Arginine dependence of Acute Myeloid Leukaemia blast proliferation: a novel therapeutic target.

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Key Points: Arginase depletion with BCT-100 pegylated recombinant human arginase is cytotoxic to AML blasts.
Abstract

Acute Myeloid Leukaemia (AML) is one of the most common acute leukaemias in adults and children, yet significant numbers of patients relapse and die of disease. In this study we identify the dependence of AML blasts on arginine for proliferation. We show AML blasts constitutively express the arginine transporters CAT-1 and CAT-2B, and that the majority of newly diagnosed patients’ blasts have deficiencies in the arginine recycling pathway enzymes arginosuccinate synthase (ASS) and ornithine transcarbamylase (OTC), making them arginine auxotrophic. BCT-100, a pegylated human recombinant arginase, leads to a rapid depletion in extracellular and intracellular arginine concentrations, resulting in arrest of AML blast proliferation and a reduction in AML engraftment in vivo. BCT-100 as a single agent causes significant death of AML blasts from adults and children, and acts synergistically in combination with cytarabine. Using RNA-sequencing 20 further candidate genes which correlated with resistance have been identified. Thus AML blasts are dependent on arginine for survival and proliferation, and depletion of arginine with BCT-100 of clinical value in the treatment of AML.
Introduction

Treatment for Acute Myeloid Leukaemia (AML) has seen significant progress, but overall survival rates have plateaued and significant numbers of patients continue to die of the disease.\textsuperscript{1,2} New therapeutic approaches are needed that complement standard chemotherapy without increasing the burden of toxicity. Arginine is an amino acid metabolized by cells to provide precursors for cell cycle activity, protein synthesis, and a number of other cell functions. In certain circumstances there can be a high demand for arginine, including rapid growth periods during development, inflammation, organ dysfunction and tumour growth. Arginine requirements may not be met by synthesis from citrulline alone, thus requiring arginine from the diet, leading to its classification as a semi-essential amino acid.\textsuperscript{3-5} Cancers also pose a unique demand on nutrient requirements, including dependency on arginine supplementation to sustain growth, i.e. arginine auxotrophism.\textsuperscript{6} Thus control over arginine availability and metabolism represents a potential therapeutic approach that can be exploited (see below).

BCT-100 is a clinical grade pegylated (PEG) recombinant human arginase that catalyses the conversion of arginine to ornithine and urea, leading to arginine depletion.\textsuperscript{7-10} BCT-100 has shown significant benefit against solid tumours in preclinical studies and early phase clinical trials.\textsuperscript{8} Here we characterise the mechanisms of dependence of AML blasts on arginine and the potential for arginine depletion with BCT-100 as a therapeutic approach.
Methods

AML patient samples

Blood samples were obtained from 20 patients with newly diagnosed or newly relapsed AML, before the start of treatment, at the Birmingham Children’s Hospital, University Hospitals Birmingham, or Heartlands Hospital Birmingham (Table 1). The cells were separated from fresh samples as previously described. AML samples were investigated within 12 hours of blood sampling from patients and only samples with >98% viability by trypan blue staining were used. Bone marrow samples from 39 newly diagnosed AML patients were obtained from the Chinese Hospital, Hong Kong.

Cytotoxicity assay

Cell lines or sorted AML blasts from patients were re-suspended in complete media and 2x10⁵ AML blasts or 0.5x10⁵ cell lines were added to each well of 96 well plates. On day 1, BCT-100 was added at final concentrations of 0, 200, 400, 600, 800, 1000, 1500, 2000 or 4000 ng/mL to triplicate wells. The cytotoxicity of cytarabine (500 ng/mL) was also tested in combination with BCT-100. Cells were incubated for a further 72 hours. The effect of arginine deprivation was similarly tested by culturing AML cell lines and patients’ blasts in SILAC arginine free RPMI-1640 (Fisher Scientific), 10% heat-inactivated arginine free fetal bovine serum (Fisher Scientific), glutamine (1x) (Sigma) and sodium pyruvate (1x) (Sigma).

Flow cytometric analysis

Cells from cell lines and patient samples were collected and labelled with propidium iodide (PI) to assess viability by flow cytometry. The relative percentage of viable cells at the end of the assay (72 hours) was calculated using the following formula: (mean no. of viable blasts recovered in treatment wells/mean no. of viable blasts in untreated wells x 100). Apoptosis
was estimated by cells being re-suspended in 1x Annexin Binding Buffer and labelled with 7-AAD and Annexin conjugated to phycocyanin (FITC) (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen, Cat. No. 556547). The cells were analyzed with a Cyan flow analyser (Beckman Coulter) using FlowJo software (Tree Star Inc.). The 50% inhibitory concentration (IC_{50}) is defined as the concentration of BCT-100 that killed 50% of the viable cells at the termination of the assay.

Cell cycle analysis was performed using propidium iodide (PI) staining and flow cytometry. 1x10^6 cells/well incubated with RPMI 10% with or without BCT-100 (600 ng/mL) in 24 well plates for 72 hours were harvested, washed twice in PBS and fixed in cold ethanol for 1h at 4°C. Following washing with PBS, cells were stained with PI solution and 50µl of RNase A stock solution (10µg/mL, Invitrogen) at 4°C for 3 hours before analysis with a Cyan flow analyser in combination with ModFit software.

To investigate effects of AML blast proliferation, Carboxyfluorescein succinimidyl ester (CFSE) labelled AML blasts, were cultured in the presence or absence of BCT-100 (600ng/ml) for 72 h. Propidium iodide added to allow viable cells to be gated on flow cytometry. Cell proliferation was determined according to CFSE dilution.

**AML murine xenografts**

NOD/Shi-scid/IL-2R SCIDγnull (NOG) mice aged 10-14 weeks were irradiated with 1.25 Gy. One day later 5x10^6 HL60 leukaemia cells were injected into the tail vein. 5mg/kg BCT-100 was injected intraperitoneally (i.p.) twice weekly. A second group of mice were treated with 25mg/kg cytarabine (i.p. once weekly). Bone marrow was harvested from the leg bones of mice sacrificed after 5 weeks of treatment. AML engraftment was defined by the detection of human CD45+ cells using flow cytometry.
Immunoblotting

Following cell lysis (20nM Tris-Hcl pH7.5, 150nM NaCl, 2mM EDTA, 1.0% triton X-100 and protease and phosphatase inhibitors Roche Applied Science, Indianapolis, IL) equal amounts of protein were loaded onto 12% Tris-Glycine SDS-PAGE (BioRad) gels and transferred to PVDF membranes. Hybridisation was carried out using antibodies to PARP, caspases -3, and -9, LC3 (Cell Signalling), and actin (Sigma). HRP-conjugated secondary antibodies, goat anti-rabbit (Cell Signalling), and sheep anti-mouse (GE Healthcare) were used for blots, which were developed with ECL substrate (BioRad) and exposed on Kodak film.

Transmission Electron microscopy

AML blasts were treated with BCT-100 (600ng/ml) in culture for 72hours. Following harvesting they were fixed in 2.5% glutaraldehyde followed by 1% osmium tetroxide. The samples were dehydrated through ethanol and embedded in propylene oxide/resin mixture at 60°C for 16h prior to sectioning at 80 nm in thickness and placement on 300 mesh copper slot grids for examination by transmission electron microscopy.

Immunohistochemistry

Paraffin-embedded tissue sections of bone marrow trephines from AML patients at diagnosis were deparaffinised and rehydrated. Antigen were demasked was performed in 50 mM Tris/2 mM EDTA pH 9.0 using a Philips Whirlpool Sixth Sense microwave on a steaming program. Staining with anti-human argininosuccinate synthase (ASS; Abcam) and anti-human ornithine transcarbamylase (OTC; Abcam) using the Novolink Polymer Detection System (RE7280-K, Leica). Primary antibody incubation was performed overnight in a cold room. Sections were counterstained with Gill Nr 3 haematoxylin (Sigma Aldrich) and mounted in Aquatex (Merck).

RNA sequencing
RNA was derived from 6 sensitive (P1, P3, P8, P9, P10, P12) and 6 (P11, P6, P4, P5, P7, P13) resistant AML patients’ blasts, as identified by IC_{50}. Samples were prepared with the Illumina TruSeq RNA Sample Preparation Kit v2 by Oxford Gene Technologies (Oxford, UK). They were sequenced on the Illumina HiSeq2000 platform using TruSeq v3 chemistry, over 100 cycles. Read files (Fastq) were generated via the manufacturer’s proprietary software. Reads were mapped by their location to the appropriate Illumina iGenomes built using Bowtie version 2.02. Splice junctions were identified using Tophat v2.0.9. Cufflinks (version 2.1.1) was used to perform transcript assembly. Visualisation of differential expression results used Cummerbund. RNA-Seq alignment metrics were generated using Picard. A table of arginine related genes, concerned with arginine recycling and transport and associated pathways were constructed based on current knowledge of arginine metabolism. Genes were compared to demonstrate differences in Fragments Per Kilobase of transcript per Million mapped reads (FPKM) between resistant and sensitive cells and subtracted to demonstrate change and direction in FPKM. Genes of interest were highlighted if the different in FPKM was +/- 1.96 (2SD from mean).

**Statistical analysis**

A Wilcoxon-rank-sum test was used to determine the statistical significance of the difference in unpaired observations between 2 groups (GraphPad Prism, USA). Correlations between parameters were evaluated using Spearman rank correlation analyses. *p* values are 2-tailed and where values were <0.05, they were considered statistically significant. For combination studies of BCT-100 with cytarabine, the interaction effect of the two drugs was tested in a two-way analysis of variance (ANOVA). Analysis of synergism was assessed according to the Chou & Talalay method, using CompuSyn software (ComboSyn Inc, NJ, USA). AML blasts from patients were cultured with BCT-100 alone (0, 200, 400, 600, 800, 1000ng/mL), cytarabine (0,
200, 400, 600, 800, 1000 ng/mL) or both for 72 hours. The percentage of viable cells relative to control after 72 hours was measured by flow cytometry. Using this method a Combination Index (CI) at IC_{50} for individual patient samples is calculated, synergism is defined as CI < 1, while antagonism is CI > 1, and an additive effect is considered as CI = 1.

**Study approval**

In accordance with the Declaration of Helsinki, patient samples were obtained after written, informed consent prior to inclusion in the study. Regional Ethics Committee (REC Number 10/H0501/39) and local hospital trust research approval for the study was granted for United Kingdom hospitals and at the Chinese University Hospital, Hong Kong. The Birmingham Biomedical Ethics Review Subcommittee (BERSC) approved all animal protocols in this study. Procedures were carried out in accordance with UK Home Office Guidelines.

**Results**

*AML proliferation is dependent on arginine*
AML blasts create an immunosuppressive microenvironment through arginase activity and release, contributing to AML growth and pathogenesis.\textsuperscript{14} However, the dependence of AML blasts on arginine for survival has not been reported. Arginine deprivation resulted in a profound decrease in the number of viable AML blasts, providing proof of principle that this amino acid plays a key role in blast viability (Figure 1a).

Blood arginine levels are maintained by dietary consumption, protein turnover and endogenous synthesis from citrulline through an intestinal-renal arginine cycle.\textsuperscript{15-18} In mammalian cells arginine is imported from the microenvironment predominantly by a Na\textsuperscript{+}-independent (System y\textsuperscript{+}) family of transmembrane cationic amino acid transporters (CAT1, CAT2A, CAT2B, CAT3) with tissue-specific expression patterns.\textsuperscript{19} We identified that AML blasts express CAT-1 and CAT2B, regardless of blast subtype, thus allowing AML blasts to utilise extracellular arginine (Figure 1b).

To understand if AML blasts express the key arginine recycling enzymes ASS and OTC we examined 39 diagnostic patient samples by immunohistochemistry (Figure 1c, Figure 1d). Of 29 adult AML samples 10\% had no staining, 45\% showed low, 24\% showed moderate, and 21\% showed high ASS expression; whilst 0\% had no staining 35\% showed low, 55\% showed moderate, and 10\% showed high OTC expression. Of 10 paediatric AML samples 20\% had no staining, 60\% had low, and 20\% had moderate ASS expression; whilst 20\% had no staining, 0\% had low, 50\% had moderate, and 30\% had high OTC expression. One paediatric sample received a 0 score for both ASS and OTC.

AML blasts can therefore lack one or both, of the important enzymes for endogenous arginine synthesis. As such their dependence on extracellular arginine levels should become rate-
limiting on many metabolic reactions and protein synthesis thereby preventing proliferation. Proliferation of cultured AML blasts was arrested when arginine fell below 10μM. Consistent with these findings in newly diagnosed patients, where AML disease burden is highest, plasma arginine concentrations is significantly lower than in healthy controls (mean: 131 μM healthy vs 9.0 μM AML patients; p=<0.0001) (Figure 1e). Together these findings highlight the dependency of AML blasts on extracellular arginine - arginine auxotrophism.

BCT-100 reduces the number of AML blasts in vitro and in vivo
BCT-100 is a pegylated recombinant human arginase being investigated for the treatment of solid tumours. We first demonstrated BCT-100 catalyses a dose-dependent reduction in media arginine concentrations in vitro (Sup. Figure 1a). Arginine was reduced to <2μM within 8 h at concentrations of 600ng/mL BCT-100 or higher.

AML cell lines are sensitive to BCT-100 arginine depletion: IC\textsubscript{50}s ranged between 50 and 180 ng/mL (Sup. Figure 1b). No further decrease in cell number was seen by increasing concentrations of BCT-100 above 600ng/mL, consistent with the depletion of arginine at this drug concentration and the specificity of drug action. In vivo experiments previously demonstrated that a single BCT-100 dose results in a sustained depletion of plasma arginine levels persisting for 6 days. We confirmed that BCT-100 led to a significant decrease in plasma arginine (mean: 20 μM healthy vs 8.5 μM AML mice; p=0.0244) (Figure 2a) and treated mice had a significant reduction in AML engraftment (human CD45+ cells: median 21% untreated vs 5% BCT treated, p=0.029) (Figure 2b), which was approximately equivalent to 25mg/kg cytarabine (median 6%). No evidence of toxicity or weight loss was observed (Figure 2c).
BCT-100 demonstrates activity against primary AML blasts from patients

The activity of BCT-100 was tested against sorted, fresh blasts from 20 AML patients. Samples varied in response, ranging from >95% cell death to completely resistant (Figure 3a). IC\textsubscript{50}s ranged from 100ng/mL to 2000ng/mL (Figure 3b). In 5 samples < 50% death occurred, and no increase in activity was seen even with doses up to 16,000ng/mL, confirming that BCT-100 does not act through off-target toxicity. Sensitivity to BCT-100 did not correlate with clinical characteristics. BCT-100 was also cytotoxic to 3 of the 4 relapse samples (P7, P14, P19, P20). This is the first report of the efficacy of arginine depletion against human AML blasts.

BCT-100 synergises with cytarabine against AML blasts from patients

Cytarabine is a key agent in AML chemotherapy protocols for adults and children\textsuperscript{20,21} However patient AML blasts can develop cytarabine resistance. Therefore a new drug which re-sensitises blasts to cytarabine may play a key role in future therapy.\textsuperscript{22,23} When BCT-100 was combined with cytarabine, cytotoxicity that is greater than the sum of the 2 individual compounds alone was seen in AML samples (F\textsubscript{(1,57)} = 6.405, p<0.0001) (Figure 3c). Analysis of individual patient samples, showed that BCT-100 synergised with cytarabine (Combination Index <1) for almost all samples (Table 2). BCT-100 sensitivity correlated moderately with sensitivity to cytarabine (r=0.5182, p=0.0280), suggesting complementary mechanisms of activity of these two drugs. (Figure 3d).

BCT-100 reduces intracellular arginine concentrations

Having demonstrated above that BCT-100 reduces local arginine concentrations, we also showed that BCT-100 led to a significant decrease in AML intracellular arginine (p<0.0007) (Figure 4a). Although BCT-100 is a relatively large molecule and acts extracellularly, some
BCT-100 molecules could also be internalised. Using fluorescently labelled BCT-100, this drug conjugate bound to the cell surface of AML blasts and was internalised (Figure 4b, Sup. Fig 2). The percentage of surface bound BCT-100 increased moderately over time (Sup Figure 3). No correlation was identified between the sensitivity of AML blasts and the percentage of internalised BCT-100.

*BCT-100 induces cell cycle arrest and death by necrosis in AML blasts*

Conventional chemotherapy agents lead to a reduction in leukaemia cell numbers through either cell cycle arrest or cell death. To investigate BCT-100 cytotoxicity and its mechanism, we show that arginine depletion leads to a significant arrest of AML proliferation (Figure 5a,b). Cell cycle analysis showed an increase in the percentage of cells in G0/G1, with decreases of those in S phase (Figure 5c and Figure 5d). G0/G1 arrest was confirmed in the blasts of AML patients treated with BCT-100, by the relative increase in Cyclin A expression, and decreases in cyclin B and E, compared to untreated cells. (Figure 6a).

Cell cycle arrest may result in either a steady-state of viable cells or in cell death. Examining cell morphology by TEM, BCT-100 caused cell death of AML blasts, with features more consistent with necrosis – including cell membrane permeabilisation and organelle enlargement (Figure 6b, and Supp Figure 4 top panel). In contrast cytarabine treated AML blasts showed nuclear fragmentation bodies characteristic of apoptosis. There was no evidence of cell death in treated normal T cells or monocytes, confirming the low toxicity against non-malignant haematopoietic cells seen in adult early phase trials of BCT-100 (Supplementary Figure 4).
Non-specific growth factor withdrawal has been associated with induction of the intrinsic pathway of apoptosis in AML.\textsuperscript{25} BCT-100 did not induce any significant activation of the requisite effector caspases -9 and -3 or PARP cleavage (Supplementary Figure 5a).\textsuperscript{26} In addition no significant increase in PI+/Annexin+ cells were seen following BCT-100 treatment (Sup Figure 5b and 5c). Amino acid deprivation has also been associated with the induction of death by autophagy in AML, identified by the conversion of cytoplasmic LC3-I to autophagosomic LC3-II.\textsuperscript{27-30} No increase in LC3-II was seen following BCT-100 treatment, confirming the absence of autophagy seen in EM (Supplementary Figure 5d). Amino acid deprivation can induce the rapid production of reactive oxygen species which induce cell death, but no evidence for this was found following BCT treatment (Sup Fig 6a, data not shown).\textsuperscript{31}

**Biomarkers of sensitivity to BCT**

Understanding characteristics of AML blasts which correlate with drug sensitivity is important for patient risk stratification. Of the 20 AML samples tested for sensitivity to BCT-100 in vitro, ASS and OTC expression did not correlate with response to BCT-100, consistent with our previous reports in adult solid tumours (Sup Fig 6b). CAT 1 and CAT 2B expression did not correlate strongly with BCT sensitivity (CAT1 $r=0.11$, $p=0.74$; CAT2B $r=-0.31$, $p=0.41$) (Figure 6c, 6d, Sup Fig 6c).

As multiple pathways, other than arginine recycling may be important in AML pathogenesis, we used RNA-sequencing to identify genes predictive of BCT-100 response. RNA was isolated from 6 sensitive and 6 resistant patient samples, based on their IC\textsubscript{50}s post treatment with BCT-100. (Figure 3b). We identified 20 genes which were differentially expressed between resistant and sensitive samples (Table 3) The top differentially expressed gene was *EREG* (epiregulin), which codes for a ligand of the epidermal growth factor family (EGF) capable of binding to
the EGF receptor and the ERBB family of tyrosine kinase receptors. Other genes of interest included ZIC5, which have a zinc finger protein known to be an upstream regulator of the Wnt pathway; EN2, a homeobox gene that regulates the forkhead box transcription factor FOXA2; HSPA6, a heat shock associated protein that seems to be associated with drug sensitivity in AML; and RGL3, a paralog of RASGRF2.

In comparing FPKM between resistant and sensitive cells (Table 4), there were 2 differentially expressed arginine pathway associated genes. Over-expression of the AKT1 gene (ΔFPKM = 7.1) was observed in sensitive cells, although there was no change in MTOR gene expression (ΔFPKM=-0.704). Type I arginase (ARG1, ΔFPKM=8.87) was also overexpressed in sensitive cells as compared to resistant cells. No changes in arginine transporter genes (SLC7A1-4) or ASS and OTC were identified. These findings identify pathways that might be predictive of sensitivity to therapeutic arginine depletion in AML, and shed light on new aspects of AML disease biology.

**Discussion**

Normal myeloid cells differ in their requirement for arginine, ranging from maintenance of neutrophil activity, through to the consumption of arginine by Myeloid Derived Suppressor Cells and inflammatory macrophages.32-34 We previously showed that AML blasts have a high
arginase activity, creating an immunosuppressive microenvironment. However the mechanism by which arginine is imported from the microenvironment by AML blasts had not been described. We identify that human AML blasts predominantly express the CAT-1 and CAT-2B isoforms. Interrogation of the 'R2: microarray analysis and visualization platform’ (http://r2.amc.nl) confirms the expression of these transporters across all FAB subtypes of AML. The role of the CAT family of proteins in haematopoiesis has only received limited study, identifying CAT-1 and CAT-2B as the main transporters responsible for arginine uptake in in vitro models of non-malignant myeloid and erythroid cells.

Arginine can be produced within healthy cells, by the ornithine-citrulline-arginine cycle. We identified that the majority of patients’ blasts are deficient in either ASS or OTC enzymes, strongly suggesting that AML blasts are reliant on extracellular arginine availability. The contribution of bone marrow stroma in producing arginine to support AML expansion is unknown, but analogous mechanisms exist. For example CNS neurons are reliant on astrocyte derived arginine, and asparaginase is produced by bone marrow stromal cells in cases of ALL. Additionally it has been shown that ASS has a tumour suppressor function in sarcoma, perhaps suggesting ASS may play a more fundamental role in the malignant transformation of blasts.

These findings are consistent with the auxotrophic requirement of arginine by AML blasts. We identified that the need for arginine by AML blasts lead to a significant decrease in arginine concentrations both locally, but also in the plasma of patients at diagnosis. To our knowledge this is the first report of a fall in plasma arginine due to a haematological malignancy and plasma arginine may correlate with AML disease burden. Decreases in plasma arginine concentrations occur in cervical cancer and renal cell carcinoma patients. Physiological compensation through protein breakdown, contributing to cancer induced cachexia, and
arginine recycling by the intestinal-renal axis may try to compensate for lowered arginine levels.\textsuperscript{46}

Since arginine is essential for proliferation and the maintenance of AML viability, arginine starvation by the pegylated recombinant arginase BCT-100 should be cytotoxic to AML, as in other tumours types. Normal human arginase has limited clinical value because of a short plasma-half life.\textsuperscript{7} The addition of a 5000 MW polyethylene glycol molecule (PEG) significantly increases the plasma half-life of arginase 1 from 10-20 min to up to 3.5 days in man with minimal loss of enzyme activity.\textsuperscript{8}

We report for the first time that the majority of AML patients’ blasts from children and adults, including those at relapse, are sensitive to arginine depletion, leading to necrotic cell death. BCT-100 may be internalised by AML blasts, consistent with other pegylated molecules in myeloid cells, which would further contribute to low intracellular arginine concentrations.\textsuperscript{47} BCT-100 can work in combination with cytarabine. Cytarabine in its triphosphorylated form is a substrate for DNA polymerases and is incorporated into phosphodiester linkages in the DNA strand. The addition of subsequent deoxynucleotides are inhibited, resulting in S-phase arrest and cell death.\textsuperscript{20,21} As BCT-100 may induce G0/G1 arrest, the synergistic effect of the two agents may be due to their targeting of different phases of the cell cycle. Similar findings have been described against T-ALL.\textsuperscript{48} Non-malignant cells show little cytotoxicity to BCT-100 because the cells tend to become quiescent, a state they can survive in for prolonged periods, due to intact restriction (R) checkpoints.\textsuperscript{49,50} However tumour cells are less tolerant of this condition, and metabolic stressors may induce G1 cell cycle arrest and ultimately death by necrosis in tumour cells.\textsuperscript{51} Tumour-specific arginine requirements and the concurrent use of drugs to drive cell death along a particular mechanistic pathway may explain tumour-specific effects of arginine depletion.\textsuperscript{52-54}
RNA sequencing of sensitive and resistant samples identified 20 genes which predict response to arginine depletion. Pathway analysis confirmed that expression of arginine recycling or transport molecules did not correlate with sensitivity to arginine depletion. Most intriguing was the finding that epiregulin (EREG), was differentially expressed, with arginase sensitive AML overexpressing EREG. Overexpressed EREG has been linked to dysregulation of MAPK signalling via the EGF pathway as well as the ERBB pathway, and this overexpression in the sensitive cells may reflect an underlying drive towards the MAPK/ERBB pathways.55,56 Conversely, in the arginase resistant cells, over-expression of the heat shock protein HSPA6 was demonstrated, suggesting that this may be a mechanism by which these cells attain resistance, especially in light of the finding that HSP inhibition is cytotoxic to AML.57 EREG and EGFR signalling, as well as the heat shock protein family, play key roles in solid tumour cell proliferation. Small molecule inhibitors are currently under development in AML to target these pathways, providing the potential for rationale combinations of other drugs with BCT-100 for maximal anti-leukaemia effect.58,59

The findings could important translational consequences for a disease which is in desperate need of new therapies. Although small molecule arginase pathway inhibitors are available for laboratory use (N\textsuperscript{G}-hydroxy-L-arginine: NOHA and L-N\textsuperscript{G}-monomethyl arginine: L-NMMA),14 their clinical application has been limited by the requirement for the molecules to be given together and by off-target toxicity in vivo. An alternative approach is through the depletion of arginine from the microenvironment, thus starving AML blasts of this key amino acid.

Pre-clinically BCT-100 has demonstrable activity against hepatocellular, melanoma and prostate carcinoma. A Phase I and II clinical trial has been completed in adults with refractory hepatocellular carcinoma, in which 1600U/kg BCT-100 (OBD) resulted in plasma arginine falling below 8µM (Adequate Arginine Depletion - ADD) and can be maintained for up to 5
or more days after a single treatment (the maintenance level of arginine in human blood is ~40µM). Interim analysis of patients enrolled on a Phase II trial in hepatocellular carcinoma suggest that patients experience significant improvements in overall survival, comparable to the standard of care (personal communication Dr P. Cheng, BCTI). For both paediatric and elderly AML patients in particular, concerns over treatment side-effects limits the type of therapies that can be given safely. In this study we show that arginine depletion is not cytotoxic to T cells and monocytes and is well tolerated in vivo. Clinically this is supported by the excellent toxicity profile of BCT-100 in trial with no evidence of increased patient infections.

Two alternative arginine depleting enzymes are also undergoing early preclinical and clinical evaluation, but have been subject to a number of limiting toxicities. The first is ADI-PEG, a pegylated form of mycoplasma derived arginine deiminase. ADI converts arginine into citrulline and ammonia, potentially leading to toxic hyperammonemia, and ensuing neutropenia. The bacterial origin of the molecule leads to neutralising antibody formation and intramuscular injection site hypersensitivity reactions, limiting continued drug administration and a failure to sustain adequately low plasma arginine. An alternative pegylated human arginase has also been described, in which the enzyme co-factor has been replaced with cobalt to increase arginase activity, but unfortunately seems from in early preclinical studies to be significantly more toxic. Thus the natural enzyme seems to be the best option.

A similar paradigm of bacterial versus recombinant protein therapy occurred in paediatric ALL, with PEG-asparaginase. The bacterial derivative (Erwinia) was eventually superseded by recombinant asparaginase, due to side-effects such as immunogenicity. Recombinant asparaginase has resulted in significant improvements in overall survival for children and its incorporation into upfront treatment protocols.
The findings of our study highlight a role for arginine in AML pathogenesis and support the ongoing development of BCT-100 and similar arginase preparations in early Phase trials for patients with AML.

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Authorship

Contribution: F.M. and C.D.S designed the study, performed research, analysed data and wrote the manuscript. F.M. additionally secured ethical approval and was chief investigator of the study. S.E designed and performed research, J.H-J performed research, T.P. and P.K. provided access to murine xenografts, A.B performed RNA-sequencing analysis, E.O. performed confocal microscopy, J.L. provided patient samples, G.P. provided patient samples, A.L. and M.N. provided patient samples and performed immunohistochemistry, P.C. and K.P.U. provided BCT-100.

Conflict of Interest Disclosure

The authors declare no competing financial interests.

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**Table 1: Table of patient characteristics**

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Table 2: Combination Index calculated according to the Chou-Talalay Method, using Compusyn software.13
Table 3: Table of ranked gene expression in arginase sensitive vs. resistant cells as determined via RNA-seq

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<th>Rank</th>
<th>Gene</th>
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<th>Name</th>
<th>log2(fold_change)</th>
<th>p_value</th>
<th>q_value</th>
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Table 4: Table of Fragments Per Kilobase of transcript per Million mapped reads (FPKM) for resistant vs. sensitive AML lines for arginine related genes

Key: FPKM = Fragments Per Kilobase of transcript per Million mapped reads
Figure 1: AML blasts are auxotrophic for arginine

a) AML patients’ blasts and AML cell lines were cultured in complete or arginine depleted media. The viability of AML blasts from patients and cell lines was assessed by flow cytometry after 72 hours. Arginine depletion leads to a decrease percentage of viable blasts. Representative of two independent experiments b) Expression of CAT-1, CAT-2A, and CAT-2B in blasts from 10 patients was confirmed by qPCR. Patients are identified by unique symbols, which are used consistently throughout the manuscript. c) Staining of 39 bone marrow samples from AML patients at diagnosis with haematoxylin eosin (left panel), anti-OTC (centre panel), anti-MTOR (middle panel), and anti-AKT1 (right panel).
panel), and anti-ASS (right panel). Representative marrows from two patients showing positive antigen staining (upper) and negative antigen staining (lower). d) Histograms of ASS and OTC staining in adult and paediatric AML bone marrow samples. e) Plasma from 20 AML patients at diagnosis and 16 healthy donors were analysed for arginine concentration by ELISA. Plasma arginine levels are significantly lower in newly diagnosed patients (p<0.0001).

**Figure 2: BCT-100 arginine depletion reduces the number of viable AML blasts in vitro and in vivo**

a) Plasma from control and BCT-100 treated NOG mice were collected after 14 days. The concentration of arginine was determined by ELISA. BCT-100 significantly lowers the plasma arginine concentration in vivo (p<0.0244). b) NOG mice were injected with HL-60 AML blasts. BCT-100 (5mg/kg) or cytarabine (25mg/kg) was given i.p. injection twice a week. Bone marrow was sampled from the femurs after 5 weeks to assess hCD45+ cells by flow cytometry. BCT-100 leads to significantly lower AML engraftment (p=0.029), equivalent to cytarabine treatment. Data are representative of 2 independent experiments. c) Untreated and BCT-100 NOG mice engrafted with HL-60 AML showed no significant difference in body weight in response to treatment.

**Figure 3: BCT-100 is cytotoxic against primary blasts from patients**

a) AML blasts from 20 newly diagnosed patients were cultured with BCT-100 (0-4000ng/mL) for 72hours. The percentage of viable blasts relative to untreated was determined by flow cytometry. BCT-100 leads to a dose-dependent decrease in AML blast viability. b) IC_{50} values for the activity of BCT-100 against AML patient blasts are shown. c) AML blasts from patients were cultured with 600ng/mL BCT-100 (OBD) alone, 500ng/mL cytarabine or both for 72hours. The percentage of viable cells relative to control after 72hours was measured by flow cytometry. BCT-100 cytotoxicity is synergistic in combination with cytarabine (BCT vs combination p=0.0054; cytarabine vs combination p=0.0059.2-Way ANOVA: F_{(1,57)} = 6.405, p<0.0001). d) The percentage of viable cells following treatment with 600ng/mL BCT-100 and 500ng/mL cytarabine was correlated. Sensitivity to BCT-100 correlates moderately with sensitivity to cytarabine (r=0.5128, p=0.0208).

**Figure 4: BCT-100 depletes arginine intracellularly**

a) Cell lines or patient samples were cultured with BCT-100 (600ng/mL) for 72hours. Intracellular arginine concentrations were measure by ELISA. BCT-100 causes a depletion of intracellular arginine. Data are representative of 2 independent experiments. b) Internalisation of BCT-100-AF647. AML blasts (top panel) and HL60 cells (lower panel) were incubated with fluorescently labelled BCT-100 for 8 hours. Unbound drug was removed with stripping buffer and extensive washing. Nucleus was stained with DAPI. Images of representative cells were collected by LSM510 system (Zeiss). Arrows indicate intracellular localisation of labelled
BCT-100; arrowheads indicate surface-bound drug. Scale 10um. Representative patient sample of 3 different patient samples.

Figure 5: BCT-100 halts proliferation and cell cycle arrest

a) BCT-100 halts AML cell division. CFSE labelled cell lines were cultured in the presence of 600ng/mL BCT-100. Representative histogram plots shown. Independent experiments were performed on two separate occasions. b) Cell lines were cultured with BCT-100 (0-2000ng/mL) for 72hours. AML proliferation was measured by 3H-thymidine incorporation after 72hours. Data are representative of 2 independent experiments. BCT-100 causes a dose-dependent decrease in AML proliferation. c) AML cell lines were cultured with 600ng/ml BCT-100. Cell cycle analysis was performed after 72 hours. BCT-100 increases the percentage of cells in G0/G1 arrest. Representative histogram plots for untreated and treated HL-60 shown. Independent experiments were performed on four separate occasions. d) Table showing the relative percentages of cells in G0/G1, S, G2/M based on flow cytometry cell cycle analysis.

Figure 6: BCT-100 induced cell cycle arrest leads to necrotic cell death

a) Relative expression of cyclins A, B, E in BCT-100 treated AML patient blasts compared to untreated controls (hashed line) were investigated by qPCR. Representative data of 4 patients shown. b) AML blasts from patients were treated with BCT-100 (600ng/mL) or cytarabine (500ng/mL) for 72 hours. Analysis of cell death was performed by transmission electron microscopy. Representative micrographs of 2 out of 5 patients shown. Left panel – untreated cells. Middle panels – post treatment with 600ng/mL BCT-100. Features consistent with organelle enlargement and cell membrane permeabilisation. Right panels – post treatment with 500ng/mL cytarabine. Features consistent with nuclear fragmentation bodies and preserved membrane integrity. Experiments performed on 3 separate occasions. c) Sensitivity to BCT-100 does not correlate with CAT1 expression (d) and only mildly with CAT-2B expression (r=-0.31, p=0.41)
BCT-100 ARGINASE THERAPY FOR AML

Figure 1
Figure 2
Figure 3
Figure 4
Supplemental Methods

Cell lines

Cell lines were cultured in RPMI-1640 (Invitrogen, CA, USA) with 10% heat-inactivated fetal bovine serum, glutamine (1x), sodium pyruvate (1x) and Penicillin-Streptomycin (RPMI 10%) using T-75 flasks kept in a humidified air atmosphere with 5% CO₂ at 37°C.

Arginine ELISA

Complete media was treated with BCT-100 as described above. Aliquots were collected after 8, 24, 48, and 72h. The concentration of arginine within the media was quantified using a competitive enzyme linked immunoassay (Immunodiagnostik K7733) according to the manufacturers’ instructions. In brief this assay uses a competitive enzyme immunoassay in which L-arginine is derivatized from samples, and competes with an L-arginine-tracer for binding of polyclonal antibodies, in the microtiter wells. The concentration of the tracer-bound antibody is inversely proportional to the L-arginine concentration in the samples. Plasma collected from the blood of NOG AML murine xenografts was similarly tested. For intracellular concentrations of arginine, AML blasts were treated with BCT-100 (600ng/ml) for 72hours. Cells were collected, washed twice in PBS and counted. Equal numbers of treated and untreated cells were lysed using lysis buffer (20nM Tris-Hcl pH7.5, 150nM NaCl, 2mM EDTA, 1.0% triton X-100) and lysates tested for arginine concentration as above.
$^{3}$H-thymidine incorporation

The effect of BCT-100 on AML cell line proliferation was tested by $^{3}$H-thymidine incorporation. Cells were plated as above with BCT-100 for 50h and then 1µCi/well $^{3}$H-thymidine (Perkin Elmer Life Sciences, Beaconsfield, UK) was added for 12-16 hours. $^{3}$H-thymidine incorporation was measured using a Wallac Microbeta Jet 1450 reader (Perkin Elmer).

PCR analysis

RT-PCR was used to detect ASS and OTC in patient-derived AML blasts. RNA was extracted using an RNeasy Mini kit (Qiagen). cDNA was prepared using SuperScriptTM III Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions. The PCR products separated by electrophoresis on a 2% agarose gel were visualised by staining with ethidium bromide. Primers sequence (Eurofin) were: OTC forward 5'-tcccaattatcaatgggctg-3' and reverse 5'-catgcttatccaaagtcttg-3', ASS: forward 5'-GGGGTCCCTGTGAAGGTGACC-3' and reverse 5'-CGTTCATGCTCACCAGCTC-3'.

mRNA levels following treatment of blasts with BCT-100, were measured using Q-PCR. cDNA was generated from RNA of untreated and treated AML blasts from patients using the protocol described above. RT-Q-PCR was done in duplicate using FAST SYBR Green Master Mix (Applied Biosystems) and the Applied Biosystems 7500 Fast Real-Time PCR system. Analysis of gene expression was
calculated according to $2^{-\Delta T}$ method described by Livak et al. plotted as arbitrary units of mRNA relative to GAPDH. Primer sequences (Eurofins) were:

Cyclin A: forward 5'-AATGGGCAGTACAGGAGGAC-3'  
reverse 5'-CCACAGTCAGGGAGTGCTTT-3',  
Cyclin B1: forward 5'-CATGGTGCACTTTTCTCCTT-3'  
Reverse 5'-AGGTAATGTGTAGAGTTGGTGTC-3',  
Cyclin E: forward 5'-GgccAAAATCGACAGGAC-3'  
reverse 5'-GGGTCTGCACAGACTGCAT-3',  
CAT1: forward 5'-ATGGGTGGAAACGCTGATGATAC-3'  
reverse 5'-ACCTTG CCTGTATAAGTCTGGTG-3',  
CAT2A: forward 5'-TTAACACTTATGATGCCGTACT-3'  
reverse 5'-GCAACTGGTGACTGCCTCTTACT-3',  
CAT 2B: forward 5'-ATGCCTCGTGTAATCTATGCTATG-3  
reverse 5'-ACTGCACCCGATGATAAGTAGC-3';  
GAPDH: forward 5'-CCAGCCGAGCCACATCGCTC-3'  
reverse 5'-ATGAGCCCCAGCCTTCTC-3'

**Immunohistochemistry scoring**

Antigen expression in immunohistochemistry sections were assigned independently by 2 experienced pathologists, as described by Nenutil et al.\textsuperscript{11} Briefly, to evaluate the immunostaining intensity each slide was examined on an Olympus BX51 microscope. Representative 400x magnification fields of at least 100 tumor cells were selected and photographed with an Olympus DP70 camera and accompanying image software. Fields were assigned an antigen staining intensity score of 0 = negative, 1 = weak, 2 = moderate, 3 = strong.
The product of the percent positive cells and staining intensity was then derived to create a histoscore of 0-300 for each high power field. A final histoscore was then given to each specimen for each antigen.

BCT-100 Internalisation and Confocal Microscopy

BCT-100 was labeled with Alexa 647 using the Alexa-Fluro 647 Protein Labelling Kit according to manufacturer’s instructions (Molecular Probes A20173, Invitrogen, Carlsbad, CA). To measure the internalisation BCT-100, 2x10^5 AML cells were suspended in 200µL of RPMI-10% with 1µg/ml BCT-Alexa-647 on ice for 30 minutes to saturate the cell surface. The cells were placed on a 37º C heat block and incubated for 0, 1, 2, 4, and 8h. They were washed with FACS buffer and stripped with 0.2M glycine-HCl (pH 2.2, with 1mg/ml BSA) on ice for 15 minutes, to remove unbound BCT-Alexa-647. After resuspension in 200mL of FACS buffer they were analysed by flow cytometry. To confirm internalization of BCT-Alexa-647, stripped AML cells were cytospun (1000 rpm) for 5 minutes, fixed for 30 minutes in 2% paraformaldeyde (Sigma), washed and mounted onto glass slides using DAPI-Fluromount G (Southern Biotech). Z-stacks of 0.7µm sections were collected at 0.5 µm intervals using LSM510-META confocal system with 63x/NA1.4 oil objective. 2.5D projections were produced using ZEN2009 software from Zeiss. XZ and YZ sections through Z-stacks were produced using ImageJ software. In instances where images of single cells are presented, these images are representative of the population of cells studied.
**Reactive Oxygen Species (ROS) detection**

To measure ROS production, cells were cultured in the presence of 2.5 μM DCFDA (Molecular Probes/Invitrogen) for 30 min prior to analysis by flow cytometry as described above. The Greiss Reaction (Cayman) was used to estimate reactive nitrate/nitric species.

**Supplementary Figures:**

**S1: BCT-100 leads to arginine depletion and reduction in AML blast viability**

a) Complete media was treated with BCT-100 (0-4000ng/mL). A dose- and time-dependent decrease in arginine concentration was determined after 72 hours by ELISA. The OBD was determined as 600ng/mL in vitro. Representative of 2 independent experiments. b) Cell lines were cultured with BCT-100 (0-2000ng/mL) for 72 hours. The percentage of viable cells relative to untreated controls was determined by flow cytometry. BCT-100 leads to a dose-dependent decrease in AML cell line viability.

**S2: Internalisation of BCT-100-ALEXA647 into AML cells**

Internalisation of BCT-100-AF647 was confirmed by confocal microscopy. Z-stacks of 0.7μm sections through individual cells were collected: HL60 (a) and AML5 (b). Three sections at various distances from the cell surface indicate intracellular localisation of BCT-100. 2.5D projections and XZ and YZ sections through the slices confirm intracellular distribution of the drug.

**S3: BCT-100 is internalised into AML blasts over time**

Internalisation of BCT-100-ALEXA647 into AML cell lines was confirmed by flow cytometry. Cell lines were incubated with labelled BCT-100 for 0,1,2,4, 8 hours. Unbound drug was removed with stripping buffer and washing. Intracellular accumulation of labelled BCT was measured by flow cytometry.

**S4: Arginine depletion by BCT-100 is not cytotoxic to non-malignant haematopoietic cells.**

a) Cell lines, and patient-derived monocytes and T cells were treated with BCT-100 (600ng/mL) for 72 hours. Analysis of cell death was performed by electron microscopy. Representative micrographs. Left panel – untreated cells. Right panels – post treatment with 600ng/mL BCT-100. HL60 post-treatment undergoing necrotic cell death. Monocytes and T cells display no features of cell death. Experiments performed on 3 separate occasions.
S5: BCT-100 does not activate apoptotic or autophagy pathways

a) Cell lines were cultured alone (A), PEG (B), or BCT-100 (C). Expression of PARP, Caspase 9, and Caspase 3 in whole cell lysates was determined by immunoblotting. Actin was used as the housekeeping gene to ensure equal loading. No significant cleavage of PARP, Caspase 9 or 3 is seen, indicating the apoptosis pathway was not activated. b) AML cell lines were treated with BCT-100 for 72 hours and stained with Annexin-PI. Percentages of Annexin and PI positive cells, compared to untreated controls, is shown, as tested by flow cytometry. No significant increase in Annexin+PI+ cells is seen, confirming the lack of apoptosis. Representative of 3 independent experiments c) AML patient blasts were treated with BCT-100 for 72 hours and stained with Annexin-PI. Percentages of Annexin and PI positive cells, compared to untreated controls, is shown, as tested by flow cytometry. No significant increase in Annexin+PI+ cells is seen, confirming the lack of apoptosis. One representative patient sample of 5. d) Cell lines were treated with BCT-100 in the presence or absence of bafilomycin. Whole cell lysates were tested for LC3-I and LC3-II turnover by immunoblotting at 0H, 24H, 48H, 72H of culture. No increase in conversion of LC3-I to LC3-II is seen, confirming the lack of autophagy induction above baseline.

S6: BCT-100 activity is not related to reactive oxygen species or arginine recycling enzyme expression

a) AML patient blasts were treated with BCT-100 for 72 hours and stained with DCFDA. No increase ROS species evidence by DCFDA staining is seen. Representative of 2 independent experiments b) ASS and OTC expression was determined by RT-PCR. Representative data from eleven patients are shown. GAPDH was used as the housekeeping gene to ensure equal loading. c) Relative expression of CAT 1 and CAT2B in BCT-100 treated AML cell lines or patient blasts compared to untreated controls were investigated by qPCR. Representative data of 4 cell lines and 7 patients shown.
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4
Supplementary Figure 5
Supplementary Figure 6