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Intracellular ara-CTP in circulating blasts post-treatment predicts remission status in patients with Acute Myeloid Leukaemia.

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Highlights

Intracellular ara-CTP is quantifiable in circulating AML blasts post-treatment

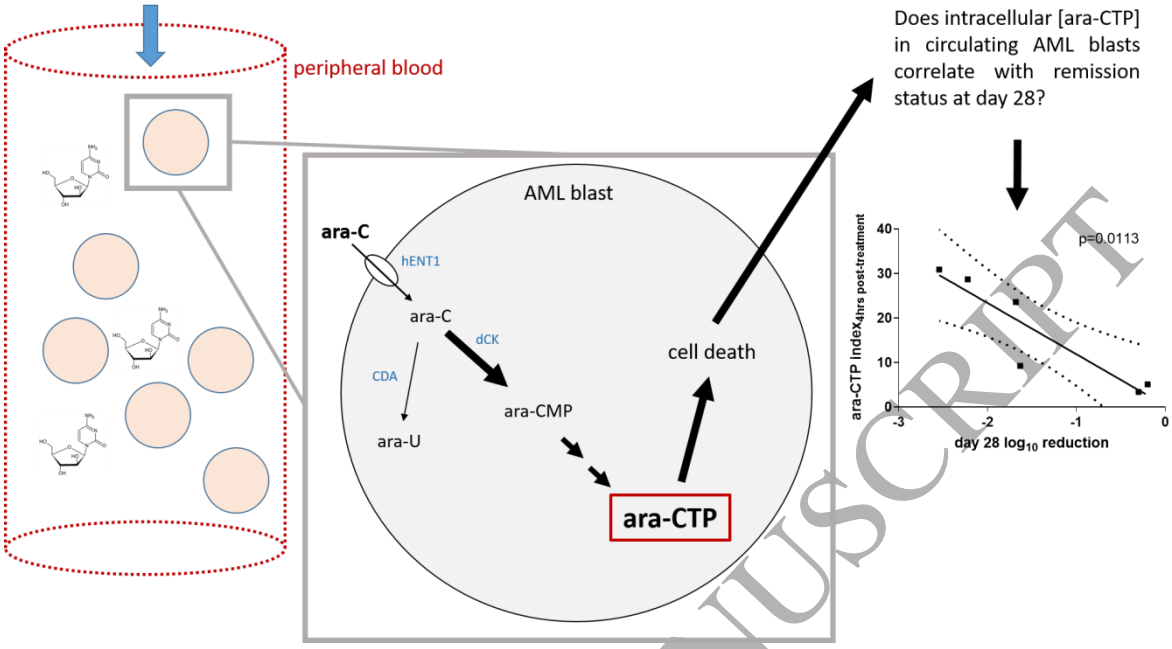
Intracellular ara-CTP 4 hours post-treatment correlates with day 28 burden decrease

Sampling for analysis can be integrated on to a busy haemato-oncology ward

Cytarabine remains the backbone of therapy in acute myeloid leukaemia (AML). The ability to assess intracellular ara-CTP levels in patients receiving cytarabine represents a major goal in the prediction of treatment response. This study, conducted within a clinical setting, aimed to assess ara-CTP levels in circulating peripheral blasts from non-M3 AML patients receiving cytarabine at one of three dosing levels, using a novel biosensor assay. Results from the initial 72 hours post-commencement were correlated with day 28 remission status, with feasibility parameters concurrently assessed.
Intracellular ara-CTP was detectable in *ex vivo* blasts post-treatment for standard-dose (SD) and high-dose (HD) patients ($p<0.05$), and quantification revealed a 27-fold increase in intracellular steady-state concentration between the two dosing levels. For low-dose cytarabine, high rates of patient discharge and low intracellular concentrations limited analysis; however, it was achievable to assess intracellular ara-CTP concentration in a dwindling population of blasts for SD and HD treatment cohorts, with 4 hours post-treatment commencement potentially being most predictive of clinical response (r=-0.912, $p=0.0113$). Concurrent assessment of peripheral leukaemia-associated immunophenotype (LAIP)- positive cells revealed a decline in burden (0-72 hours), which correlated with remission status ($p<0.05$).
Unexpectedly high rates of night sampling lead to challenges associated with sampling rates, but did not impact on patient compliance. Additional training of night staff improved feasibility substantially. Multiple peripheral sampling during the initial 72 hours of

treatment is feasible in newly diagnosed patients, and ara-CTP is detectable over the initial 24 hours, facilitating prediction of chemo-sensitivity of leukaemic blasts to cytarabine.

Graphical Abstract



Introduction

Prediction of response to therapy and risk stratification is a major goal in the early treatment of acute myeloid leukaemia (AML). Whilst complete remission (CR) rates have greatly improved in younger patients with AML, high relapse rates and poor disease-free survival still pose a significant challenge (Bose *et al*., 2017). The backbone of AML therapy is cytarabine (ara-C), given at one of three dosing levels, depending on stage of disease (induction or relapse) and patient fitness (intensive or non-intensive therapy). Due to rapid commencement of therapy after diagnosis, often prior to prognostic information being available, early assessment predicting response to cytarabine could be an important clinical decision-making tool. The relationship between intracellular ara-CTP accumulation and disease response has long been controversial, with some studies advocating a link (Rustum and Preisler, 1979; Karp *et al*., 1987) and others dismissing it (Koehl *et al*., 2007; Gruber *et al*., 1995). This single-centre, observational study used a previously validated biosensor technology (Anderson *et al*., 2014) to assess *in vivo* intracellular ara-CTP concentration in circulating blasts over the initial 72 hours post-treatment. This was used to predict remission status in AML patients (*n*=26) after treatment with low-, standard- and high-dose (LD, SD, HD) cytarabine.

Materials and Methods

Patient samples and dosing

Samples were gathered under informed consent (15/WM/0415) from non-M3 AML participants (≥18 years) receiving cytarabine therapy (*n*=26) (Table 1). Patients received one of three dosing levels as judged by the Consultant Haematologist according to current UK guidelines (SD: 200 mg/m^2 infused in divided doses twice daily; HD: 1.5-3 g/m^2 infused in divided doses twice daily; LD: 20 mg twice daily by subcutaneous injection) (Milligan *et al.*,

2006). Fresh peripheral blood samples (≤4 mL) were collected at pre-treatment (t=0) and post-treatment commencement (t=2, 4, 8, 12, 24, 48 and 72 hours ±30 mins), with standard-of-care assessment of remission/non-remission status (CR/NR) performed at day 28 as per current UK guidelines. Where a sampling time-point coincided with an infusion time-point, the blood sample was removed pre-infusion.

Isolation of primary AML blasts & lysate preparation

Peripheral blood mononuclear cells (PBMC) fractions were isolated from whole blood samples by density gradient centrifugation (Histopaque®-1077, Sigma-Aldrich, Gillingham, UK) within 1 hour of withdrawal. PBMCs were pelleted at 300*x*g (5 mins), washed in RPMI 1640 medium (10 mL), re-suspended in red cell lysis buffer (0.15 mM ammonium chloride, 0.01 mM potassium bicarbonate, 0.001 mM EDTA, pH7.2-7.4) for 5 mins, and washed in RPMI 1640 medium (without phenol red) (10 mL). Lysates were prepared and stored at -80°C until biosensor analysis as per Alloush *et al*. (2010).

Flow cytometry

Leukaemia-associated immunophenotype (LAIP)-positive absolute counts were determined on baseline peripheral blood, and every 24 hours thereafter for 72 hours. Clearance of peripheral blasts was calculated by conversion of daily blast count to logarithmic scale, and subtraction from baseline.

Preparation of ara-CTP standard curves

Ara-CTP stock solution (10 mM, Jena Biosciences, Jena, Germany) was diluted (0-0.5 μM) in cell lysate as per Alloush *et al*. (2010). Limits of detection (LOD) and quantitation (LOQ) were calculated from the standard deviation of the blank (*n*=6) as per Shrivastava *et al*. (2011). Results indistinguishable from background were reported as <LOD and removed from the analysis.

Statistical Analysis

Analysis performed used GraphPad Prism 7® (GraphPad, USA) including two-way ANOVA with Tukey's (Figure 1A) and Sidak's (Figure 2B) post-hoc tests, and analysis of co-variance (ANCOVA) of body mass index (BMI) versus sensitivity index (SI%). Linear regression of $\log_{10}$ reduction in day 28 bone marrow (BM) blast burden versus diagnostic BM (flow cytometry) compared to SI% values, with Pearson coefficient (*r*) calculated at each time-point assessable (SD cohort). Feasibility calculated as the percentage of samples successfully taken versus patients (*n*) in the group. Any patient suffering treatment-related mortality was removed from subsequent feasibility assessments.

	Regimen		
Patient	**LD**	**SD**	**HD**
n	7	13	6
Median age (years, range)	77 (63-79)	67 (47-76)	41 (22-55)
Gender			
Male	6	10	1
Female	1	3	5
Ethnicity			
White, British	6	11	4
White, non-British	0	0	2
not known	1	2	0
FAB			
M0-M1	3	2	2
M2	1	4	3
M4-5	0	3	1
M6-7	0	0	0
MDS/therapy-related MDS	2	1	0
Not known	1	3	0
Cytogenetic risk group			
Favourable	1	1	3
Intermediate	6	6	1
Adverse	0	5	2
Not known	0	1	0
Prior MDS (*n*)	4	3	0
Presenting WBC count ($x10^9$/L)	8.89 (2.26-88.4)	8.76 (0.63-118.52)	9.175 (2.98-24.48)
Dose (mg) (range)	20 (20-20)	200 (160-220)	4100 (3900-4600)

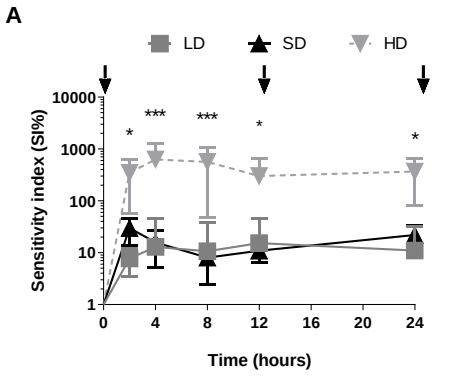
Relevant co-medication			
Daunorubicin		13/13	
Mylotarg		12/13	5/6
FLAG-IDA			6/6
Tosedostat	2/7		
Lenalidomide	2/7		
BSA (m^2) (median, range)	2.11 (1.74-2.26)	2.02 (1.64-2.22)	2.02 (1.74-2.31)
BM burden at presentation (%median, range)	24.7 (3.8-91.8)	42 (21.3-90.0)	65.3 (11.0-85.0)
Peripheral blasts at presentation (%median, range)	8.6 (0.2-97.1)	25.3 (0.7-88.0)	54.2 (30-78.4)
Day 28 BM burden (%median, range)	10.75 (0.1-72) [6]	0.90 (0.12-60) [12]	1.75 (0.4-2.5)[5]

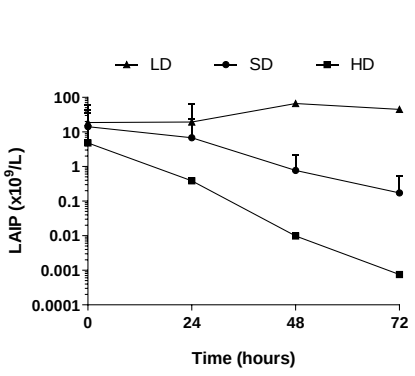
Table 1: Patient characteristics. LD, low-dose cytarabine; SD, standard-dose cytarabine; HD, high-dose cytarabine; FLAG-IDA, fludarabine, cytarabine, GM-CSF, idarubicin; FAB, French-American-British classification; WBC, white cell count; BM, bone marrow; MDS, myelodysplastic syndrome; BSA, body surface area.

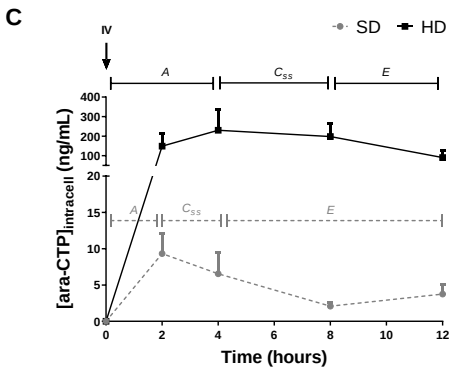
Results and Discussion

Measurement of circulating blast intracellular ara-CTP concentration post-treatment (expressed as SI%) was possible in all treatment groups over the initial 24 hours (Figure 1A), with the greatest discrimination observed between HD (max *n*=6) and SD (max *n*=11) patients ($p<0.05$ at all time-points collected). Peripheral LAIP counts were sufficient for ara-CTP analysis from a single vacutainer (4 mL, $\geq 2x10^6$ blasts) in all patient groups over the initial 24 hours (LD:100%; SD:80%; HD:77%) (Figure A.1A), with a dose-dependent decrease in LAIP over the initial 72 hours of therapy (Figure 1B). Limitations to assessment were: high rates of night-time sampling across all treatment groups; decreasing peripheral burden in HD patients (Figure 1B); a high proportion of LD patients with results below the limit of detection of the assay (4/7); and high rates of discharge of LD patients at 48 hours (1/7) and 72 hours (2/7) (Figure A.1A), as these patients can self-administer cytarabine once stabilised.

For a proportion of patients (SD: max *n*=9; HD: max *n*=5), it was possible to calculate the intracellular ara-CTP concentration (ng/mL) (Figure 1C) using a standard curve across a therapeutically-achievable ara-CTP range (0-500 nM) (Fig. A.1B). The LOD for the assay was 1.58 ng/mL, and the LOQ was 4.81 ng/mL. HD patients achieved C_{ss} of ara-CTP from t=4 hours post-infusion, with t_{max} at 4 hours, and 111-fold higher area-under-the-curve (AUC) compared to SD patients (Figure 1D). In comparison, SD patients achieved C_{ss} 2 hours earlier but with a less prolonged duration (HD, 4 hours; SD, 2 hours) and 27-fold lower concentration than HD patients (Figure 1C). These results show similar trends and timings to previous assessment of ara-CTP by HPLC-MS/MS (Liang *et al*., 2014), but required $1/10^{th}$ cell density necessary for HPLC analysis, albeit lower concentrations of ara-CTP (ng/mL) were observed herein. This represents the first quantitative analysis of post-treatment intracellular ara-CTP levels in AML patients using the biosensor, conducted within a working clinical environment.







D

	SD (*n*=9) mean ±95%CI	HD (*n*=6)* mean ±95%CI
Dose range (mg)	160-220	3900-4600
C_{ss} (ng/mL)	7.94 ±2.4	214.18 ±28.5
AUC (ng hr/mL)	94.23 ±163.2	10477.9 ±19950.7
C_{max} (ng/mL)	10.7 ±8.2	268.0 ±248.2
t_{max} (hours)	2	4

Fig. 1: (A) Assessment of intracellular ara-CTP (expressed as sensitivity index SI% on log_{10} axis) in patients undergoing three different regimens (SD max *n*=11, HD max *n*=6, LD max *n*=3) (for *n* per time-point see Figure A.2-4), (B) leukaemia-associated immunophenotype cells (LAIP) over time by dose assessed at 24, 48 and 72 hours post-commencement of treatment (SD max *n*=7, HD max *n*=2, LD max *n*=5), (C) peripheral blast intracellular ara-CTP concentration (ng/mL) by dosing regimen (SD max *n*=9, HD max *n*=6), (D) pharmacokinetic parameters over initial 12 hours post-commencement of infusion with ara-C. Arrowheads indicate timing of SD and HD dosing. Mean SI% and 95% CI are shown for each regimen throughout. SD versus HD: **p*<0.05, ****p*<0.001. LAIP, leukaemia-associated immunophenotype assessed by flow cytometry (% of total nucleated cells). C_{ss}, steady-state concentration; A, absorption; E, elimination; AUC, area under the curve; C_{max}, maximum concentration; T_{max}, time to maximum concentration. *HD $AUC_{0\text{-}12hrs}$ (*n*=5) (Figure A.3).

Initial analysis of prediction of outcome at day 28 versus ara-CTP accumulation was performed at each time-point for the SD cohort (Figure 2A-E). The most striking correlation was observed at t=4 hours post-treatment commencement, where a higher SI% correlated with day 28 log_{10} bone marrow blast reduction (Figure 2A) (*n*=6, *p*=0.0113). Correlation was only possible in 6/7 patients at this time-point as one patient was not assessable for burden at day 28 (Figure A.2). A significant relationship was notable at t=8 hours (*n*=4, *p*=0.0280) (Figure 2C), however the *n*-value limits interpretation. No significant correlation was observed with t=2 (Figure 2A) or t=12 hours post-treatment commencement SI% (Figure 2D) or $AUC_{0\text{-}12\ hours}$ (Figure 2E). For any future study, infusion time +4 hours may derive the most predictive time-point as the majority of SD patients showed declining ara-CTP levels after the 4 hour time-point (Figure 1C). Concurrent LAIP assessment (0-72 hours) versus day 28 outcome performed in SD patients (*n*=7) (Figure 2F) replicated previous findings from day 14 outcome correlation versus circulating blast burden decline over the initial days post-

treatment (Gianfaldoni *et al*., 2006; Elliott *et al*., 2007). Herein, non-standard flow sampling (t=24, 48 & 72 hours) suffered <50% mean completion rate (Figure A.1A), limiting correlation notably for HD and LD cohorts. Intervention with additional training of night-staff improved the completion rate substantially after interim identification of the issue (0% versus 62.5%).

Recruitment exceeded targets in all treatment groups (Figure A.5A), with no patients electing to withdraw from the study, however sampling was dependent on both time of treatment start (Figure A.5B), and consequently the proportion of sampling by day- versus night-staff (Figure A.5C). As expected, LD patients showed the highest proportion of start times between 7am-7pm, as advance notice of treatment commencement was possible in this group (typically 1 week). Unexpectedly, the majority of SD and HD patients commenced treatment between 7pm-1am (~50%), with a significant proportion starting between 1am-7am (SD:8%; HD:16%). This in turn led to a high rate of night sampling in these treatment groups (Figure A.5C), and challenges associated with training and continuity of night-staff. As well as affecting sampling rates for flow cytometry, the presence of analytical staff frequently prompted sampling by clinical night-staff, potentially inflating the true feasibility of peripheral sampling at night. A further study investigating key time-points is planned, benefiting from the feasibility observations reported herein.

Interestingly high median body surface area (BSA) of patients was noted in this study (Table 1), with BMI indicating a large proportion of patients in the overweight or obese categories (Figure A.5D). Whilst no significant association was observed between BMI and SI% herein, a higher BMI was associated with lower initial intracellular ara-CTP accumulation in SD patients (p=0.0169, n=10). No association was observed in LD (n=3) or HD (n=6) cohorts. This is an interesting finding given the argument for dose-capping in obese patients (Tavitian *et al*., 2016).

Inter-individual variation in ara-CTP levels has previously been identified as attributing to clinical variability in response (Lamba, 2009). Previous groups have attempted to retrospectively predict response using expression analysis of key genes (e.g. hENT1, deoxycytidine kinase) involved in cytarabine uptake, metabolism and mitochondrial signalling (Lamba *et al*., 2011, Abraham *et al*., 2015, Yan *et al*., 2017). Whilst valuable prediction tools, a phenotypic assessment of post-treatment ara-CTP accumulation, incorporating systemic and intracellular handling of cytarabine, could allow more rapid and specific treatment tailoring, identifying both those at risk of adverse reaction, and treatment failure.

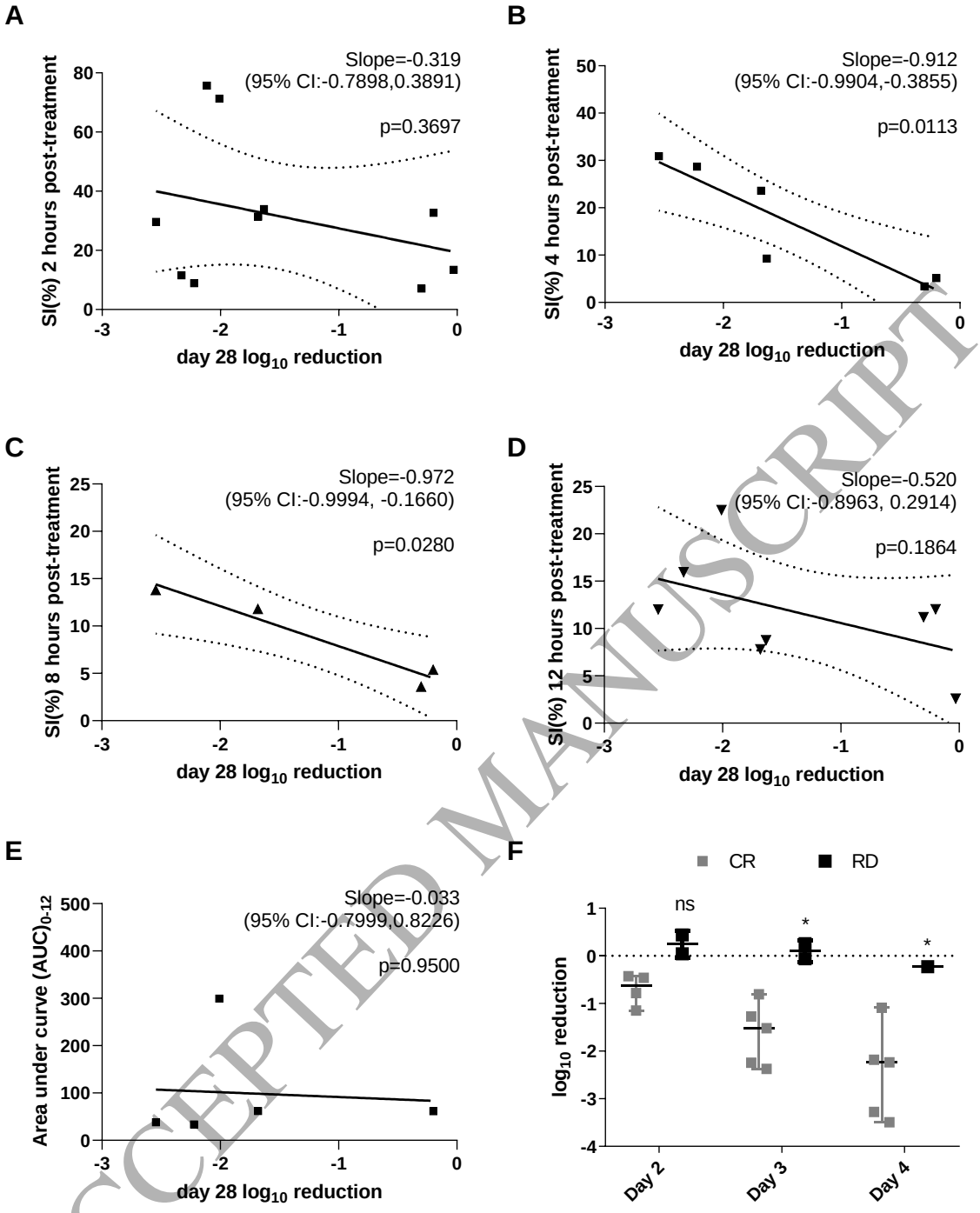


Fig. 2: (A) Correlation between day 28 bone marrow log_{10} reduction and post-treatment commencement intracellular ara-CTP (expressed as sensitivity index SI%) at t=2 (*n*=10) (A), t=4 (*n*=6) (B), t=8 hour (*n*=4) (C), t=12 hour (*n*=8) (D) and area under the curve 0-12 hours (AUC) (*n*=5) (E), and (F) peripheral blast log_{10} reduction per treatment day in day 28 complete remission (CR) (*n*=5) and residual disease (RD) patients (*n*=2). SI% calculated as per Alloush *et al*. (2010). Peripheral blast burden assessed by flow cytometry every 24 hours post commencement of treatment. Day 28 log_{10} reduction calculated versus diagnostic flow sample. Mean and 95% CI are shown, ns, not significant; *p<0.05.

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Authorship contributions

EA, AP, VS, RP & PM designed the research. EA, BR, EF, JH[1] & JH[2] performed the research. EA, RG & PM analysed the data. EA wrote the paper.

Conflicts of interest

This work was funded by a charitable grant from Above and Beyond, Bristol.

Abbreviations: ara-C, cytosine arabinoside/cytarabine; ara-CTP, cytosine arabinoside triphosphate; SI%, sensitivity index; hENT, human equilibrative nucleoside transporter; CR, complete remission (day 28); RD, residual disease; C_{ss}, steady-state concentration; AUC, area-under-the-curve; A, absorption; E, elimination; LD, low-dose cytarabine; SD, standard-dose cytarabine; HD, high-dose cytarabine; LAIP, leukaemia-associated immunophenotype.