiological levels, knockdown or knockout of *POR* in cell lines causes little or no decrease in evofosfamide cytotoxicity under hypoxia (Su, Gu, *et al.*, 2013; Hunter *et al.*, 2015; Hong *et al.*, 2018). Moreover, the promiscuity and redundancy of flavoreductases as xenobiotic metabolising enzymes has obstructed identification of the proteins that activate evofosfamide in tumours. Here, we use integrated functional CRISPR and shRNA screens and gene expression analysis to interrogate genetic modifiers of evofosfamide activity. We present evidence for a role of mitochondrial electron transport in the reductive activation of evofosfamide, with implications for the utility of reductase profiling in the use of this agent for precision cancer medicine.

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## Materials and Methods

**Compounds.** Evofosfamide was gifted by Threshold Pharmaceuticals (South San Francisco, CA). PR-104A, SN30000 and deuterated standards for evofosfamide metabolism assays were synthesised at the Auckland Cancer Society Research Centre (Auckland, New Zealand). The purity of compounds (>95%) was assessed by HPLC and DMSO stock solutions stored at  $-80^{\circ}\text{C}$ .

**Cell lines and Culture.** 786-O, A375, A549, ACHN, BxPC-3, Caki-1, Calu-6, DU 145, H460, H82, HCT 116, Hs766T, HT-1080, HT-29, IGROV-1, KHOS/NP, LNCaP, Malme-3M, MDA-MB-231, MIA PaCa-2, PANC-1, PC-3, PLC/PRF/5, RPMI 8226, SiHa, SK-BR-3, SK-MEL-2, SK-MEL-28, SK-MEL-5, SU.86.86, T-47D and U-87 MG cell lines were sourced as reported (Meng *et al.*, 2012) and cultured as recommended by the American Type Culture Collection (Manassas, VA). KBM-7 cells were procured from Haplogen (Vienna, Austria) and cultured in IMDM + 5% FCS. UT-SCC-74B cells were gifted by Prof. Reidar Grénman (University of Turku, Finland) and cultured in MEM + 10% FCS,  $4.5\text{ mg}\cdot\text{mL}^{-1}$  D-glucose,  $1.9\text{ mg}\cdot\text{mL}^{-1}$  sodium bicarbonate,  $1\text{ mM}$  sodium pyruvate and  $20\text{ mM}$  HEPES. 143B  $\rho^0$  and parental 143B cells were gifted by Prof. Mike Berridge (Malaghan Institute, Wellington, New Zealand) and cultured in MEM + 10% FCS,  $3.5\text{ mg}\cdot\text{mL}^{-1}$  D-glucose,  $20\text{ mM}$  HEPES,  $1\text{ mM}$  sodium pyruvate, and  $50\text{ }\mu\text{g}\cdot\text{mL}^{-1}$  uridine. Rho zero status was confirmed by PCR using the *MT-TL1* primers GAT-GGC-AGA-GCC-CGG-TAA-TCG-C and TAA-GCA-TTA-GGA-ATG-CCA-TTG-CG and the *GAPDH* primers ACG-GGA-AGC-TTG-TCA-TCA-AT and TGG-ACT-CCA-CGA-CGT-ACT-CA. All cell lines were propagated from cryopreserved vials authenticated by short-tandem repeat analysis and confirmed to be mycoplasma-free by Plasmotest (InvivoGen, San Diego, CA).

**Antiproliferative (IC<sub>50</sub>) Assays.** The sensitivity of cell lines to drugs was assessed by antiproliferative (IC<sub>50</sub>) assay. For the cancer cell line panel (Fig. 1), 2,000 cells were seeded in 0.5 mL per well in 24-well plates and allowed to attach over 24 h. The plates were then placed under anoxia (<10 ppm gas-phase O<sub>2</sub>) inside an H<sub>2</sub>-scrubbed glove port chamber (Hypoxystation) and the medium exchanged with pre-equilibrated anoxic medium containing a dilution series of evofosfamide. Cells were exposed to evofosfamide over 2 h, then drug washed out by two medium changes. Parallel plates were challenged with evofosfamide under 20% O<sub>2</sub>. The cells were cultured for 7 d prior to assessing viability using alamarBlue (Thermo Fisher Scientific; Waltham, MA). For the 143B and p<sup>0</sup> lines, 300 or 800 cells, respectively, were seeded in 0.1 mL per well in 96-well plates under anoxia (Coy anaerobic chamber) and allowed to attach over 2 h. Drugs were then added to the plates in dilution series and exposed over 4 h. Parallel plates were challenged under 20% O<sub>2</sub>. Drug washout was effected by three medium changes and the plates were then incubated for 5 d prior to assessing culture density by sulphorhodamine B staining. In both cases, four-parameter variable slope functions were fitted to the concentration-response data using least squares and solved to define the drug concentrations for 50% inhibition of cell growth relative to vehicle-treated control wells on the same plate. The parameters in these functions (Hill slope, EC<sub>50</sub>, minimum and maximum response) were unconstrained. IC<sub>50</sub> data are presented as the mean ± SEM for ≥3 independent experiments per cell line. Hypoxic selectivity was quantified as the ratio of mean IC<sub>50</sub> values under normoxia and anoxia.

**Evofosfamide Metabolism Assays.** Reductive activation of evofosfamide in cell lines was assessed by liquid chromatography-tandem mass spectrometry as the concentrations of Br-IPM, Cl-IPM and Tr-H metabolites produced in anoxic cell cultures exposed to 30 μM

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evofosfamide for 1 h. Metabolite concentrations in the intra- and extracellular fractions were measured separately then summed for statistical analysis. The cell culture and bioanalytical methods have been reported in detail (Hong *et al.*, 2018).

**RNA Sequencing.** RNA sequencing (RNAseq) data for the cancer cell line panel were retrieved from the Cancer Cell Line Encyclopedia. For 143B and  $\rho^0$  cells, RNA was extracted from cultures in logarithmic growth ( $n=3$  independent cultures per line) and stranded mRNA libraries generated using a NEXTflex Rapid Directional kit (Perkin Elmer; Waltham, MA) with v4 chemistry. Final libraries were quantified using a Qubit high-sensitivity DNA Assay Kit (Thermo Fisher Scientific; Waltham, MA) and quality was assessed with a TapeStation 4200 (Agilent; Santa Clara, CA). Libraries were normalised, pooled equimolarly and sequenced on a NextSeq500 using a 75 bp single-end flow cell (Illumina; San Diego, CA). Reads were aligned to hg19 with STAR and mRNA abundance estimated using RSEM. For both datasets, expected counts were  $\log_2$  transformed and quantile normalised before analysis. Differential expression analysis, correlation with  $IC_{50}$  data and hierarchical clustering were performed in R and used the *limma*, Pearson and ward.D methods with Euclidean distance. Statistical enrichment of gene ontology (GO) and pathway classifications among gene lists was assessed using GeneSetDB ([genesetdb.auckland.ac.nz](http://genesetdb.auckland.ac.nz)).

**Whole-Genome CRISPR Knockout Screen.** KBM-7 cells were stably transduced with *Streptococcus pyogenes* Cas9 (lentiCas9-Blast vector) at an MOI of 0.02 and expression confirmed by immunoblotting (mouse anti-Cas9 monoclonal antibody clone 7A9 diluted 1:1000; Diagenode, Belgium). The resulting pool was transduced with the GeCKOv2 single guide RNA (sgRNA) library (lentiGuide-Puro vector) at an MOI of 0.25 and selected in puromycin for 7 d. Triplicate cultures of  $10^8$  cells (i.e. 810 cells per sgRNA in the GeCKOv2



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library) were exposed to 0.013  $\mu\text{M}$  evofosfamide as stirred suspensions ( $10^6$  cells.mL<sup>-1</sup>) for 1 h under anoxia (Hypoxystation; Don Whitley Scientific), with a prior 30-min drug-free incubation to deplete O<sub>2</sub>. Evofosfamide was removed by centrifugation and the cultures maintained under 20% O<sub>2</sub> with daily assessment of regrowth (Coulter particle counter). Two cycles of drug challenge were imposed. Triplicate vehicle-treated cultures were exposed to anoxia without evofosfamide and maintained in parallel. Genomic DNA was isolated from cells at screen endpoint (day 26) using QIAamp DNA Blood Maxi kits (Qiagen; Hilden, Germany) and sgRNA sequences PCR-amplified as described (Sanjana *et al.*, 2014). Sequencing was performed on a NextSeq500 (Illumina; San Diego, CA) using a high-output, 150 bp paired-end flow cell. The screens were deconvolved and statistical significance of sgRNA enrichment or depletion in evofosfamide-treated cultures relative to controls was assessed using the MAGeCK (sourceforge.net/p/mageck) and PinAPL-Py (pinapl-py.ucsd.edu) algorithms. Genes that were enriched or depleted at a statistical significance threshold of  $P < 0.005$  according to one or both algorithms were considered of interest.

**Reductase-Focused shRNA Screen.** UT-SCC-74B cells were transduced at an MOI of 0.38 with a previously-reported (Hunter *et al.*, 2015) custom pool of 1,821 shRNA constructs (pLKO.1 vector) targeting 359 genes enriched for oxidoreductases and covering the annotated human flavoproteome. Triplicate transduced cultures, each of  $150 \times 10^6$  cells, were treated with 0.75  $\mu\text{M}$  evofosfamide as stirred single-cell suspensions ( $10^6$  cells.mL<sup>-1</sup>) for 1 h under anoxia and subsequently regrown under 20% O<sub>2</sub>. Triplicate, vehicle-treated control cultures were maintained in parallel. Cells were seeded for assessment of plating efficiency immediately prior to and following evofosfamide challenge and at screen endpoint (i.e. complete recovery evident 18 d after treatment by phase-contrast microscopy). Genomic DNA was extracted from cells and shRNA barcodes PCR-amplified, sequenced and scored as reported (Hunter *et al.*, 2015).

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Statistical significance of shRNA enrichment in evofosfamide-treated cultures relative to controls was assessed using the MAGeCK and RIGER methods.

**FSL-61 Metabolism Assays.** Reductive activation of the fluorogenic probe FSL-61 was measured as described (Su, Guise, *et al.*, 2013). Briefly, anoxic cell suspensions ( $2 \times 10^6 \text{ mL}^{-1}$ ) in Phenol Red-free medium were labelled with 300 or 600  $\mu\text{M}$  FSL-61 for 3 h then analysed using a BD Accuri C6 flow cytometer with excitation and emission wavelengths of 355 and 425-475 nm, respectively. The distributions of fluorescence area events for  $\rho^0$  and 143B cells were compared to cells not labelled with FSL-61 but otherwise handled identically.

**EF5 Metabolism Assays.** EF5 binding was assayed as described (Wang *et al.*, 2012) with modifications. Briefly,  $10^6$  cells were pre-incubated under anoxic conditions in 10 mL Phenol Red-free MEM $\alpha$  with 5% FCS for 30 min with continuous stirring before being exposed to 120  $\mu\text{M}$  EF5 for 2 h. Cells were then centrifuged ( $1000 \times g$ , 5 min) and fixed in cold 4% paraformaldehyde for 1 h. The fixed cells were incubated in blocking buffer (PBST with 20% low-fat milk, 1.5% lipid-free albumin and 5% mouse serum) at 4°C for 30 min then stained overnight at 4°C with 100  $\mu\text{g mL}^{-1}$  Alexa488-conjugated anti-EF5 antibody (supplied by Prof. Cameron Koch, University of Pennsylvania, PA). Samples were washed twice with PBST and once with PBS then analysed using BD Accuri C6 or BD LSR II flow cytometers.

**Cellular Physiology Assays.** Mitochondrial respiration and ROS production were assessed using OROBOROS™ oxygraphs (O2k; Innsbruck, Austria) and analysed in real time with DatLab 7.1 software after instrument calibration and back-flux correction. Substrate–inhibitor–uncoupler protocols were employed to deconvolute the characteristics of the mitochondrial electron transport system components. Cells ( $5 \times 10^6$  in 2 mL) were

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introduced in the O2k chamber containing fully-aerated PBS at 37°C. After signal stabilisation, permeabilisation was effected by titrating digitonin to 10 µg/10<sup>6</sup> cells. Respiratory substrates were then added at saturation (5 mM pyruvate, 2.5 mM malate, 10 mM glutamate, 10 mM succinate and 2.5 mM ADP) to achieve OXPHOS state (maximum respiration attributed to oxidative phosphorylation). Evofosfamide (0-200 µM) or vehicle (DMSO equivalent volume) were titrated on permeabilised cells at OXPHOS state. The maximum contribution of succinate dehydrogenase (complex II) to OXPHOS was determined with the addition of the complex I inhibitor rotenone (0.5 µM). Respiration not efficiently directed to OXPHOS but dissipated to proton leak (LEAK) was determined with the addition of the ATP FOF1 synthase inhibitor oligomycin (2 µg.mL<sup>-1</sup>) on cells at OXPHOS state. The maximum activity of cytochrome *c* oxidase (complex IV) was measured with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD, 0.5 mM) and ascorbate (2 mM) to protect against TMPD auto-oxidation. Potassium cyanide (1 mM) was added at the end of respirometry assays to (determine TMPD auto-oxidation and induce the maximal reductive state of mitochondrial cytochromes.

ROS production was assessed concurrently with respiration by Amplex UltraRed assay as previously described (Pham *et al.*, 2014). Cytochrome spectra were also obtained simultaneously using purpose-build light-emitting diodes (370-750 nm) placed adjacent to the O2k chamber. Reflected light was collected via a 1 mm optic fibre connected to a USB4000 spectrophotometer (Ocean Optics, Inc.). Absorbance was measured with SpectraSuite 2.0.162. The change in absorption mediated by evofosfamide was obtained by reference to the absorption spectra prior to drug titration at OXPHOS state. The relative contribution of each cytochrome (*a*, *b* and *c*) to the absorbance was determined using their respective extinction coefficients. Normalisation of spectra was made using KCN to maximally reduce mitochondrial cytochromes.

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**Statistics.** Statistical tests were performed in R, Python or GraphPad Prism v7 and were two-tailed where applicable. The specific tests used, the number of experimental replicates and representations of dispersion and central tendency are described in figure legends. Protein-protein interaction networks were defined using the STRING database ([string-db.org](http://string-db.org)), while overrepresentation of GO terms in gene lists was assessed using PANTHER ([pantherdb.org](http://pantherdb.org)), DAVID ([david.ncifcrf.gov](http://david.ncifcrf.gov)) and GeneSetDB ([genesetdb.auckland.ac.nz](http://genesetdb.auckland.ac.nz)).  $P < 0.05$  is denoted as ‘\*’,  $P < 0.01$  as ‘\*\*’ and  $P < 0.001$  as ‘\*\*\*’.

## Results

**Evofosfamide Sensitivity Correlates with Expression of Mitochondrial Genes in Cancer Cell Lines.** To investigate sources of variation in the sensitivity of cancer cells to evofosfamide,  $IC_{50}$  was measured under anoxia (“N<sub>2</sub>”) and normoxia (“air”) in a panel of 32 histologically-diverse cell lines (Fig. 1B). Drug sensitivity data were then related to gene expression profiles from RNAseq. This  $IC_{50}$  study recapitulated a prior dataset in the same panel (Meng *et al.*, 2012), except that a longer regrowth endpoint was used (7-d vs 3-d) to more closely reflect clonogenic survival than acute antiproliferative effects. Evofosfamide showed low-micromolar potency under anoxia with a 67-fold range in  $IC_{50}$  values – less than the 900-fold range observed using the 3-d endpoint (Meng *et al.*, 2012). The two datasets were generally well-correlated with the exception of Hs766T and IGROV-1, which were markedly more sensitive to evofosfamide than in the 3-d assay (Fig. 1C). The prodrug was strongly inactivated by ambient O<sub>2</sub>, conferring a median Air–N<sub>2</sub>  $IC_{50}$  ratio of 48 (range 16-260; Fig. 1B), while the patterns of sensitivity were highly correlated under both conditions (Supplemental Fig. 1A). To explore biological determinants of evofosfamide sensitivity, RNAseq data available from the Cancer Cell Line Encyclopedia for 30 of the cell lines (Fig. 2A) were correlated with  $IC_{50}$  measures. The 173 genes with expression values that inversely correlated with evofosfamide  $IC_{50}$  under anoxia ( $R \leq -0.4$ ) were overrepresented for GO annotations relating to mitochondrial localisation and function (Table 1, Supplemental Fig. 2). To assess the extent to which a signature derived from these genes might associate with evofosfamide sensitivity, we computed the first principal component (PC1) of expression values for these genes and identified a quantitative association between the rank order of cell lines by PC1 and evofosfamide sensitivity (Fig. 2B). Moreover, dichotomising the cell line panel by the PC1 median defined groups with significantly different drug sensitivity distributions (Supplemental Fig. 1B).

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Network analysis of the correlating genes revealed an interacting cluster with annotations relating mitochondrial inner membrane localisation, mitochondrial protein biosynthesis, electron transport and metabolism (Fig. 2C).

**Whole-genome CRISPR Knockout and Reductase-Focused shRNA Screens Identify Mitochondrial Involvement in Evofosfamide Activity.** As an orthogonal line of investigation, a genome-scale CRISPR knockout screen was performed in near-haploid KBM-7 cells transduced with the GeCKOv2 sgRNA library (Fig. 3A). Two treatments with 0.013  $\mu$ M evofosfamide under anoxia, which was the measured  $IC_{40}$  in KBM-7 (Supplemental Fig. 3), on days 0 and 7 inhibited total population growth by a factor of  $10^4$  relative to vehicle-treated cultures at screen termination (Fig. 3B). Evofosfamide-challenged cells acquired bulk resistance to the agent relative to drug-naïve cultures, with a dose-modifying factor (DMF) of 1.6 (Fig. 3C). The screen was deconvolved using the MAGeCK ([sourceforge.net/p/mageck/](http://sourceforge.net/p/mageck/)) and PinAPL-Py (<http://pinapl-py.ucsd.edu/>) algorithms to identify sgRNA enriched or depleted by evofosfamide treatment and to aggregate the multiple guides specific for each gene to output a gene level score. As Among the most significant hits in the screen were the mitochondrial factor *YME1L1* and the DNA damage response and repair factors *C10orf90* (*FATS*), *SLX4IP* (*C20orf94*) and *SLFN11* (Fig. 3D-E). Considering all sgRNA targets positively selected by evofosfamide (i.e. sgRNA representation increased following drug challenge;  $P < 0.005$  by MAGeCK, PinAPL-Py or both, Fig. 3E) defined a gene list overrepresented in GO terms relating to mitochondrial respiration and electron transfer from NADH to ubiquinone (Table 2). Among these positively selected sgRNA targets was an interacting protein network encompassing subunits and regulators of mitochondrial respiratory complex I (Fig. 3F).

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A parallel screen was performed in the lingual squamous cell carcinoma line UT-SCC-74B using a previously-reported custom pool of 1,821 shRNA targeting 359 genes, including the majority of annotated human flavoproteins (Hunter *et al.*, 2015). This screen was analogous to the KBM-7 study (Fig. 3A), except that a single instance of acute evofosfamide treatment was imposed, resulting in three logs of clonogenic cell killing (Fig. 4A). Deconvoluting the screen identified factors putatively involved in sensitivity to evofosfamide, including constituents of mitochondrial complexes I and III (Fig. 4B). The resulting list of candidate genes was overrepresented for GO annotations relating to mitochondrial localisation and respiratory electron transport, even after correcting for the reductase-enriched target-space of the shRNA pool (Table 3). The full list of positively-selected shRNA targets ( $P < 0.05$  by MAGeCK, RIGER or PinAFL-Py) encompassed a cluster of interacting proteins functioning in mitochondrial electron transport (Fig. 4C).

**Rho Zero Cells Demonstrate Enhanced Reductive Metabolism of Bioreductive Prodrugs and Global Transcriptional Changes.** Mitochondrial involvement in the reductive activation of evofosfamide was investigated in rho zero ( $\rho^0$ ) cells derived from the 143B osteosarcoma line by protracted treatment with ethidium bromide to deplete mitochondrial DNA (King and Attardi, 1989). Rho zero status was confirmed by PCR for mitochondrial DNA-encoded *MT-TL1* (Fig. 5A). Functionally,  $\rho^0$  cells showed profound loss of mitochondrial  $O_2$  flux in the OXPHOS, CII, LEAK and CIV states (Fig. 5B). Despite lacking a functional respiratory transport chain,  $\rho^0$  cells demonstrated elevated generation of the evofosfamide reduction metabolites Br-IPM, Cl-IPM and 2-nitroimidazole fragmentation product (1,5-dimethyl-2-nitroimidazole, 'Tr-H', Fig. 5C). Rho zero cells also showed elevated reductive activation of the fluorogenic 6-nitroquinolone FSL-61 (Su, Guise, *et al.*, 2013) and the bioreductive probe EF5, which shares a 2-nitroimidazole moiety with evofosfamide (Fig. 5D).

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Rho zero cells were correspondingly more sensitive to evofosfamide and the additional hypoxia-activated prodrugs PR-104A and SN30000 specifically under anoxia (Fig. 5E), with a commensurate increase in selectivity (Supplemental Fig. 4). Given these surprising observations, gene expression features of the  $\rho^0$  line were investigated by RNAseq. Relative to parental 143B cells, the  $\rho^0$  line (all analysed in triplicate) showed widespread transcriptional changes encompassing a broad array of molecular pathways, with 6,741 transcripts differentially expressed beyond a Benjamini–Hochberg adjusted  $P$ -value of 0.05 (Fig. 6A). Notably,  $\rho^0$  cells showed higher expression of genes with GO annotations relating to DNA repair (adjusted  $p < 10^{-7}$ ; Fig. 6B) but lower expression of genes with GO annotations relating to DNA damage response ( $P$ -value after adjusting for multiple comparisons  $< 0.01$ ; Fig. 6C). The expression of a number of flavoreductases, including the evofosfamide-activating enzyme P450 oxidoreductase (POR), was also higher in  $\rho^0$  cells (Fig. 6D).

**Evofosfamide Oxidises Mitochondrial Cytochromes and Inhibits Respiration.** To directly examine the involvement of mitochondrial electron transport in evofosfamide reduction, key components of mitochondrial function were assessed in permeabilised UT-SCC-74B cells exposed to evofosfamide (5–200  $\mu\text{M}$ ). Shifts in absorption spectra confirmed the oxidation of mitochondrial cytochromes *a*, *b* and *c* by evofosfamide with a clear shift in the Soret band from 410 to 400 nm, characteristic of cytochrome *c* oxidation (Vanderkooi *et al.*, 1980) (Fig. 7A). All cytochromes appeared to be equally affected, with maximum oxidation reached at approximately 25  $\mu\text{M}$  evofosfamide (Fig. 7B). Since components of the mitochondrial transport system were affected by the prodrug, the efficiency of electron transport to oxygen and electron leakage to ROS production were assessed concurrently. We thereby assessed whether reduction of evofosfamide by electron carriers in the transport chain would compete with leakage of electrons to generate superoxide, and hence  $\text{H}_2\text{O}_2$ . However,



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electron capture by the prodrug did not appear to be sourced from mitochondrial ROS (Fig. 7C), but rather from respiration, which was inhibited by evofosfamide in a concentration-dependent manner (Fig. 7D). Respiration was equally affected in all mitochondrial respiration states, with specific respiration rates approximately halved in the presence of 200  $\mu$ M evofosfamide relative to vehicle controls (Fig. 7E).

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## Discussion

Severe hypoxia is a prevalent and specific feature of the tumour microenvironment that contributes to aggressive and refractory disease (Wilson and Hay, 2011). Despite its strong rationale as a target, the most widely explored strategy for addressing tumour hypoxia – bioreductive prodrugs – has had a chequered clinical development history. Agents such as tirapazamine, evofosfamide, PR-104, apaziquone (EO9), banoxantrone (AQ4N) and tarloxotinib have each had trials (in some cases, major registrational studies) that either failed to meet efficacy or safety endpoints or were discontinued for commercial reasons. Recognised obstacles in the development of bioreductive prodrugs include toxicological interactions with standards of care (DiSilvestro *et al.*, 2014), stringent micropharmacokinetic requirements (Hicks *et al.*, 2003) and challenges in the application of diagnostic tools to select patients whose tumours express the target of these drugs (Hunter *et al.*, 2016). The target in question is multifactorial and encompasses both hypoxia and the intrinsic sensitivity of malignant cells to the active drug species. The principal challenge has been in clinically assessing hypoxia itself, as PET–CT imaging with nitroimidazole radiopharmaceuticals and analysis of hypoxia markers in tissue samples have been confounded by scalability and macroregional heterogeneity in tumour hypoxia, respectively. An example of intrinsic sensitivity is deficiency in homologous recombination repair of DNA double-strand breaks, which sensitises model tumours to prodrugs that release DNA crosslinking agents (Hunter, Hsu, *et al.*, 2014). Indeed, the present study identified sgRNA targeted to *SLX4IP* (*C20orf94*) to be negatively selected by evofosfamide. *SLX4IP* forms a Holliday junction resolvase in complex with *SLX4*, *ERCC4*, *ERCC1*, *MUS81*, *EME1* and *SLX1* and thus has a putative role in DNA crosslink repair (Svendsen *et al.*, 2009). The gene is frequently mutated in paediatric acute lymphoblastic leukaemia (Meissner *et al.*, 2010), suggesting that these neoplasms may be sensitive to

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evofosfamide. Similarly, positive selection of sgRNA targeting *C10orf90* (*FATS*) and *SLFN11* by evofosfamide is consistent with the function of these genes in DNA damage response (Zoppoli *et al.*, 2012; Smurnyy *et al.*, 2014) and platinum sensitivity (Tian *et al.*, 2012; Nogales *et al.*, 2015).

Another aspect of the bioreductive prodrug target is the facility of prodrug activation given a state of hypoxia, i.e. the bioreductive capacity of the hypoxic cancer cell. Accordingly, identifying the reductases responsible for prodrug activation has been the subject of substantial effort, with the ultimate goal of profiling their expression in tumours to aid in predicting sensitivity. Prodrugs that require initial one-electron reduction are activated by flavoproteins, a family of approximately 100 oxidoreductases that catalyse electron transfer from NAD(P)H via FMN and FAD to substrates. Candidate-based screening of subsets of these enzymes by overexpression in cell lines has identified POR, MTRR, NDOR1, NOS2A, FOXRED2 and CYB5R3 as capable of prodrug metabolism (Patterson *et al.*, 1997; Papadopoulou *et al.*, 2003; Guise *et al.*, 2007, 2012; Chandor *et al.*, 2008; Hunter, Jaiswal, *et al.*, 2014; Wang *et al.*, 2015). In the case of evofosfamide, this has only been demonstrated for POR and FOXRED2 (Hunter *et al.*, 2012; Meng *et al.*, 2012; Hunter, Jaiswal, *et al.*, 2014). However, flavoreductases are promiscuous in their xenobiotic metabolism, and the more relevant question is the complement of enzymes actually responsible for prodrug activation in tumours at native expression levels. This calls for correlative or high-throughput, loss-of-function discovery approaches. Using gene expression analysis, functional CRISPR and shRNA screens, we present evidence for the mitochondrial electron transport chain (ETC) as a key source of reducing equivalents in the activation of evofosfamide, where several flavoproteins serve as constituents of complexes in the electron transport chain (ETC). While the actual electron donors are not identified in this study (and could be flavoproteins, cytochromes or Fe-S centres), the cell physiological data

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presented are consistent with evofosfamide intercepting electrons at multiple nodes of the ETC (Fig. 7F). Although we note that there is a limited precedent for mitochondrial reduction of nitro compounds (Köchli *et al.*, 1980; Bironaite *et al.*, 1991), this represents a conceptually new model of the reductive activation of evofosfamide. While the oxidation of all cytochromes was affected by the prodrug, respiratory data suggested that complex II negligibly contributes to respiration (i.e. only marginally fuels the mitochondrial transport system with electrons) in the UT-SCC-74B model investigated and that the overall effect of the prodrug is primarily mediated by the decrease in electron transport prior to complex III (containing cytochrome *b*). This is evidenced by the fact that all components with the exception of complex I and the ubiquinone pool were affected independently of mitochondrial state. Interestingly, although not explicitly investigated here, the model suggests a possible mechanism of hypoxia selectivity (in addition to oxidation of the prodrug radical by O<sub>2</sub>) as ETC redox centres are fully reduced under hypoxia due to the absence of O<sub>2</sub> as terminal electron acceptor. Our model also implies that the increased activity of evofosfamide in tumour models conferred by pre-treatment with pyruvate (Takakusagi *et al.*, 2014) may reflect enhanced mitochondrial electron flux (and thus prodrug activation) in addition to enhancing hypoxia through oxygen consumption. Similarly, our observation that evofosfamide inhibits cellular respiration implies that this prodrug may decrease tumour hypoxia both by direct ablation of hypoxic regions and by suppressing oxygen consumption, contributing to its efficacy in combination with radiotherapy that we and others have reported (Nytko *et al.*, 2017; Jamieson *et al.*, 2018; Takakusagi *et al.*, 2018).

It is notable that the mitochondrial signature we observed in the present study, which spanned multiple cancer cell lineages, contrasts with the proliferation signature that we previously observed to associate with evofosfamide sensitivity specifically in human papillomavirus-negative head and neck squamous cell carcinoma (Jamieson *et al.*, 2018). This

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distinction may arise from the fact that rates of evofosfamide activation were more homogeneous in models of the latter indication, with evofosfamide sensitivity principally determined by cellular phenotypes downstream of prodrug activation.

Curiously, we found mitochondria-deficient 143B  $\rho^0$  cells to show enhanced activation of evofosfamide and other prodrugs, although massive transcriptional reprogramming in these cells – which included upregulation of a number of non-mitochondrial flavoreductases such as *POR* – suggests that these cells are far from an isogenic system and have limited utility in dissecting the contribution of mitochondria to bio-reductive prodrug activation. Nonetheless, the observation underscores the view that mitochondrial electron transport is not the sole source of evofosfamide reduction. Indeed,  $\rho^0$  cells have been reported to show increased cytosolic NADH/NAD<sup>+</sup> and NADPH/NADP<sup>+</sup> ratios (Naviaux, 2008), which would be consistent with enhanced electron flux via non-mitochondrial NAD(P)H-dependent flavoreductases. Of interest,  $\rho^0$  cells have been reported to show altered one-carbon metabolism, with increased S-adenosyl methionine synthesis and consequent epigenetic silencing via CpG methylation (Smiraglia *et al.*, 2008). This may be one mechanism for the transcriptional reprogramming we observed.

If the mitochondrial ETC plays a significant role in tumour activation of evofosfamide, differences in mitochondrial biology between malignancies may be a contributor to evofosfamide sensitivity. Warburg's original contention that the fundamental lesion in cancer cells is mitochondrial dysfunction is no longer accepted, but mitochondrial changes are common in many tumours and mitochondrial DNA copy number and mass vary widely (Vyas *et al.*, 2016). In addition, hypoxia modulates mitochondrial function through multiple mechanisms including HIF-1-dependent transcription of pyruvate dehydrogenase kinase 1

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(PDK1), which inhibits pyruvate dehydrogenase and thereby mitochondrial O<sub>2</sub> consumption (Kim *et al.*, 2006; Papandreou *et al.*, 2006). Thus, suppressed ETC flux in the hypoxic cells that evofosfamide seeks to target could compromise its metabolic activation, although under severe hypoxia lack of O<sub>2</sub> as the terminal electron acceptor could favour competing reduction of the prodrug.

A notable feature of our study was the absence of canonical prodrug reductases among the genes identified in the screens, with *POR* only modestly selected in the CRISPR screen (MAGeCK P=0.004; median increase in depth-normalised sgRNA read counts 1.7-fold) and completely absent in the shRNA screen. This contrasts with our earlier study, which utilised the same shRNA library to identify *POR* as the predominant activating reductase and a major sensitivity determinant for the benzotriazine di-*N*-oxide SN30000 (Hunter *et al.*, 2015). Our data suggest a minor role at most for *POR* in evofosfamide activation and provide further evidence for the view that the complement of activating enzymes is non-identical for different classes of prodrugs (Su, Gu, *et al.*, 2013), even if the sum bioreductive metabolism of chemically-distinct pharmacophores can be correlated across tumour models (Wang *et al.*, 2012). These findings, our description of involvement of mitochondrial electron transport in the reduction of evofosfamide and the curious enhanced sensitivity of rho zero cells to the same highlight the pleiotropic mechanisms of bioreductive prodrug activation. Moreover, they suggest that attempts to predict the facility of evofosfamide in tumours by measuring the expression of one or several reductases is likely to prove futile for patient stratification. Indeed, as we have advanced previously (Wang *et al.*, 2012; Hunter *et al.*, 2016), use of diagnostic 2-nitroimidazole probes such as EF5 that are activated by the same complement of reductases may be the only tractable means of predicting activation facility. Alternatively, biomarker strategies that focus on robustly stratifying patients according to tumour hypoxia may be more

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successful. Nonetheless, it will be of interest to investigate mitochondrial involvement in the activation of other hypoxia-activated prodrugs and more broadly in the metabolism of nitro compounds, quinones and other xenobiotics with high one-electron reduction potentials.

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## Authorship Contributions

*Conducted experiments:* Hunter, Devaux, Meng, Hong, Khan, Ketela, Sharma, Kakadia, Marastoni, Shalev.

*Performed data analysis:* Hunter, Devaux, Meng, Hong, Ketela.

*Performed bioinformatic analyses:* Hunter, Tsai, Print, Bohlander.

*Participated in research study design:* Hunter, Hickey, Print, Bohlander, Hart, Wouters, Wilson.

*Wrote or contributed to writing the manuscript:* Hunter, Devaux, Meng, Hong, Khan, Tsai, Ketela, Sharma, Kakadia, Marastoni, Shalev, Hickey, Print, Bohlander, Hart, Wouters, Wilson.

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## Footnotes

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**Meeting Abstract.** Hunter et al., 2017. Abstract 169: Preclinical efficacy and sensitivity determinants of evofosfamide in molecularly defined models of head and neck squamous cell carcinoma. *Cancer Research* 77(13 Supplement):169-169. Proceedings: AACR Annual Meeting 2017; April 1-5, 2017; Washington, DC.

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## Figure Legends

**Fig. 1.** Antiproliferative activity of evofosfamide in cancer cell lines. (A) Schema of the mechanism of reductive activation and hypoxia selectivity of evofosfamide. The 2-nitroimidazole moiety of evofosfamide undergoes enzymatic one-electron reduction to yield a short-lived radical anion. In the absence of oxygen, the latter fragments (frag.) to release the DNA-crosslinking effector bromo-*iso*-phosphoramidate (Br-IPM), which is spontaneously converted to Cl-IPM by halide exchange. In the presence of oxygen, the radical is back-oxidised to the parent prodrug in kinetic competition with fragmentation. (B) Antiproliferative activity and hypoxic selectivity of evofosfamide in a diverse panel of 32 human cancer cell lines. Cells in 24-well plates were exposed to a dilution series of evofosfamide for 2 h under anoxia (N<sub>2</sub>) or 20% O<sub>2</sub> with 5% CO<sub>2</sub> (Air) then cultured aerobically for 7 d in drug-free medium prior to assessment of culture density by alamarBlue assay. The drug concentrations for half-maximal inhibition of cell growth relative to vehicle-treated control wells on the same plate (IC<sub>50</sub>) were computed by four-parameter functions to the data. Data are presented as the mean + SEM of three independent experiments. (C) Correlation between the antiproliferative potency of evofosfamide as assessed in cancer cell lines using 3-d or 7-d regrowth assays. The former dataset has been published previously (Meng *et al.*, 2012). Data are the mean ± SEM of three independent experiments.

**Fig. 2.** The expression of genes implicated in mitochondrial biology correlates with evofosfamide sensitivity in cancer cell lines. (A) Pairwise correlation structure of transcriptional profiles in 30 cancer cell lines also tested for *in vitro* sensitivity to evofosfamide. RNAseq data were sourced from the Cancer Cell Line Encyclopedia and RSEM counts were log<sub>2</sub> transformed and quantile normalised. Pairwise Spearman correlation coefficients for the resulting count distributions are plotted in the heatmap. (B) The relationship between gene expression features and evofosfamide sensitivity in cancer cell lines. The heatmap illustrates the expression of the 173 genes inversely correlated with evofosfamide sensitivity under anoxia with a Pearson coefficient ≤ -0.4. The cell lines in the cluster are ranked in ascending order by the first principal component of expression values for these 173 genes, with the relationship between

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this variable and evofosfamide  $IC_{50}$  under anoxia indicated. (C) An interacting protein network among genes correlated with evofosfamide potency as per (B). The network was generated using the STRING database ([string-db.org](http://string-db.org)) and gene functions of clusters within the network annotated using GeneSetDB ([genesetdb.auckland.ac.nz](http://genesetdb.auckland.ac.nz)). The number of edges (protein-protein interactions) in the network ( $n=166$ ) was significantly greater than expected by random sampling ( $E(n)=82$ ;  $p<10^{-15}$ ).

**Fig. 3.** A whole-genome CRISPR knockout screen for modifiers of evofosfamide sensitivity identifies mitochondrial respiration and DNA damage response. (A) Abstracted workflow for functional screens using lentiviral whole-genome sgRNA and focused shRNA libraries (results of the latter are reported in Fig. 4) to identify genetic modifiers of sensitivity to evofosfamide. (B) Growth kinetics of KBM-7 cultures transduced with the GeCKOv2 sgRNA library and treated with evofosfamide or control vehicle. Data points are the mean  $\pm$  SEM of the cumulative fold increase in population size relative to starting cultures for three biological replicates of each condition. Red arrows denote the times of challenge with 0.013  $\mu$ M evofosfamide. The growth of evofosfamide-treated cultures was inhibited by a factor of  $10^4$  relative to vehicle control. (C) Assessment of evofosfamide sensitivity by  $IC_{50}$  assay in drug-challenged and naïve KBM-7 knockout libraries at the endpoint of the CRISPR screen. Data points are the mean  $\pm$  SEM of cell viability determinations at each evofosfamide concentration for three replicate cultures from the CRISPR screen. Dose-modifying factor (DMF) was defined as the ratio of  $IC_{50}$  values in evofosfamide-challenged relative to naïve cultures. (D) Deconvolution of the CRISPR screen using the MAGeCK statistical algorithm. The statistical significance of positive or negative selection of sgRNA targets is plotted as a function of the median  $\log_2$  fold change in the representation of sgRNA against each target. Select high ranking findings are highlighted. (E) Comparison of hits called in positive selection in the CRISPR screen using the MAGeCK and PinAPL-Py algorithms. Targets called as positively selected beyond a statistical threshold of  $P<0.005$  by either method are coloured red, with select high ranking hits further highlighted. (F) An interacting protein network among gene knockouts positively selected by evofosfamide treatment in the CRISPR screen (targets in red in E). The network was generated using the STRING database ([string-db.org](http://string-db.org)) and gene functions of clusters within the network annotated using GeneSetDB ([genesetdb.auckland.ac.nz](http://genesetdb.auckland.ac.nz)). The number of edges (protein-protein

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interactions) in the network ( $n=37$ ) was significantly greater than expected by random sampling ( $E(n)=18$ ;  $p<10^{-4}$ ).

**Fig. 4.** An oxidoreductase-focused shRNA screen identifies mitochondria-related genes potentially involved in determining evofosfamide sensitivity. (A) Plating efficiency of UT-SCC-74B cells immediately prior to the addition of evofosfamide (“baseline”), immediately after the completion of evofosfamide exposure (“post-EVO”) and after recovery of the cultures. The surviving fraction of  $10^{-3}$  was determined as the ratio of plating efficiencies after and before evofosfamide exposure. Data points are from 2-3 determinations and the mean  $\pm$  SEM is shown. (B) Deconvolution of the shRNA screen using the MAGeCK and RIGER algorithms, the latter using weighted-sum aggregation to generate gene-level significance scores. The statistical significance of positive selection hits is plotted for the two deconvolution methods, with select high ranking candidates highlighted. (C) An interacting protein network among gene knockouts positively selected by evofosfamide in the shRNA screen ( $P<0.05$  by MAGeCK, RIGER or PinAPL-Py). The network was generated using the STRING database ([string-db.org](http://string-db.org)) and gene functions of clusters within the network annotated using GeneSetDB ([genesetdb.auckland.ac.nz](http://genesetdb.auckland.ac.nz)). The number of edges (protein-protein interactions) in the network ( $n=82$ ) was significantly greater than expected by random sampling ( $E(n)=11$ ;  $p<10^{-16}$ ).

**Fig. 5.** Rho zero cells deficient in mitochondrial DNA show enhanced bioreductive prodrug activation and sensitivity. (A) Confirmation of the absence of mitochondrial DNA in  $\rho^0$  cells derived from the 143B osteosarcoma cell line. Endpoint PCR for amplification of mitochondrial-encoded *MT-TL1* (tRNA leucine 1) relative to nuclear-encoded *GAPDH* was used to demonstrate the absence of mitochondrial genomes in  $\rho^0$  cells. (B) Mitochondrial respiration rates in 143B and  $\rho^0$  cells in LEAK state (respiration attributed to proton leak in non-phosphorylating mitochondria), OXPHOS (maximum rate of oxidative phosphorylation), CII (maximum electron entry at complex II from succinate forced by complex I inhibition with rotenone) and CIV (maximum activity of complex IV with TMPD and ascorbate). Data are mean  $\pm$  SEM from five independent experiments and statistical significance of differences in respiration rates for each state was assessed using way-way ANOVA with Benjamini–Hochberg

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adjustment for multiple comparisons. (C) Assessment of reductive activation of evofosfamide in 143B and  $\rho^0$  cells by LC-MS/MS measurement of total Br-IPM, Cl-IPM and the 2-nitroimidazole fragmentation product ('Tr-H') concentrations at the endpoint of one-hour exposures to 30  $\mu$ M evofosfamide under anoxia (24-well format,  $10^6$  cells/0.5 mL in each well). Data are the mean + SEM of metabolite concentrations for the sum of intracellular and extracellular metabolites (measured separately then summed), per culture, from five independent experiments. Statistical significance was assessed using Student's t-test operating on the sum of Br-IPM and Cl-IPM concentrations. (D) Enhanced reductive activation of the fluorogenic 6-nitroquinolone FSL-61 and the 2-nitroimidazole probe EF5 by  $\rho^0$  cells under anoxia. The flow cytometry histograms illustrate fluorescence area event distributions for 143B,  $\rho^0$  and unstained  $\rho^0$  cells, where fluorescence originated from the reduced product of FSL-61 (Su, Guise, *et al.*, 2013) or an Alexa488-conjugated secondary antibody against cell-bound EF5 metabolites. The figures are representative of three independent experiments performed. The nitro moieties that are substrates for bioreduction are coloured red in each structure. (E) Enhanced sensitivity to bioreductive prodrugs in  $\rho^0$  cells under anoxia. IC<sub>50</sub> values for parental 143B cells and  $\rho^0$  cells treated under normoxia ("air") and anoxia ("N<sub>2</sub>") are shown for evofosfamide, the nitroaromatic mustard prodrug PR-104A and the benzotriazine di-*N*-oxide prodrug SN30000. Data points are from three independent experiments and the mean  $\pm$  SEM is shown.

**Fig. 6.** Widespread changes in gene expression in 143B  $\rho^0$  cells. (A) Heatmap of 6,740 genes determined to be differentially expressed in  $\rho^0$  cells relative to parental 143B cells using the *limma* method ( $P < 0.05$  after adjustment for multiple comparisons using the Benjamini–Hochberg method). Functions enriched (Benjamini–Hochberg adjusted  $P$ -values  $< 0.05$ ) among differentially expressed gene clusters were annotated using GeneSetDB ([genesetdb.auckland.ac.nz](http://genesetdb.auckland.ac.nz)) and the PANTHER database ([pantherdb.org](http://pantherdb.org)). (B) Reduced and increased (C) expression of genes involved DNA damage response and repair genes in  $\rho^0$  cells. (D) Increased expression of flavoproteins in  $\rho^0$  cells. For A-D, triplicate  $\rho^0$  and 143B samples, were hierarchically clustered without supervision using the ward.D method with Euclidean distance. The heatmap scales denote row Z-scores for the triplicate samples arrayed in columns, where rows correspond to individual genes, for quantile-normalised, log<sub>2</sub>-transformed RSEM counts.

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**Fig. 7.** Evofosfamide oxidises mitochondrial cytochromes and inhibits respiration in permeabilised UT-SCC-74B cells. (A) Absorption spectra of the mitochondrial cytochromes by spectrophotometry in permeabilised UT-SCC-74B cells exposed to graded evofosfamide with ambient O<sub>2</sub> relative to untreated controls. (B) Concentration-dependent oxidation of cytochromes *a*, *b* and *c* in permeabilised UT-SCC-74B cells exposed to evofosfamide. The relative contribution of each cytochrome to the absorbance in (A) was determined using extinction coefficients and the resulting oxidation state was normalised by the maximum reduction state of each cytochrome (induced using KCN). Curves represent independent experiments. (C) Assessment of ROS production by Amplex UltraRed assay in permeabilised UT-SCC-74B cells exposed to increasing concentrations of evofosfamide or corresponding volumes of DMSO vehicle. Data points are the mean ± SEM of determinations from at least four independent experiments. (D) Concentration-dependent inhibition of mitochondrial respiration in permeabilised UT-SCC-74B cells. Data points are the mean ± SEM of determinations from five independent experiments. (E) Inhibition of respiration by evofosfamide (200 μM) in permeabilised UT-SCC-74B cells in the LEAK (attributed to proton leak in non-phosphorylating mitochondria), OXPHOS (fully-active electron transport chain), CII (electron entry at complex II from succinate forced by inhibiting complex I with rotenone) and CIV (maximum capacity of CIV to reduce O<sub>2</sub>) states. Data are mean + SEM of determinations from five independent experiments and statistical significance of differences in respiration rates for each state was assessed using two-way ANOVA with Benjamini–Hochberg adjustment for multiple comparisons. (F) Potential mechanism for reduction of evofosfamide via the interception of electrons from the mitochondrial transport chain.

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## Tables

TABLE 1

GO terms overrepresented among expressed genes that correlated with evofosfamide IC<sub>50</sub> in cancer cell lines treated under anoxia

Genes with expression levels that were inversely correlated with evofosfamide IC<sub>50</sub> values under anoxia (Pearson coefficient  $\leq -0.4$ , which defined 173 genes) were assessed for overrepresentation of GO annotations using Fisher's exact tests. The resulting *P*-values were adjusted for multiple comparisons (i.e. all possible GO terms) using the Benjamini–Hochberg method. The fold enrichment of each significant (adjusted *P*-value < 0.05) GO annotation (i.e. the ratio of the actual to the expected number of genes on the list with the annotation given random sampling) is denoted.

GO term	Fold enrichment	Adj. <i>P</i> -value
GO0005743: Mitochondrial inner membrane	5.29	<10 <sup>-7</sup>
GO0005759: Mitochondrial matrix	5.10	10 <sup>-4</sup>
GO0005840: Ribosome	6.69	10 <sup>-3</sup>
GO0044822: Poly(A) RNA binding	2.34	0.002
GO0005739: Mitochondrion	2.45	0.009
GO0046872: Metal ion binding	1.93	0.009
GO0070125: Mitochondrial translational elongation	2.39	0.02
GO0070126: Mitochondrial translational termination	11.58	0.03



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TABLE 2

GO terms overrepresented among sgRNA targets positively selected following evofosfamide selection of KBM-7 cells in a whole-genome CRISPR knockout screen

Gene targets called to be positively selected at a statistical significance threshold of  $P \leq 0.005$  using the MAGeCK or PinAPL-Py screen deconvolution algorithms, or both, were included in the analysis. *P*-values for GO enrichment from Fisher's exact tests were adjusted for multiple comparisons using the Benjamini–Hochberg method.

GO term	Fold enrichment	Adj. <i>P</i> -value
GO0005747: Mitochondrial respiratory chain complex I	17.71	0.004
GO0032981: Mitochondrial respiratory chain complex I assembly	15.29	0.004
GO0008137: NADH dehydrogenase (ubiquinone) activity	17.16	0.008
GO0006120: Mitochondrial electron transport, NADH to ubiquinone	16.85	0.008

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TABLE 3

GO terms overrepresented among shRNA targets positively selected following evofosfamide selection of UT-SCC-74B cells in a reductase-focused RNAi screen

Gene targets called to be positively selected at a statistical significance threshold of  $P < 0.05$  using the MAGeCK, PinAPL-Py or RIGER screen deconvolution algorithms were included in the analysis. *P*-values for GO enrichment arising from Fisher's exact tests were adjusted using the Benjamini–Hochberg method.

GO term	Fold enrichment	Adj. <i>P</i> -value
GO0005740: Mitochondrial envelope	2.80	0.002
GO0044455: Mitochondrial membrane part	3.94	0.002
GO0070469: Respiratory chain	3.78	0.002
GO0031967: Organelle envelope	2.65	0.003
GO0031975: Envelope	2.65	0.003
GO0005746: Mitochondrial respiratory chain	3.90	0.004
GO0031966: Mitochondrial membrane	2.61	0.006
GO0006091: Generation of precursor metabolites and energy	3.08	0.01
GO0005743: Mitochondrial inner membrane	2.54	0.01
GO0019866: Organelle inner membrane	2.54	0.01
GO0044429: Mitochondrial part	2.18	0.01
GO0005747: Mitochondrial respiratory chain complex I	4.62	0.01
GO0045271: Respiratory chain complex I	4.62	0.01
GO0030964: NADH dehydrogenase complex	4.62	0.01
GO0022900: Electron transport chain	3.15	0.02

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GO0051536: Iron-sulfur cluster binding	5.69	0.02
GO0051540: Metal cluster binding	5.69	0.02
GO0045333: Cellular respiration	3.94	0.03
GO0005739: Mitochondrion	1.73	0.03
GO0015980: Energy derivation by oxidation of organic compounds	3.78	0.03
GO0050136: NADH dehydrogenase (quinone) activity	5.25	0.03
GO0008137: NADH dehydrogenase (ubiquinone) activity	5.25	0.03
GO0003954: NADH dehydrogenase activity	5.25	0.03
GO0006119: Oxidative phosphorylation	4.05	0.03
GO0031090: Organelle membrane	1.85	0.04
GO0051539: 4 iron, 4 sulfur cluster binding	7.32	0.04

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## Figures

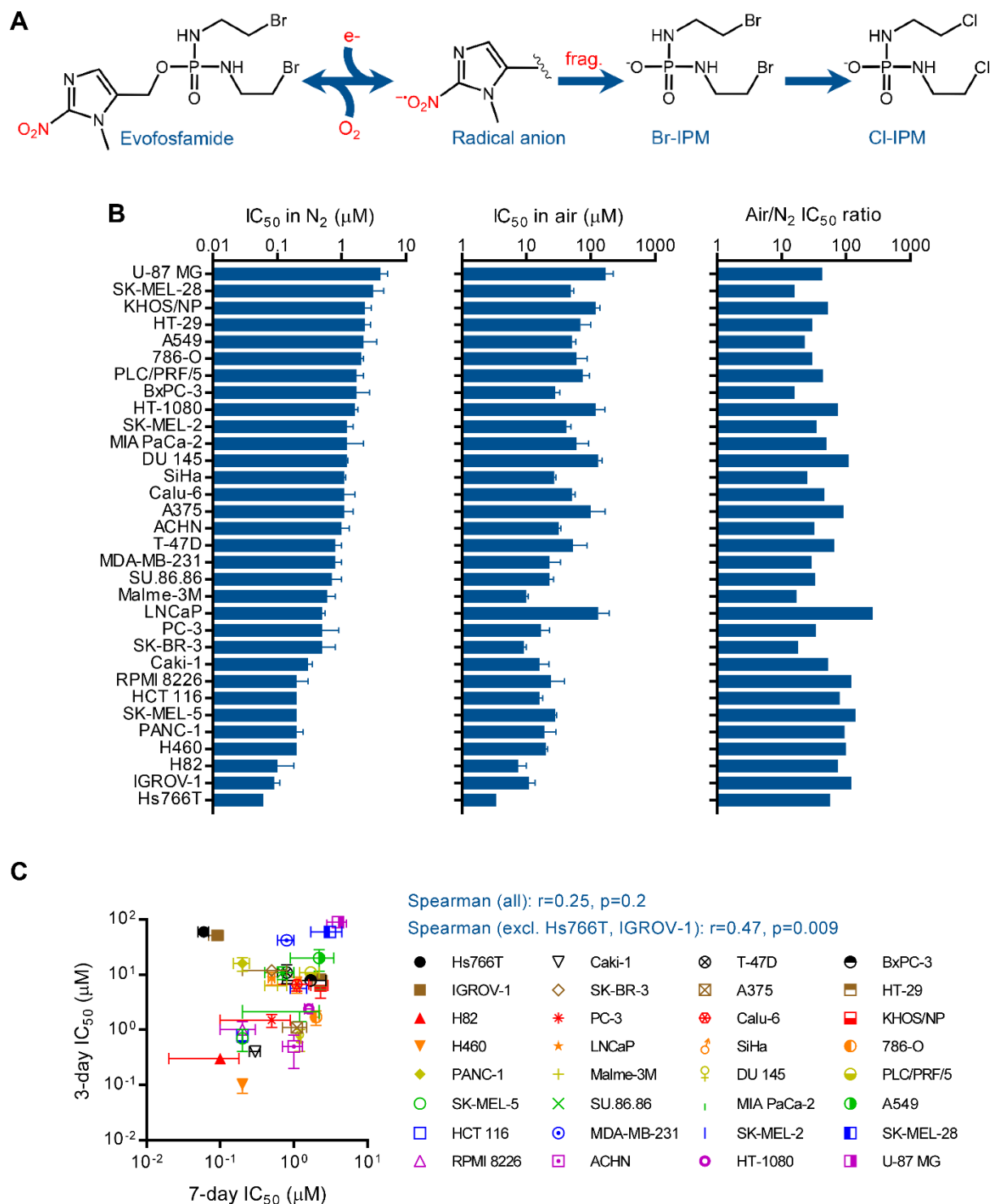


Figure 1

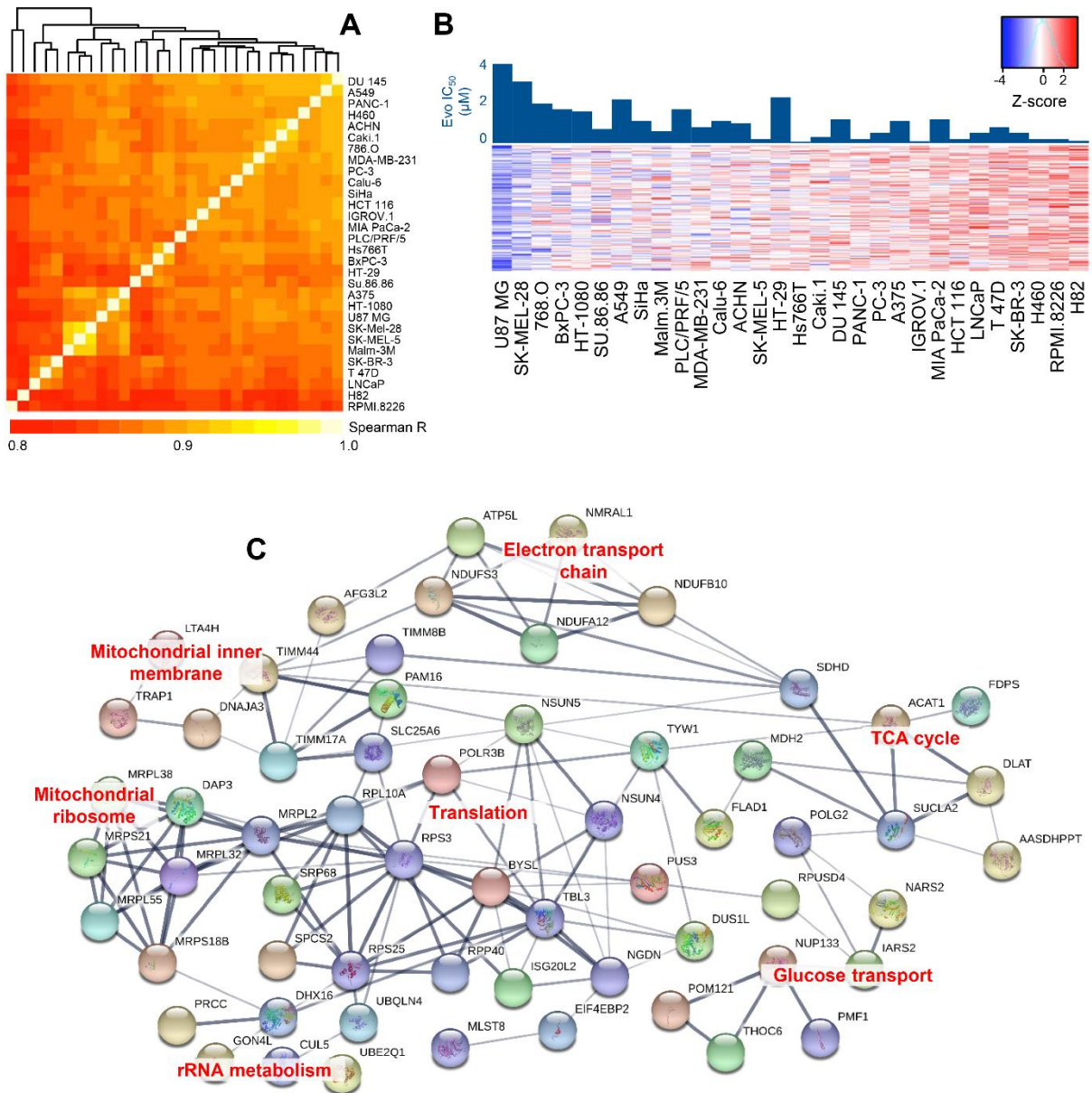


Figure 2

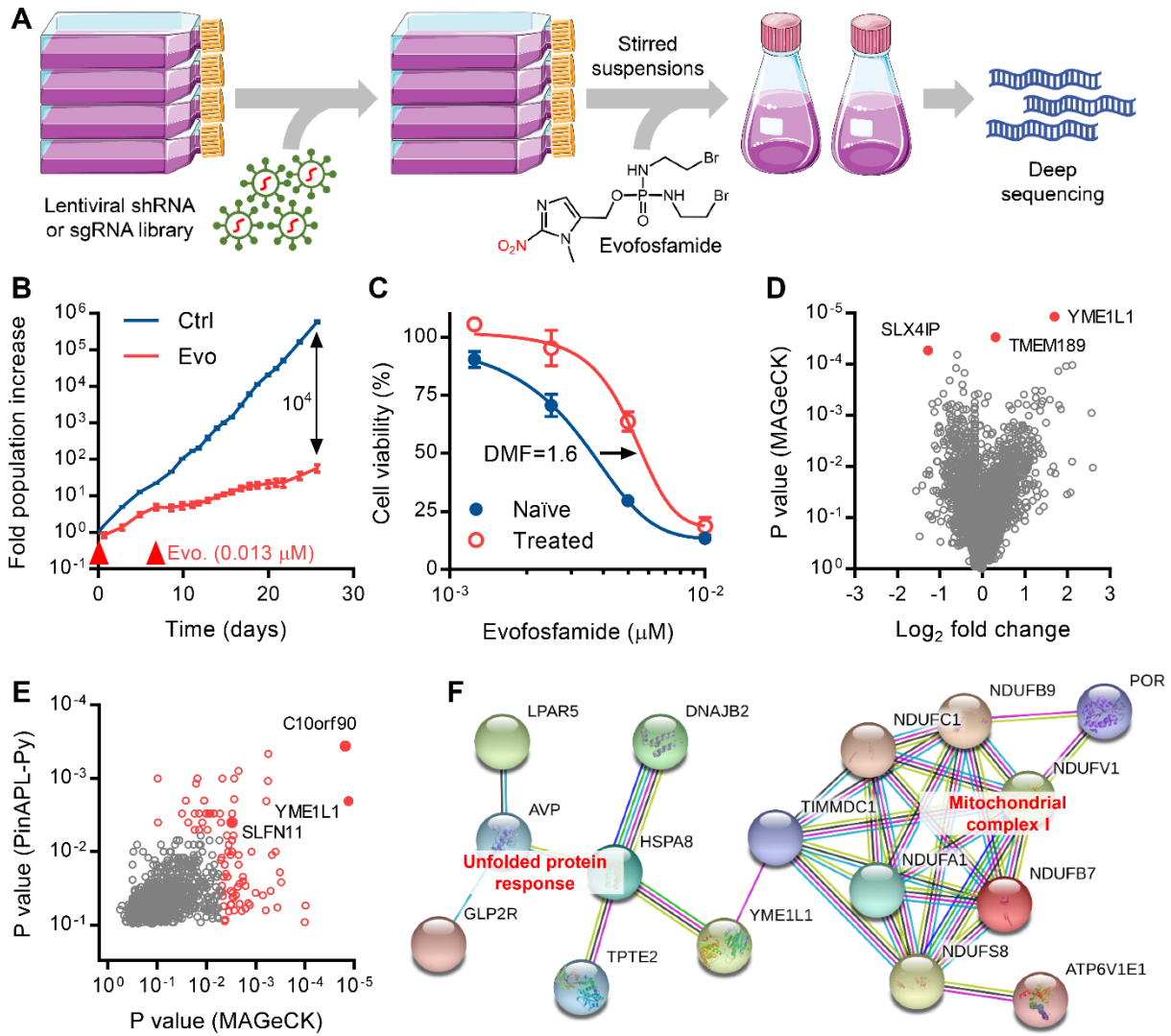


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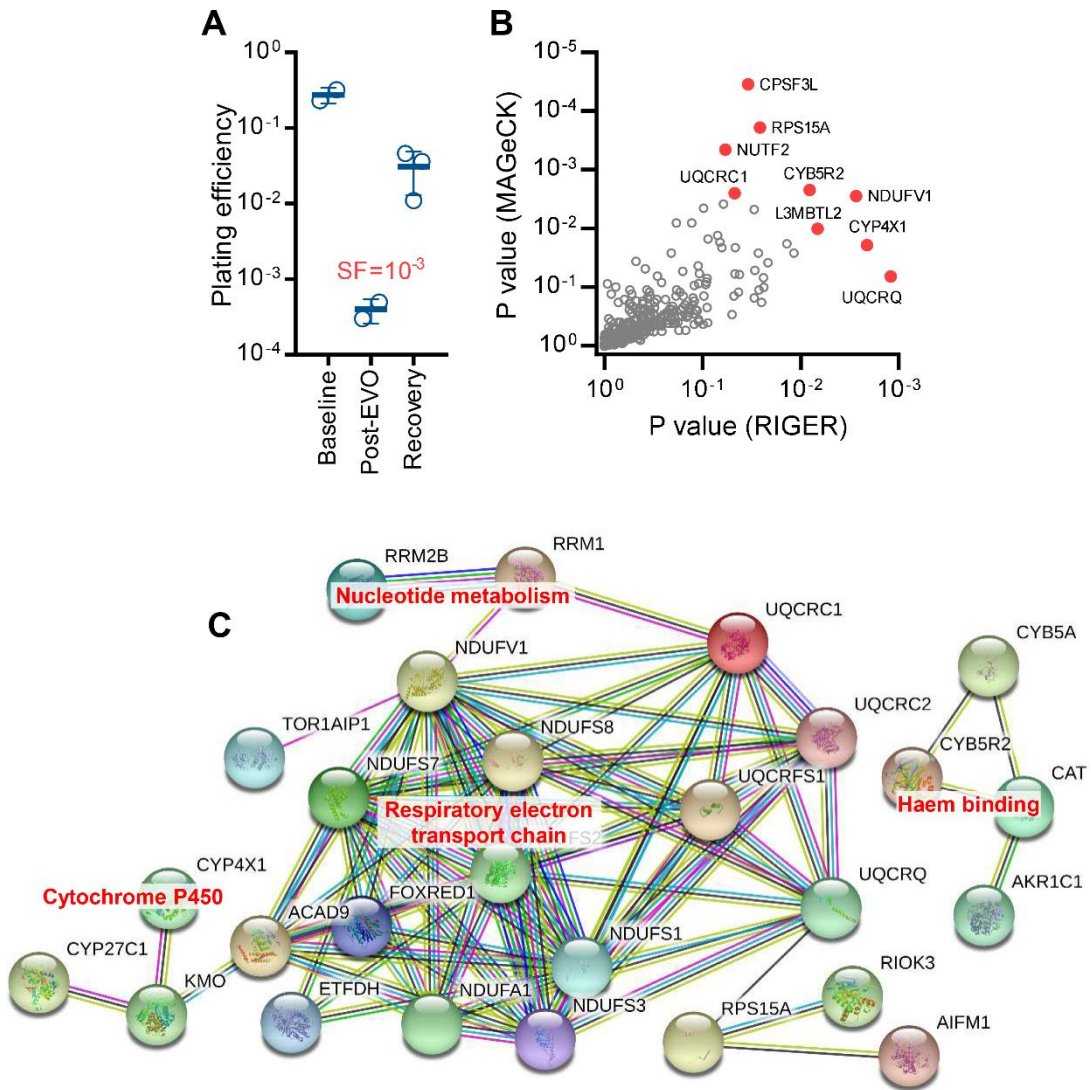


Figure 4

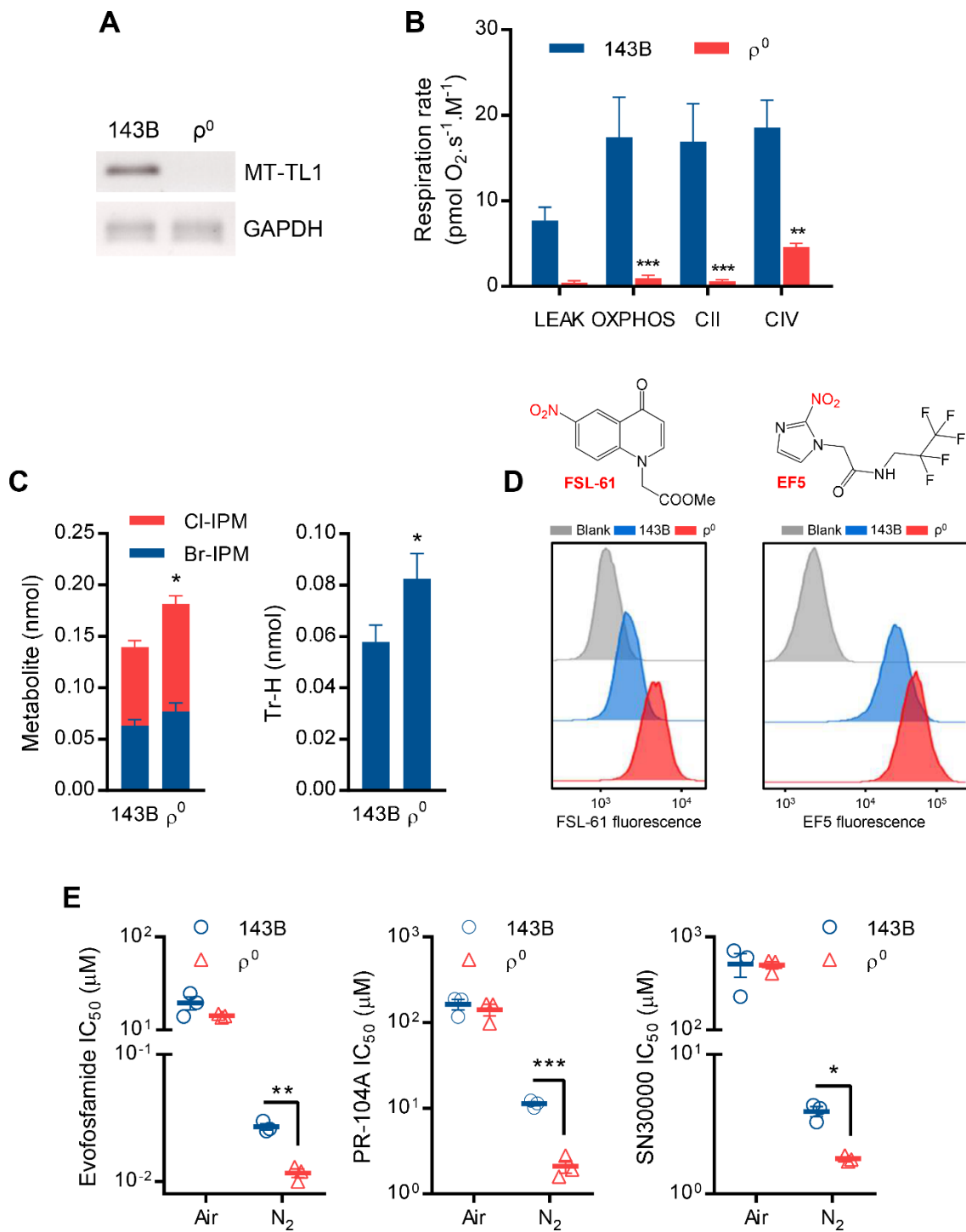


Figure 5



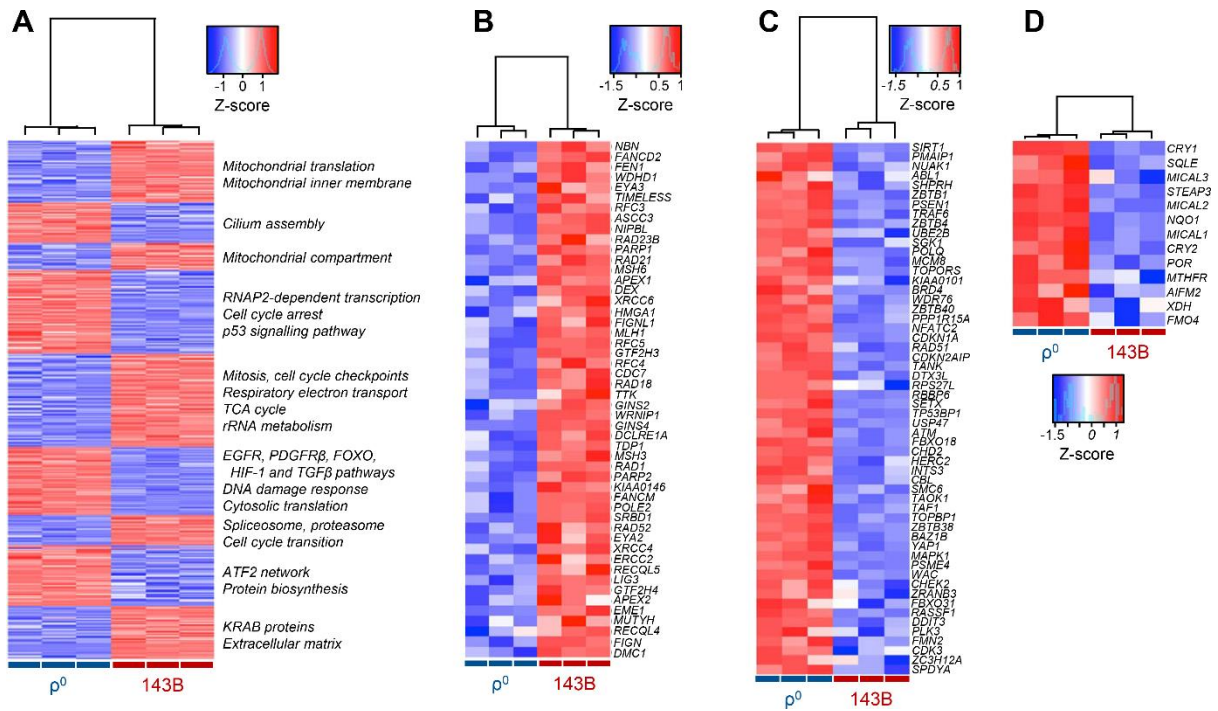


Figure 6

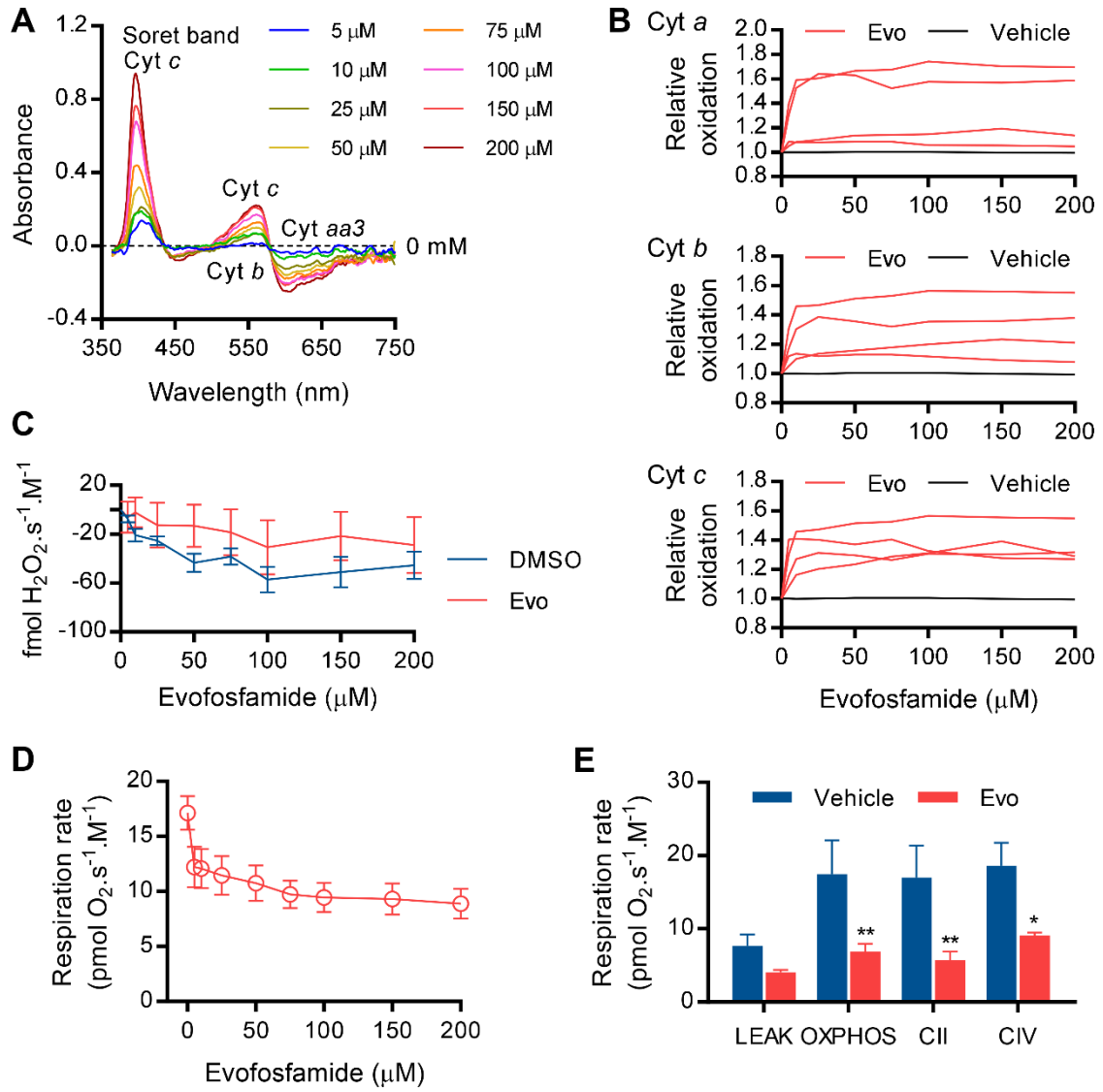


Figure 7

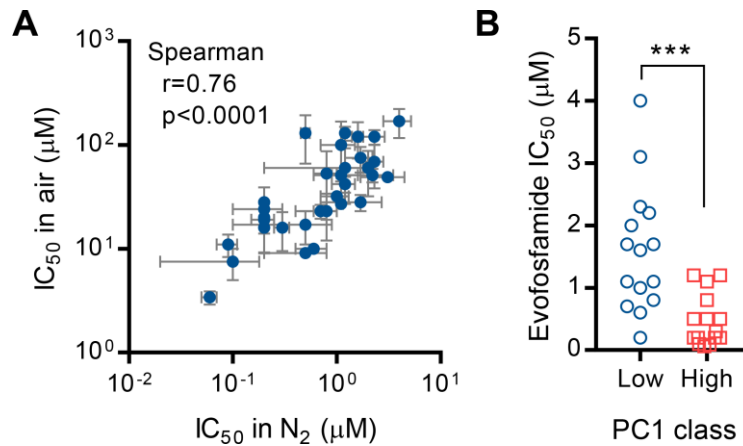
# *Molecular Pharmacology*

## Supplemental Figures for

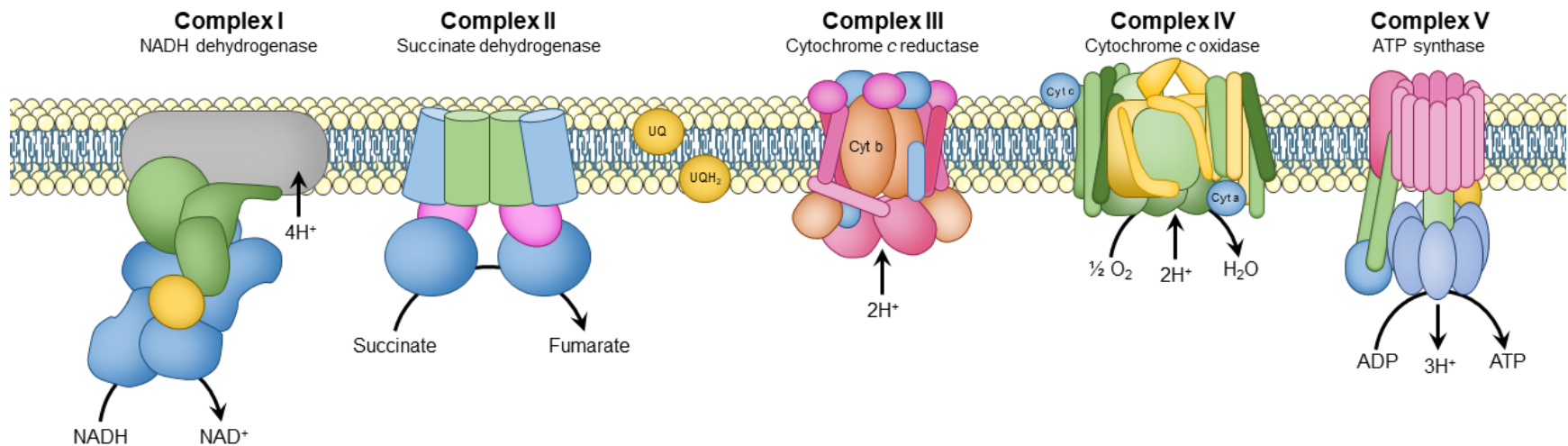
### **Functional CRISPR and shRNA screens identify involvement of mitochondrial electron transport in the activation of evofosfamide**

Francis W. Hunter, Jules B. L. Deveaux, Fanying Meng, Cho Rong Hong, Peter Tsai, Troy W. Ketela, Indumati Sharma, Purvi R. Kakadia, Aziza Khan, Stefano Marastoni, Zvi Shalev, Anthony J. R. Hickey, Charles P. Hart, Cristin G. Print, Stefan K. Bohlander, Bradly G. Wouters, William R. Wilson

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**Supplemental Figure 1.** (A) Correlation between the sensitivity of 32 cancer cell lines to evofosfamide when challenged under anoxia (“N<sub>2</sub>”) or 20% O<sub>2</sub> (“air”) and as assessed by 7-d regrowth assay. Data points are the mean + SEM of IC<sub>50</sub> determinations from three independent experiment for each cell line. Data correspond to Figure 1B in the main text. (B) Comparison of evofosfamide sensitivity under anoxia between cell lines dichotomised by the first principal component of expression values for 173 genes correlated with evofosfamide potency (Pearson coefficient <math><-0.4</math>; illustrated in Figure 2B in the main text). Statistical significance of differences in the distribution of IC<sub>50</sub> values between groups was assessed by Mann–Whitney U-test.



NADH dehydrogenase

E	ND1	ND2	ND3	ND4	ND4L	ND5	ND6										
E	Ndufs1	Ndufs2	Ndufs3	Ndufs4	Ndufs5	Ndufs6	Ndufs7	Ndufs8	Ndufv1	Ndufv2	Ndufv3						
B/A	NuoA	NuoB	NuoC	NuoD	NuoE	NuoF	NuoG	NuoH	NuoI	NuoJ	NuoK	NuoL	NuoM	NuoN			
B/A	NdhC	NdhK	NdhJ	NdhH	NdhA	NdhI	NdhG	NdhE	NdhF	NdhD	NdhB	NdhL	NdhM	NdhN	HoxE	HoxF	HoxJ
E	Ndufa1	Ndufa2	Ndufa3	Ndufa4	Ndufa5	Ndufa6	Ndufa7	Ndufa8	Ndufa9	Ndufa10	Ndufab1	Ndufa11	Ndufa12	Ndufa13			
E	Ndubf1	Ndubf2	Ndubf3	Ndubf4	Ndubf5	Ndubf6	Ndubf7	Ndubf8	Ndubf9	Ndubf10	Ndubf11	Ndufc1	Ndufc2				

Succinate dehydrogenase / Fumarate reductase

E	SDHC	SDHD	SDHA	SDHB
B/A	SdhC	SdhD	SdhA	SdhB
	FrdA	FrdB	FrdC	FrdD

Cytochrome c reductase

E/B/A	ISP	Cyt b	Cyt 1				
E	COR1	QCR2	QCR6	QCR7	QCR8	QCR9	QCR10

Cytochrome c oxidase

E	COX10	COX3	COX1	COX2	COX4	COX5A	COX5B	COX6A	COX6B	COX6C	COX7A	COX7B	COX7C	COX8	E/B/A	COX11	COX15	COX17
B/A	CyoE	CyoD	CyoC	CyoB	CyoA													
	CoxD	CoxC	CoxA	CoxB														
	QoxD	QoxC	QoxB	QoxA														

Cytochrome c oxidase, cbb3-type

B	I	II	IV	III
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Cytochrome bd complex

B/A	CydA	CydB	CydX
-----	------	------	------

F-type ATPase (Bacteria)

alpha	beta	gamma	delta	epsilon
a	b	c		

F-type ATPase (Eukaryotes)

alpha	beta	gamma	delta	epsilon	
OSCP	a	b	c	d	e
f	g	fb/h	j	k	8

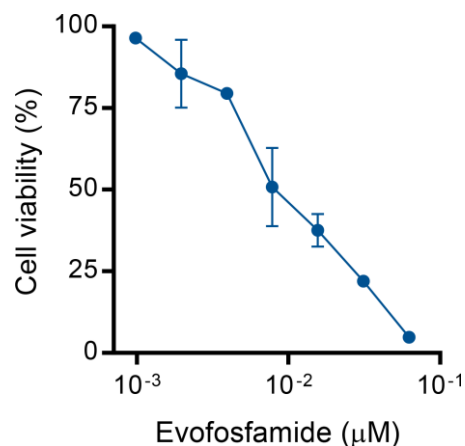
V/A-type ATPase (Bacteria, Archaeas)

A	B	C	D	E	F	G/H
I	K					

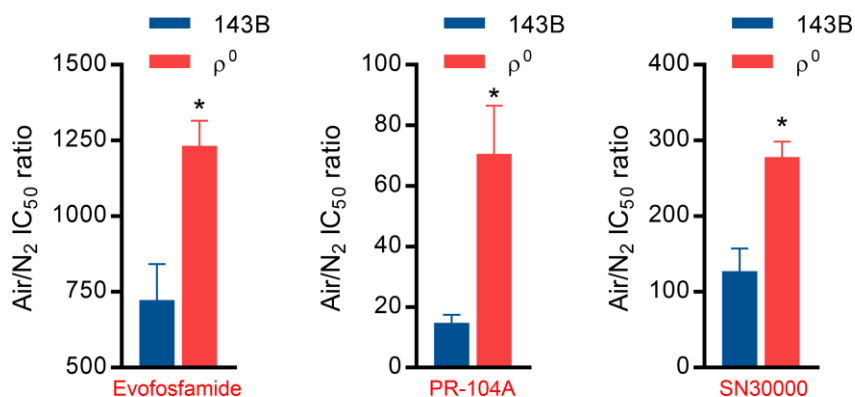
V-type ATPase (Eukaryotes)

A	B	C	D	E	F	G	H
a	c	d	e	S1			

**Supplemental Figure 2.** Mitochondrial electron transport chain components correlated with evofosfamide potency in 30 genomically diverse cancer cell lines. The expression of all genes marked with red stars correlated with evofosfamide potency (Pearson coefficient <-0.4).



**Supplemental Figure 3.** Concentration-dependent inhibition of cell viability in KBM-7 cells exposed to evofosfamide. Cells (2,000 per well in 96-well format) were challenged with an evofosfamide dilution series under anoxia for 2 h, re-oxygenated then cultured for 5 d before assessing viability using alamarBlue. Data points are the mean  $\pm$  SEM of determinations from three independent experiments.



**Supplemental Figure 4.** Hypoxia selectivity of evofosfamide, PR-104A and SN30000 in parental and  $\rho^0$  143B osteosarcoma cells. Data are the mean + SEM of the intra-experiment ratio of IC<sub>50</sub> values for cells challenged with evofosfamide under 20% O<sub>2</sub> (“air”) or anoxia (“N<sub>2</sub>”) in three independent pairs of assays. Statistical significance of differences in IC<sub>50</sub> ratios was assessed by Mann–Whitney U-test.