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BRIEF COMMUNICATION

Intracellular cytarabine triphosphate in circulating blasts post-treatment predicts remission status in patients with acute myeloid leukemia

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Cytarabine remains the backbone of therapy in acute myeloid leukemia (AML). The ability to assess intracellular cytarabine triphosphate (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, assessment of intracellular ara-CTP concentration was achievable 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 leukemia-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 led to challenges associated with sampling rates, but did not have an 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 chemosensitivity of leukemic blasts to cytarabine. © 2019 Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells.

Prediction of response to therapy and risk stratification is a major goal in the early treatment of acute myeloid leukemia (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 [1]. 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). Because of the rapid commencement of therapy after diagnosis, often prior to prognostic information being available, early

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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 [2,3] and others dismissing it [4,5]. This single-centre, observational study used a previously validated biosensor technology [6] to assess 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.

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 hematologist 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) [7]. Fresh peripheral blood samples (≤4 mL) were collected pre-treatment ($t = 0$) and post-treatment (t=2, 4, 8, 12, 24, 48, and 72 hours ± 30 min) commencement, 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 and 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*g* (5 min), washed in RPMI-1640 medium (10 mL), re-suspended in red cell lysis buffer (0.15 mmol/L ammonium chloride, 0.01 mmol/L potassium bicarbonate, 0.001 mmol/L

Table 1. Patient characteristics

Characteristic	Cytarabine regimen		
	Low dose	Standard dose	High dose
n	7	13	6
Median age, y (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			
M0–1	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			
Favorable	1	1	3
Intermediate	6	6	1
Adverse	0	5	2
Not known	0	1	0
Prior MDS	4	3	0
Presenting WBC count, × 10^9/L	8.89 (2.26–88.4)	8.76 (0.63–118.52)	9.175 (2.98–24.48)
Dose, mg	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		
Body surface area, m^2	2.11 (1.74–2.26)	2.02 (1.64–2.22)	2.02 (1.74–2.31)
Bone marrow burden at presentation, %	24.7 (3.8–91.8)	42 (21.3–90.0)	65.3 (11.0–85.0)
Peripheral blasts at presentation, %	8.6 (0.2–97.1)	25.3 (0.7–88.0)	54.2 (30–78.4)
Day 28 bone marrow burden, %	10.75 (0.1–72) [6]	0.90 (0.12–60) [12]	1.75 (0.4–2.5) [5]

Values are expressed as number of patients or median (range).

FLAG-IDA=fludarabine. cytarabine, granulocyte–macrophage colony-stimulating factor, idarubicin; FAB=French–American–British classification; WBC, white blood cell; MDS=myelodysplastic syndrome.

EDTA, pH 7.2–7.4) for 5 min, 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. [8].

Flow cytometry

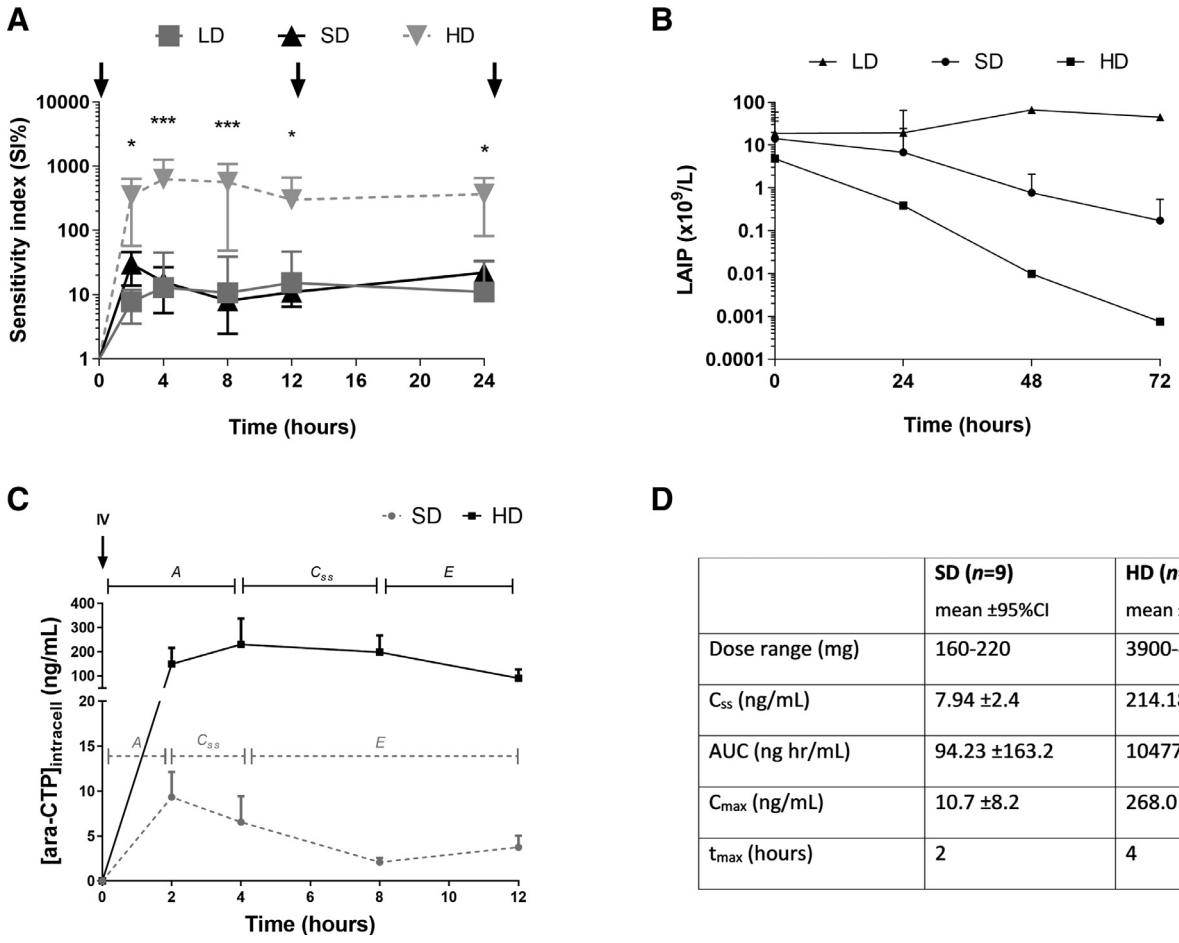
leukemia-associated immunophenotype (LAIP)-positive absolute counts were determined on baseline peripheral blood and every 24 hours thereafter for 72 hours. Clearance of peripheral blasts was calculated by conversion of daily blast count to logarithmic scale, and subtraction from baseline.

Preparation of ara-CTP standard curves

Ara-CTP stock solution (10 mmol/L, Jena Biosciences, Jena, Germany) was diluted (0–0.5 μmol/L) in cell lysate as per Alloush et al. [8]. Limits of detection (LOD) and quantitation (LOQ) were calculated from the standard deviation of the blank ($n = 6$) as per Shrivastava et al. [9]. Results indistinguishable from background were reported as <LOD and removed from the analysis.

Statistical analysis

Analysis performed used GraphPad Prism 7 (GraphPad, La Jolla, CA, USA) including two-way analysis of variance (ANOVA) with Tukey's (Figure 1A) and Sidak's 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) was compared with SI% values, with the Pearson coefficient (r) calculated at each time point assessable (SD cohort). Feasibility was 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.



	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

Figure 1. Assessment of **(A)** 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 Supplementary Figures E2–E4); **(B)** leukemia-associated immunophenotype (LAIP) cells 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); and **(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% confidence interval are shown for each regimen throughout. SD versus HD: *$p < 0.05$, ***$p < 0.001$. LAIP=leukemia-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_{0-12h} ($n = 5$) (Supplementary Figure E3).

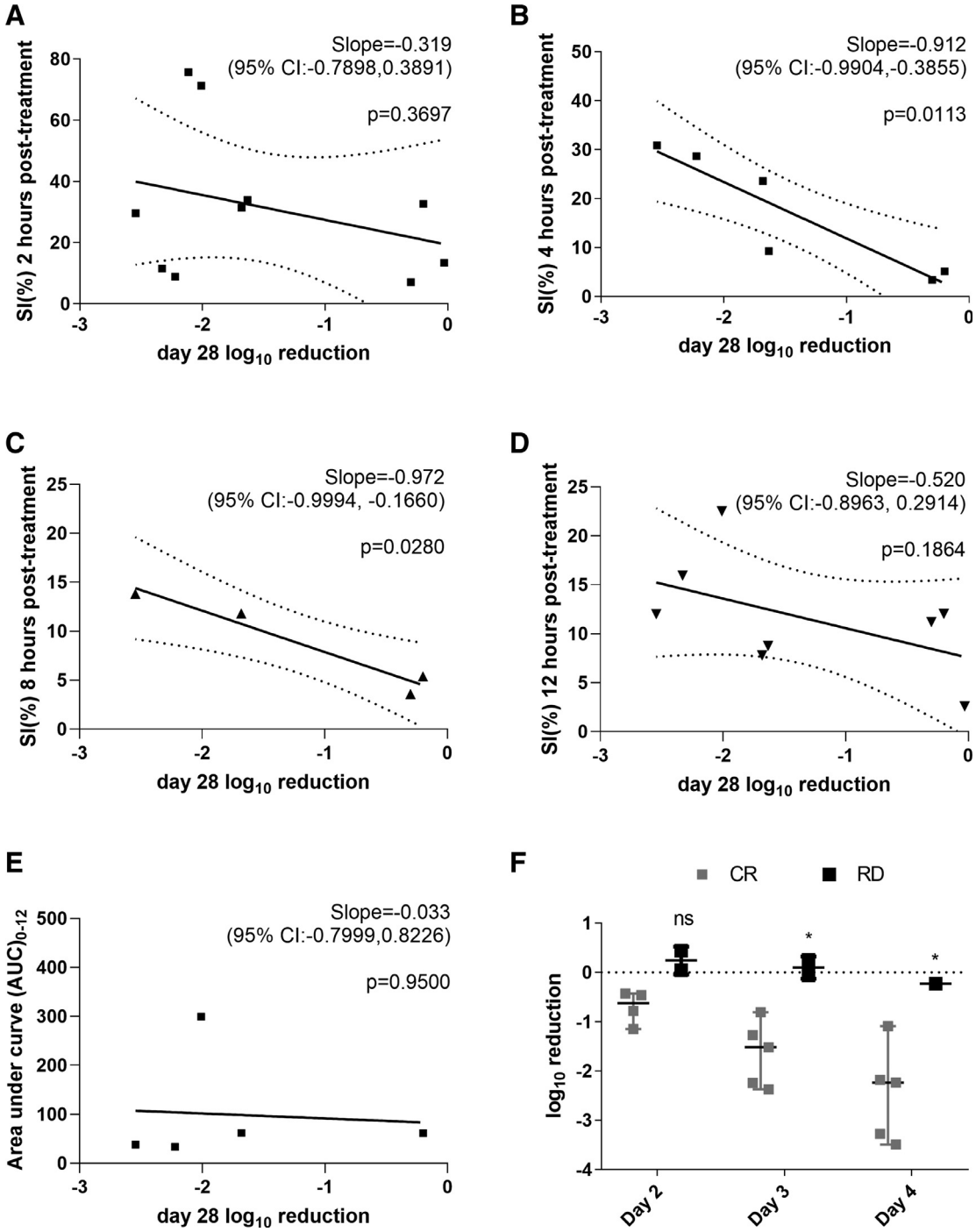


Figure 2. Correlation between day 28 bone marrow $\log_{10}$ reduction and post-treatment commencement intracellular ara-CTP (expressed as sensitivity index SI%) at $t = 2$ hours ($n = 10$) **(A)**, $t = 4$ hours ($n = 6$) **(B)**, $t = 8$ hours ($n = 4$) **(C)**, and $t = 12$ hours ($n = 8$) **(D).** Area under the curve at 0–12 hours (AUC) ($n = 5$) **(E)**. Peripheral blast $\log_{10}$ reduction per treatment day in day 28 complete remission (CR) ($n = 5$) and residual disease (RD) ($n = 2$) patients. SI% calculated as per Alloush et al. [8]. 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% confidence interval are shown. ns=not significant. *$p < 0.05$.

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, $\geq 2 \times 10^6$ blasts) in all patient groups over the initial 24 hours (LD: 100%, SD: 80%, HD: 77%) (Supplementary Figure E1A, online only, available at www.exphem.org), with a dose-dependent decrease in LAIP over the initial 72 hours of therapy (Figure 1B). Limitations to assessment were high rates of nighttime 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) (Supplementary Figure E1A), as these patients can self-administer cytarabine once stabilized.

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 nmol/L) (Supplementary Fig. E1B). The LOD for the assay was 1.58 ng/mL, and the LOQ was 4.81 ng/mL. HD patients achieved the C_{ss} of ara-CTP from $t = 4$ hours post-infusion, with a t_{max} at 4 hours, and a 111-fold higher area under the curve (AUC) compared with SD patients (Figure 1D). In comparison, SD patients achieved the 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 indicate similar trends and timings to previous assessment of ara-CTP by HPLC-MS/MS [10], but required 1/10th the 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.

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 possible in only 6 of 7 patients at this time point, as 1 patient was not assessable for burden at day 28 (Supplementary Figure E2, online only, available at www.exphem.org). 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 $AUC_{0-12\ hours}$ (Figure 2E). For any future study, infusion time +4 hours may derive the most predictive time point as the majority of SD patients exhibited 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 [11,12]. Herein, non-standard flow sampling ($t = 24$, 48, and 72 hours) had a <50% mean completion rate (Supplementary Figure E1A), 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% vs. 62.5%).

Recruitment exceeded targets in all treatment groups (Supplementary Figure E5A, online only, available at www.exphem.org), with no patients electing to withdraw from the study; however, sampling was dependent on both time of treatment start (Supplementary Figure E5B) and, consequently, the proportion of sampling by day versus night staff (Supplementary Figure E5C). As expected, LD patients showed the highest proportion of start times between 7 AM and 7 PM, 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 7 PM and 1 AM (~50%), with a significant proportion starting between 1 and 7 AM (SD: 8%, HD: 16%). This, in turn, led to a high rate of night sampling in these treatment groups (Supplementary Figure E5C) 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 body mass index (BMI) indicating a large proportion of patients in the overweight or obese category (Supplementary Figure E5D). 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 the LD ($n = 3$) or HD ($n = 6$) cohort. This is an interesting finding given the argument for dose capping in obese patients [13].

Inter-individual variation in ara-CTP levels has previously been identified as contributing to clinical variability in response [14]. 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 [15–17]. While 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 those at risk of both adverse reaction and treatment failure.

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Conflict of interest disclosure

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

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A

Time post-treatment commencement (hrs)	Regimen	Lysate	Haematology	Clinical chemistry	LAIP (flow cytometry)
	LD	100	100	88	63
	SD	77	100	100	46
24	HD	80	100	100	40
	LD	57	88	88	25
	SD	77	100	100	46
48	HD	20	100	100	40
	LD	43	75	75	25
	SD	54	100	100	38
72	HD	20	100	100	40
	LD		100	100	88
	SD		92	92	92
day 28	HD		100	100	100

B

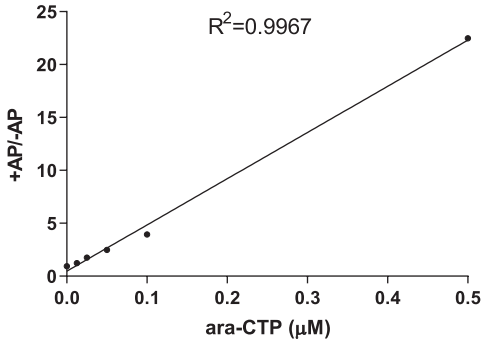

Figure E.1. (A) Feasibility of standard-of care and LAIP assessments per time-point (as % of total cohort), and (B) example ara-CTP standard curve for biosensor HA1. (A) Haematology, clinical chemistry and day 28 flow cytometry were standard-of-care. LAIP, leukaemia-associated immunophenotype assessed by flow cytometry (% of total nucleated cells). (B) Ara-CTP standard curve produced as per Methods section (equivalent to 0-242 ng/mL).

	Lysate produced (1) or no lysate produced (0)							Quantitation	Flow cytometry (day 0 vs 28)
Patient ID	t=2	t=4	t=8	t=12	t=24	t=48	t=72	AUC	log10 reduction
2	1	1	1	1	1	1	0	1	1
3	1*	1*	1*	1*	1	1*	1*	0	1
7	1	1	0	0	0	0	0	1	1
10	1	1	1 (<LOD)	1	1	1	0	curve failed	1
11	1	1	1	1	1	1	1	1	0
13	1	1	1	1	1	1	1	1	1
14	1	0	0	1	1	1	0	0	1
16	0	0	0	0	0	0	0	0	1
17	1	1	1	1	0	0	0	1	1
19	1	1 (<LOD)	1 (<LOD)	1	1	1 (<LOD)	1 (<LOD)	0	1
21	1	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	0	1
22	1	0	1 (<LOD)	1	1	1	1	1	1
23	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	0	1
n (lysate)	12	10	10	11	10	10	7		
Feasibility (%)	92	77	77	85	77	77	54		
n (lysate SI%>LOD)	11	7	5	9	8	7	6		
n (correlation)	10	6	4	8	7	6	5	5	12
n (quantitation)	9	5	4	7					

Figure E.2. Feasibility data (standard dose patients). 1, lysate produced; 0, no lysate produced; <LOD, below limit of detection; *below limit of quantitation.

	Lysate produced (1) or no lysate produced (0)							Quantitation	Flow cytometry (day 0 vs 28)
Patient ID	t=2	t=4	t=8	t=12	t=24	t=48	t=72	AUC	log10 reduction
5	1	1	1	1	1	0	0	1	1
8	1	1	1	1	w.d.	w.d.	w.d.	1	0
9	1	1	1	0	0	0	0	n.c.	1
15	1	1	1	1	1	1	1	1	1
18	1	1	1	1	1	0	0	1	1
20	1	1	1	1	1	0	0	1	1
n (lysate)	6	6	6	5	4	1	1		
Feasibility (%)	100	100	100	83	80	20	20		
n (lysate SI%>LOD)	6	6	6	5	4	1	1		
n (correlation)	5	5	5	4	4	1	1	5	5
n (quantitation)	6	6	6	5					

Figure E.3. Feasibility data (high dose patients). 1, lysate produced; 0, no lysate produced; <LOD, below limit of detection; n.c., not calculable (no 12 hour result); w.d., withdrawn/no longer able to verbally consent (removed from feasibility assessment).

		Lysate produced (1) or no lysate produced (0)							Quantitation	Flow cytometry (day 0 vs 28)
Patient ID	Diagnosis	t=2	t=4	t=8	t=12	t=24	t=48	t=72	AUC	log10 reduction
1	AML	1	1	1	0	1	1 (<LOD)	0	0	1
6	MDS	1	1	1	1	1	1	1	0	0
24	AML	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	0	0	0	1
25	AML	1	1	1	1	1	0	1	0	1
26	MDS	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	0*	0*	0	1
27	AML	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	0	1
28	AML	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	1 (<LOD)	0*	0	1
n (lysate)		7	7	7	6	7	4	3		
Feasibility (%)		100	100	100	86	100	57	43		
n (lysate SI%>LOD)		3	3	3	2	3	1	2		
n (correlation)		2	2	2	1	2	0	1	0	6

Figure E.4. Feasibility data (low dose patients). 1, lysate produced; 0, no lysate produced; <LOD, result below limit of detection; *patient discharged from hospital; MDS, myelodysplastic syndrome; AML, acute myeloid leukaemia.

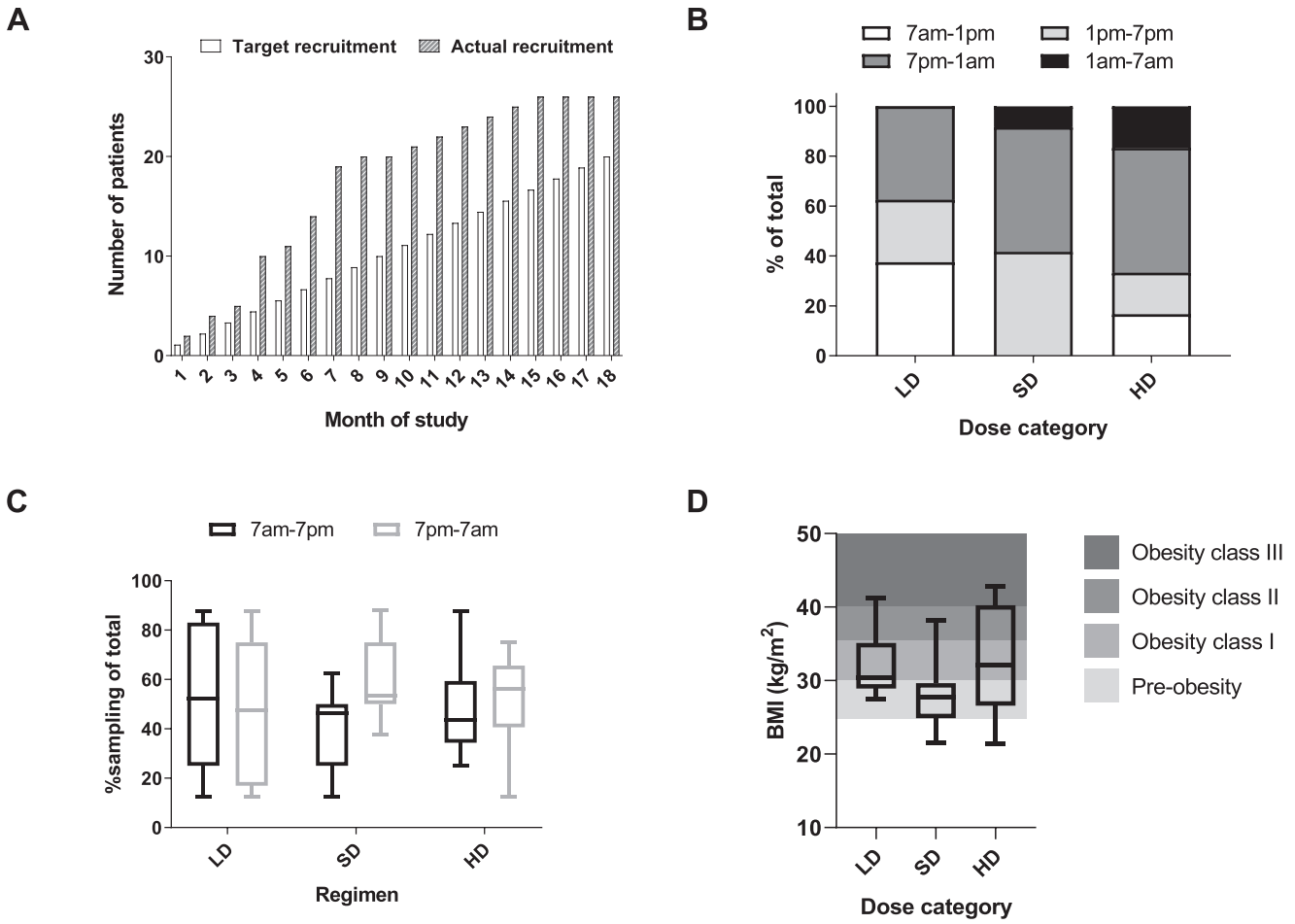

Figure E.5. (A) Patient recruitment to multiple peripheral sampling study (n=26), (B) treatment commencement time, (C) percentage of recruited patients sampled by time-point (LD n=7, SD n=13, HD n=6), and (D) body mass index (BMI) of patient cohorts against WHO criteria for obesity. (B) Time of therapy commencement as percentage of total cohort per dose category. (C) Patients treated with 20 mg/m^2 (LD), 200 mg/m^2 (SD) or 1.5 g/m^2 (HD) cytarabine-containing regimens were sampled at t=0, 2, 4, 8, 12, 24, 48 and 72 hours post–treatment commencement. Error bars represent range. (D) BMI classification as described by Poynter *et al*. (2016).