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A novel bioluminescent bacterial biosensor for measurement of Ara-CTP and cytarabine potentiation by fludarabine in seven leukaemic cell lines

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ABSTRACT

This study evaluates an *in vitro* biosensor assay capable of detecting the intracellular levels of the tri-phosphorylated form of cytarabine (Ara-CTP) within one working day. The biosensor predicted the response of seven leukaemic cell lines with varying known sensitivities to cytarabine alone and in combination with fludarabine. High-performance liquid chromatography (HPLC), 3-day assessment of cellular viable mass, and flow cytometric assessment of apoptosis were used to validate biosensor performance. A correlation between the biosensor results and Ara-CTP quantitation by HPLC was confirmed ($R = 0.972$). The biosensor was also capable of detecting enhanced accumulation of Ara-CTP following sequential pre-treatment of leukaemic cells with cytarabine ± fludarabine.

1. Introduction

The nucleoside analogue cytarabine (Ara-C), an effective and widely used agent for the treatment of acute myeloid leukaemia (AML), is sometimes used as a single agent in varying doses (low dose 20 mg/m^2/day, standard dose 200 mg/m^2/day or high dose 1.5–3 g/m^2/day) or in combination with other agents such as fludarabine and anthracyclines [1]. Although standard regimens including cytarabine induce high rates of initial response, relapse is prevalent with only 70% of newly diagnosed patients achieving a durable complete remission [2,3]. Accordingly, there is considerable interest in understanding the mechanisms of resistance to cytarabine and a validated same-day predictive test could be beneficial in selecting the most effective chemotherapeutic approach.

The cytotoxic effect of cytarabine is mainly mediated *via* its tri-phosphorylated metabolite Ara-CTP through its interference with DNA polymerase α and incorporation into DNA strands leading to chain termination and synthesis arrest. Its secondary effect involves free radical generation and induction of apoptosis [4–6]. *In vivo*, the deoxycytidine analogue cytarabine is transported into the cell *via* the human equilibrative nucleoside transporter 1 (hENT1) where it is rapidly phosphorylated by deoxycytidine kinase (dCK) to its monophosphate form (the rate-limiting step).

Ara-CMP is further phosphorylated by nucleoside kinases into its active tri-phosphorylated form, Ara-CTP [7]. Previous studies have suggested that reduced intracellular concentrations of Ara-CTP are associated with clinical resistance to cytarabine [8,9] and that intracellular Ara-CTP levels correlate with clinically observed therapeutic efficacy following treatment with high-dose cytarabine [10–15]. Cytarabine resistance has been attributed to a number of factors including, impaired entry *via* hENT1, failure of Ara-CTP formation and incorporation into DNA [16–18]. A further mechanism is the action of leukaemic cytidine deaminase (cdd) in the conversion of cytarabine to Ara-uracil and thus diversion of the agent into the RNA pool where it is harmless [19].

This study used a bioluminescent, pyrimidine-requiring, *cdd*-deficient mutant of *Escherichia coli* (*E. coli*) with enhanced sensitivity to cytarabine. The *E. coli* construct has previously been described in detail and used in an assay developed by this group to determine uptake and phosphorylation of cytarabine by leukaemic cells [20]. Light emission from bioluminescent bacteria results from the activity of the bacterial luciferase enzyme, which catalyses the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde in the presence of O_2 to produce FMN

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and acid with the emission of blue-green light. The high metabolic rate of bacterial cells compared to mammalian cells allows for fast, real-time toxicity testing. Light output from bioluminescent biosensors can be accurately measured, with no background interference, using luminometers or low-light cameras. In this study, seven leukaemic cell lines with well-established sensitivities to cytarabine, were treated with cytarabine as single agent or in combination with fludarabine to assess *in vitro* response [21]. In order to validate the biosensor, assay findings were correlated with measurement of intracellular concentration of Ara-CTP by reverse phase high performance liquid chromatography analysis (HPLC), APO2.7 staining of apoptotic cells and assessment of cell death using a commercially available 3-day *in vitro* assay for assessment of cellular viable mass.

2. Materials and methods

2.1. Cell lines

AML cell lines (K562, MV4-11, HEL, KG-1a, HL-60 and THP-1) and the T-ALL cell line (CCRF-CEM) supplied by the Health Protection Agency (HPA) or American Type Culture Collection (ATCC) were maintained as recommended in RPMI 1640 medium containing L-glutamine (2 mM) and foetal calf serum (FCS) (10%) at 37 °C with 5% CO_2. Culture reagents were purchased from Invitrogen (UK) except FCS which was purchased from Biosera (UK). Cells were sub-cultured every 3 days to maintain exponential growth and testing was carried out between passages 5 and 15 from purchase.

2.2. Biosensor assay protocol

The biosensor assay is described in detail elsewhere [20]. Briefly, 4×10^6 cells at a concentration of 2×10^6 cells/ml were incubated in RPMI 1640 for 30 min in the presence or absence of cytarabine (0–50 μM). Stock solution of cytarabine (10 mM), dissolved in sterile deionised water, was diluted in culture medium to yield doses of 0.1–50 μM. Stock solution of fludarabine (5 mM), prepared in DMSO was diluted 1:1000 in culture medium to yield a dose of 5 μM with 0.1% DMSO. Relevant diluent controls were included throughout. Cells were exposed to cytarabine alone at an equivalent clinical dose of 200 mg/m^2 in culture (25 μM) for 30 min or pre-dosed for 4 h with a clinically relevant dose of fludarabine (5 μM) equivalent to 25 mg/m^2, prior to exposure to cytarabine.

Following exposure to drugs or control conditions, cells were washed in RPMI and lysed using saponin (0.1%) in the presence of EDTA (1.5 mM). Lysate was then mixed with the biosensor [20] in the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM) with or without the addition of bovine intestinal mucosa alkaline phosphatase (AP) (1 unit per 200 μl reaction volume). Reagents and drugs were purchased from Sigma–Aldrich (UK).

Bioluminescence from the biosensor following exposure to lysates was measured every 15 min for 15 h in a luminometer (Lumistar, BMG Labtech). Results represent three separate experiments each performed in triplicate and are expressed as mean ± standard deviation ($n = 3$). Peak bioluminescence was noted consistently at 5.25 h. Addition of AP to the sample allowed conversion of any Ara-CTP produced by cells back to cytarabine that could then enter the biosensor. Concurrent analysis of an AP negative control confirms adequate washing steps. The diagnostic discriminator was the maximal difference in light output between the plus AP and minus AP curves that is representative of the level of Ara-CTP generated within the cells. This was expressed as a percentage sensitivity index (SI-value) using a formula that has provided a consistent range for samples in our previous study [20]:

$$\text{Biosensor response (SI\%)} = \left[\left(\frac{+\text{AP}}{-\text{AP}} \right)_{25\,\mu\text{M}} - \left(\frac{+\text{AP}}{-\text{AP}} \right)_{0\,\mu\text{M}} \right] \times 100$$

As the rate-limiting step in Ara-CTP formation is conversion of cytarabine to Ara-CMP [22], the assay assumes that all mono- and di-phosphorylated cytarabine would be converted to Ara-CTP, causing DNA chain termination and synthesis arrest following a protracted incubation [8]. SI% results from the biosensor were back-calculated to produce Ara-CTP concentrations (nM) using a 5-point calibration curve (data not shown) produced by spiking known amounts of Ara-CTP (Jena Bioscience, Germany) into untreated lysate.

2.3. 3-Day CellTiterGlo™ assay

A commercially available 3-day assay, CellTiterGlo™ (Promega, UK) was used to measure ATP generation by viable cells following exposure to cytarabine. Cells (2×10^4 per well) were cultured in the presence or absence of cytarabine (0–50 μM). Drug exposure for 72 h was determined as optimal in this assay (data not shown). ATP levels were measured using a Lumistar™ luminometer (BMG Labtech, Aylesbury, UK). Light units were used in the calculation of the viable mass (ATP%), with results expressed as percentage of treated over control. Results represent three separate experiments each performed in triplicate and are expressed as mean ± standard deviation ($n = 3$). LC_{50} values (concentration of a given agent that is lethal to 50% of the cells) were calculated from the dose response data ($n = 3$).

2.4. APO2.7 assessment of cytarabine cytotoxicity

Apoptosis was assessed flow cytometrically using phycoerythrin-conjugated APO2.7 monoclonal antibody (Beckman Coulter, UK). APO2.7 detects a mitochondrial protein, 7A6 that is expressed in early apoptosis [23]. Optimal 7A6 expression and commitment to apoptosis was observed after 48 h incubation with cytarabine (25 μM). Following incubation, 1×10^6 cells were fixed using 1% paraformaldehyde [23] and permeabilised using digitonin (100 μg/ml), which maintains mitochondrial integrity [24]. Results obtained using a BD Accuri C6 cytometer™ were expressed as the difference in percentage of positivity compared to negative controls. Samples were concurrently analysed using a Genetix CellReporter™.

2.5. HPLC assessment of intracellular Ara-CTP concentration

In order to detect Ara-CTP produced by leukaemic cells using HPLC, lysate prepared from 2×10^7 cells was required, which represents a 10-fold higher cell concentration than the biosensor assay. Cells were incubated with cytarabine (25 μM) with/without pre-exposure to fludarabine (5 μM) as described, and lysates prepared. Lysates were incubated with concentrated perchloric acid for 10 min on ice to remove protein and high molecular weight molecules. Clarified lysates were subsequently neutralised with potassium carbonate (5 M) and the pH adjusted to 6.0, and stored on ice prior to analysis.

HPLC analysis was performed as described by Ahlmann et al. [25]. Briefly, a reverse phase C18 column, 5 μm, 4.6 mm × 150 mm (Agilent, UK) fitted with a C18 pre-column (Phenomenex, USA) was equilibrated with mobile phase containing 0.09 M phosphate buffer, 0.01 M tetrabutyl ammonium hydrogen sulphate, 0.8% acetonitrile, pH6.0. Clarified lysate samples (100 μl) were injected onto the column and separation conducted with a flow rate of 1 ml/min. Eluted Ara-CTP was detected using a 2489 UV/vis detector (Waters, Ireland) at 270 nm. The limit of detection was 50 nm Ara-CTP. To avoid a matrix effect and due to the lack of a suitable internal standard, an untreated lysate was spiked with a known concentration of Ara-CTP and analysed subsequently with each test sample to serve as a suitable standard.

2.6. Statistical interpretation

Spearman's rank correlation was performed on results using cytarabine (25 μM) across the four assay types (GraphPad Prism 5®) (significance determined as $p < 0.05$). Dose response analysis was performed for cell lines treated with a range of cytarabine concentrations (0–50 μM) using one-way ANOVA with Bonferroni's *post hoc* test ($p < 0.05$). LC_{50} values were calculated using log (inhibitor) *versus* response curves (three parameters) with a Hill Slope of −1.0 (GraphPad Prism 5®).

3. Results

3.1. Comparison of detection of Ara-CTP production by the biosensor assay and HPLC

Table 1 compares levels of Ara-CTP in lysates detected using HPLC with sensitivity index values (SI%) from the biosensor assay following 30 min exposure to cytarabine (25 μM). Both measurements were carried out on the same lysate samples, however lysates were diluted 1 in 10 prior to biosensor analysis. The most sensitive cell line CCRF-CEM (T-ALL) assessed by both methods, produced 644 nM of Ara-CTP on HPLC testing and an SI-value of 97% (equivalent to 210 nM) with the biosensor. Of the three cell lines least capable of generating Ara-CTP, K562 and THP-1 produced 62 and 54 nM Ara-CTP when measured by HPLC, and SI-values of 32 and 35% (equivalent concentrations of 70 and 76 nM) with the biosensor. Ara-CTP production by MV4-11 cells was below the lower limit of detection of the HPLC system (<50 nM) and the biosensor (25 nM). Results from HPLC and biosensor analysis correlated statistically across the seven cell lines between the two methods of analysis ($R = 0.9722$, $p = 0.0028$) indicating that the biosensor might be capable of determining relative sensitivity to cytarabine.

Table 1
Comparison of Ara-CTP quantitation by biosensor assay and HPLC (Fig. S1) compared with LC_{50} assessment and percentage apoptosis following treatment of leukaemic cell lines with cytarabine (25 μM). Incubation times and cell densities were optimised for each assay type. Results represent mean ± standard deviation for duplicate (HPLC) and triplicate analysis (biosensor assay, CellTiterGlo™ and flow cytometry). LC_{50} values were calculated based on a 5-point dose response curve for cytarabine (0–50 μM) with 95% confidence intervals in brackets. Flow cytometry results represent the percentage of cells expressing the apoptotic marker APO2.7 following cytarabine treatment (following subtraction of untreated cell percentage) (Fig. S4).

Method	HPLC	Biosensor assay	CellTiterGlo™ assay	Flow cytometry
Analysis parameter	Ara-CTP (nM)	Sensitivity index (%)	LC_{50} (nM)	% Apoptotic (treated minus control)
Incubation time (h)	0.5	0.5	72	48
Cell density	2×10^7/ml	2×10^6/ml	2×10^5/ml	1×10^6/ml
Cell line				
CCRF-CEM	644 ± 5.7	97 ± 2.0	156 (5–4750)	46.6 ± 8.5
KG-1a	314 ± 11.7	91 ± 2.9	1468 (470–4600)	16.3 ± 6.7
HL-60	208 ± 21.9	82 ± 1.4	238 (170–320)	29.4 ± 15.1
HEL	140 ± 6.9	78 ± 7.0	3 (5–43)	52.6 ± 11.0
K562	62 ± 11.3	32 ± 2.2	4525 (50–421,600)	0.2 ± 0.2
THP-1	54 ± 1.4	35 ± 9.5	13,441 (8660–20,860)	5.1 ± 1.3
MV4-11	n.d.[a]	4 ± 4.7[b]	1821 (290–11,330)	28.1 ± 12.2

[a] Not detected, Ara-CTP was below the limit of quantitation of the HPLC system (50 nM).
[b] Ara-CTP was below the limit of quantitation of the biosensor system (25 nM). Back-calculation performed for HPLC to convert raw results to nanomolar concentrations using 5-point calibration curves.

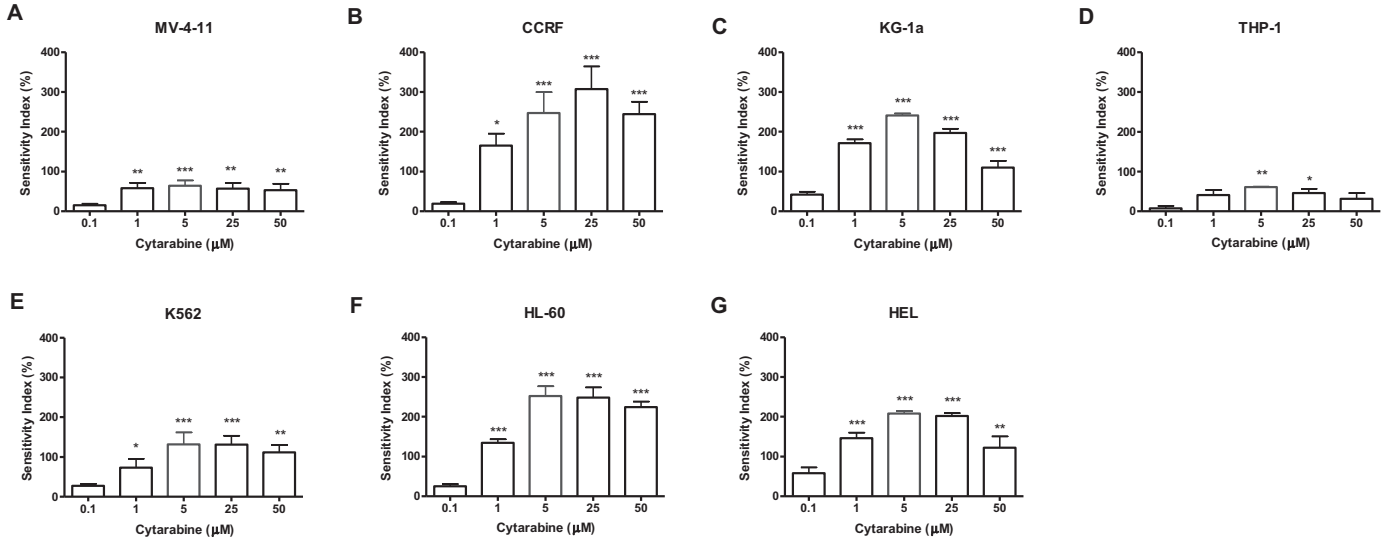

Fig. 1. Response of leukaemic cell lines to a range of concentrations of cytarabine using the biosensor assay. Cells (2×10^6/ml) were exposed to control or cytarabine and lysates were produced as described in Methods. Panels A–G represent individual cell line dose responses (0–50 μM), equivalent to *in vivo* dosing (10–1500 mg/m^2/day). Panel H shows all cell lines at the *in vitro* equivalent (25 μM) to the *in vivo* standard dose (200 mg/m^2/day). Error bars represent standard deviation calculated from three separate experiments each performed in triplicate ($n = 3$). Statistical significance was calculated using one-way ANOVA with Bonferroni's *post hoc* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.2. Ara-CTP quantitation by HPLC and biosensor assays compared with LC_{50} assessment and percentage apoptosis following treatment of leukaemic cell lines with cytarabine

Cytarabine-induced cell death in the seven leukaemic cell lines was evaluated using a commercially available viable mass assay and flow cytometric assessment of Apo 2.7 expression (Table 1). Dose response data are shown for biosensor (Fig. 1) and 72-h viable mass (Fig. 2) respectively. Levels of Ara-CTP production following exposure to cytarabine (25 μM) were highest in CCRF-CEM (644 nM), KG-1a (314 nM), HL-60 (208 nM) and HEL cell lines (140 nM) as measured by HPLC. These cell lines also exhibited the lowest LC_{50} values in the CellTiterGlo™ assay, confirming cytarabine sensitivity. This was mirrored in the flow cytometric assessment of apoptosis (Fig. S2). Table 2 shows individual correlations for each cell line when comparing intracellular Ara-CTP concentration (biosensor) and cell death (CellTiterGlo™ assay), post-dosing with cytarabine (0, 0.1, 1, 5, 25 and 50 μM). For CCRF-CEM, HL-60 and K562, correlations were >0.8, and for the remaining cell lines, <0.7.

Table 3 shows the Spearman's rank correlations of the four assay types across the leukaemic cell lines: for Ara-CTP generation measured by HPLC and the biosensor; and cell viable mass measured by CellTiterGlo™ and APO2.7 expression respectively. Significant correlation of data was observed between HPLC

Table 2
Spearman's rank correlation of dose response data by biosensor assay (Fig. S1) and CellTiterGlo™ (Fig. S3) for each leukaemic cell line. Cell lines were ranked relative to Ara-CTP generation at t = 30 min (biosensor) or ATP% at t = 72 h (CellTiterGlo™ assay) in response to a range of concentrations of cytarabine (0, 0.1, 1, 5, 25, 50 μM), and correlation coefficients and statistical significance calculated ($n = 3$).

Cell line	*R*-value	*p*
CCRF-CEM	0.9559	0.017
KG-1a	0.6000	0.242
HL-60	0.8117	0.058
HEL	0.6866	0.136
K562	0.9852	0.003
THP-1	0.4658	0.356
MV4-11	0.5508	0.297

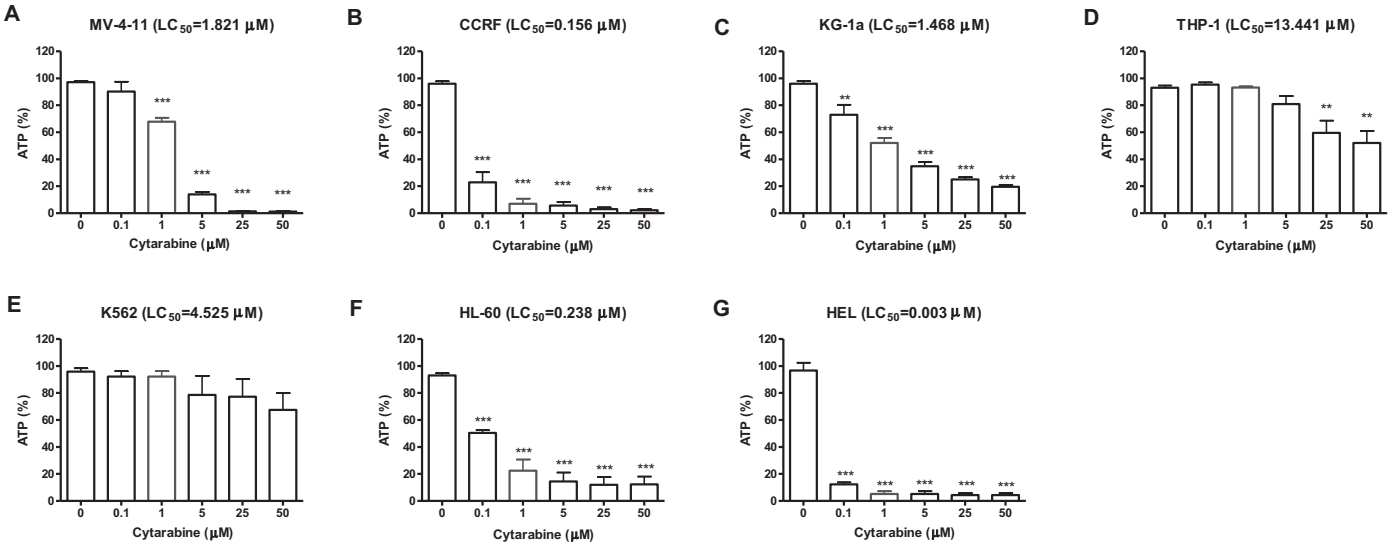

Fig. 2. Dose response of leukaemic cell lines to *ex vivo* standard dose cytarabine using 3-day cell viability assay. Panels A–G represent individual cell line dose responses (0–50 μM), equivalent to *in vivo* dosing (10–1500 mg/m^2/day). Panel H shows all cell lines at the *in vitro* equivalent (25 μM) to the *in vivo* standard dose (200 mg/m^2/day). Optimised cell densities were used (2×10^5/ml) and exposed to control or cytarabine as described in Methods. Error bars represent standard deviation calculated from three separate experiments each performed in triplicate ($n = 3$). Statistical significance was calculated using one-way ANOVA with Bonferroni's *post hoc* test ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). LC_{50} values (concentration of a given agent that is lethal to 50% of the cells) were calculated from the dose response data ($n = 3$).

and biosensor ($R = 0.9722$, $p = 0.0028$). Similarly there was significant correlation between cytarabine-induced cytotoxicity and caspase-dependent cell death ($R = 0.9818$, $p = 0.0004$). However, the correlations between Ara-CTP generation and cell death as measured by the CellTiterGlo™ assay ($R = 0.5505$, $p = 0.2000$) and APO2.7 expression ($R = 0.4129$, $p = 0.3536$) were not significant. This was particularly marked in HEL cells, which following cytarabine exposure exhibited the highest level of apoptosis (52.6%), highest cytotoxicity (LC_{50} = 3 nM), but not the greatest accumulation of Ara-CTP by HPLC (140 nM) or biosensor (SI-value of 78% equivalent to 170 nM).

3.3. Biosensor detection of Ara-CTP generation augmented by fludarabine pre-treatment of leukaemic cell lines

Augmentation of Ara-CTP accumulation following 4 h pre-incubation with fludarabine (5 μM) was assessed in the three cell lines with the lowest accumulation of Ara-CTP following treatment with cytarabine alone. Table 4 shows biosensor, CellTiterGlo™ assay and HPLC results. Significant enhancement in Ara-CTP concentration following pre-exposure to fludarabine (5 μM) was observed using the biosensor for the most resistant cell lines THP-1 ($p = 0.0032$) and K562 ($p = 0.0329$). A corresponding decrease in viable mass was observed. For THP-1, pre-treatment with fludarabine decreased ATP percentage from 38% to 4% ($p < 0.01$), and for K562 from 77% to 52% ($p < 0.05$). In MV-411, fludarabine caused an augmentation in Ara-CTP generation (not detectable to 65 nM) measured by HPLC, however the cell line was sufficiently sensitive to cytarabine alone that no decrease in viable mass with fludarabine was observed (0.4% *versus* 0.4%).

4. Discussion

We have previously described a bacterial biosensor capable of detecting accumulation of intracellular Ara-CTP in leukaemic cell lines as well as blood and bone marrow specimens from AML patients within 8 h of sample receipt [20]. The present study was to validate the assay against established research techniques, using leukaemic cell lines due to the finite nature of patient samples. The ability of the biosensor to detect Ara-CTP was compared with quantitation by HPLC, which has been used successfully in other studies to monitor Ara-CTP levels [25,26]. Herein HPLC was used to quantify the amount of Ara CTP in samples with respect to a known internal standard whereas the biosensor assay measures the SI-value of samples as a reflection of cell sensitivity to cytarabine. HPLC represents a lengthy and demanding process not suited to routine clinical laboratory practice, whereas the biosensor would be applicable to that setting with a turnaround time within one working day, enabling availability of results in a clinically useful timeframe. The effect of exposure of leukaemic cell lines to cytarabine at *in vivo*

Table 3
Spearman's rank correlation of the four assay types across the seven leukaemic cell lines. Cell lines were ranked relative to Ara-CTP generation (HPLC and biosensor assay), ATP% (CellTiterGlo™ assay) or expression of APO2.7, and correlation coefficients and statistical significance calculated. Data represents duplicate (HPLC) and triplicate analysis (biosensor assay, CellTiterGlo™ assay and APO2.7 expression).

	Biosensor	CellTiterGlo™ cytotoxicity assay	HPLC	APO2.7 expression (flow cytometry)
Biosensor		$R = 0.5413$ $p = 0.2357$	$R = 0.9722$ $p = 0.0028$	$R = 0.4312$ $p = 0.3536$
CellTiterGlo™ cytotoxicity assay	$R = 0.5413$ $p = 0.2357$		$R = 0.5505$ $p = 0.25000$	$R = 0.9818$ $p = 0.0004$
HPLC	$R = 0.9722$ $p = 0.0028$	$R = 0.5505$ $p = 0.2000$		$R = 0.4129$ $p = 0.3536$
APO2.7 expression (flow cytometry)	$R = 0.4312$ $p = 0.3536$	$R = 0.9818$ $p = 0.0004$	$R = 0.4129$ $p = 0.3536$	

Table 4
THP-1, K562 and MV4-11, the three cell lines with lowest Ara-CTP generation were tested with cytarabine alone *versus* pre-incubation with fludarabine (5 μM) for 4 h followed by cytarabine (25 μM) for 30 min. Experimental controls contained DMSO (0.1%). Lysates were analysed for Ara-CTP production using HPLC and biosensor respectively. Concurrent analysis of ATP% was performed over 72 h. HPLC results represent duplicate analysis, biosensor and viability assay results represent triplicate analysis ± standard deviation.

Cell line	Treatment	Ara-CTP (nM) ($n=2$)	Sensitivity index (%) ($n=3$)	ATP (%) at 72 h ($n=3$)
THP-1	Ara-C	54	35 ± 10	38.1 ± 2.2
	FLA	76	58 ± 6	4.0 ± 0.7
K562	Ara-C	62	32 ± 2	76.7 ± 5.5
	FLA	93	52 ± 9	52.0 ± 3.5
MV4-11	Ara-C	n.d.[a]	4 ± 5[b]	0.4 ± 0.2
	FLA	65	29 ± 6	0.4 ± 0.3

[a] Not detected, Ara-CTP was below the limit of quantitation of the HPLC system (50 nM).
[b] Ara-CTP was below the limit of quantitation of the biosensor system (25 nM).

relevant concentrations was concurrently assessed by monitoring levels of induced apoptosis and residual viable mass.

The cytotoxic effect of cytarabine is dependent on the ability of cells to produce and retain Ara-CTP, with the maximal level of Ara-CTP formation (125 pmol/10^6 cells) occurring within the first 30 min exposure of leukaemic cells to cytarabine [27]. The biosensor assay has been optimised for 30 min incubation with cytarabine, unlike other studies that have used 4 h incubation periods [25,28,29]. The biosensor has a limit of detection of 25 nM [20] and the HPLC system used herein achieved a limit of detection of Ara-CTP at 50 nM (equivalent to 5 pmol/10^6 cells), which concurs with other studies [10,25,26]. It is important to note that the biosensor (requiring 2×10^6 cells) was able to discriminate between the different sensitivities of the various cell lines to cytarabine, in one tenth the number of cells of the HPLC system (requiring 2×10^7 cells). The biosensor assay is currently being miniaturised, requiring an even lower cell density, important for patient sample analysis.

Across the seven cell lines treated with cytarabine (25 μM) there was a significant correlation between Ara-CTP levels measured using HPLC and the SI-values from the biosensor assay ($R=0.9722$, $p=0.0028$) (Table 3). Clinically, cytarabine is used at various doses ranging from low dose at 20 mg/m^2/day (2 μM), standard dose at 200 mg/m^2/day (25 μM), to high dose at 1–3 g/m$^{2/}$day (400 μM) [30,31]. In this study, the biosensor was capable of quantifying Ara-CTP levels at the *in vitro* equivalent of low (0.1–1 μM) and standard dose (25 μM) cytarabine. This was corroborated by assessment of cell viable mass at 72 h in all seven cell lines (Fig. 2). High dose equivalents (1–3 g/m^2) equating to ≥100 μM have been previously tested using the biosensor [20] and appear to lower the SI-value for the more sensitive cell lines (Fig. 1B, C and G). The biosensor is sensitive to the level of nutrient in the medium due to its requirement for pyrimidine starvation [20]. It is postulated that exposure of sensitive cell lines to very high levels of cytarabine results in cell death and hence pyrimidine release into the lysate, negatively affecting the SI-value by raising the control level; however this effect has not been observed in preliminary studies with patient samples [20]. This is due in part to the comparatively low accumulation of Ara-CTP in patient samples compared to cell lines as previously observed with regard to metabolism of cytarabine [32].

This study has also shown a significant correlation between cytarabine-induced cytotoxicity and apoptosis across the seven cell lines tested ($R=0.9818$, $p=0.0004$) (Table 3). Levels of apoptosis (Table 1) were measured using APO2.7 which detects early caspase-dependent programmed cell death [33]. This suggests that caspase-dependent apoptosis is significant in cytarabine-induced cytotoxicity.

Correlation of Ara-CTP concentration (measured by HPLC) and cell death (measured by 72 h viable mass and APO2.7 expression) was less conclusive ($R=0.5505$, $p=0.2000$ and $R=0.4129$, $p=0.3536$ respectively) (Table 3). For the most resistant cell lines K562, and THP-1, there was a consistent relationship between low Ara-CTP generation (62 and 54 nM) measured by HPLC, high LC_{50} activity (4525 and 13,441 nM) and correspondingly low levels of apoptosis (0.2 and 5.1%). However, dose response analysis (0–50 μM) of Ara-CTP generation and residual cellular viable mass at 72 h only produced correlations >0.8 for CCRF-CEM ($p=0.017$), HL-60 ($p=0.058$) and K562 cells ($p=0.003$). The lack of agreement in the remaining cell lines indicates that Ara-CTP was not exclusive in the cause of cell death (Table 2). This is corroborated by the previous observations that cytarabine can induce apoptosis independently of nuclear incorporation of the triphosphorylated metabolite [34–38]. We intend to investigate this phenomenon in future work.

The question of the prognostic value of intracellular Ara-CTP measurement has been investigated previously with conflicting results. In leukaemic cell lines, correlation between Ara-CTP levels and sensitivity to cytarabine has been observed [10,29,39]. In patients, the correlation between outcome and Ara-CTP levels has been shown to be variable. Estey et al. showed that in patients receiving high dose continuous infusion cytarabine, significantly lower Ara-CTP were observed in resistant *versus* responsive individuals (63 μM *versus* 122 μM) [28]. Another study however, showed no predictive correlation between Ara-CTP accumulation and outcome when high dose bolus cytarabine was used [10]. Nonetheless, Ara-CTP accumulation has shown predictive power in clinical outcome in several other studies [12–14,39], although again dependent on dose and delivery of cytarabine [15,40].

Treatment of relapsed and poor prognosis AML is an important area of current research, involving the use of compounds such as fludarabine that potentiate the action of cytarabine by decreasing the pool of deoxynucleotides and increasing intracellular Ara-CTP generation [41]. Fludarabine associated potentiation of Ara-CTP accumulation was observed using both the biosensor and HPLC in the THP-1, K562 and MV4 11 the cell lines (Table 4). These cell lines exhibited the lowest production of Ara-CTP following exposure to cytarabine alone, and for THP-1 and K562 an associated decrease in cellular viable mass after 72 h (Table 4). This finding suggests that the biosensor has the potential to predict the modulation of Ara-CTP metabolism by fludarabine, corroborating previous findings in these cell lines [22,42] and performing equally well compared to other techniques which can take considerably longer to generate results.

Although Ara-CTP levels represent a valid index of cytarabine-mediated cytotoxicity, it must be acknowledged that other factors affect response to the drug. Nonetheless, the biosensor may be a potentially useful tool as an adjunct to other investigations such as flow cytometry, cytogenetic analysis and molecular diagnostics for predicting functional response to cytarabine alone and in combination with fludarabine. Further studies are planned to assess clinical

correlations in patient samples being treated in UK National Cancer Research Institute (NCRI) trials.

Conflict of interest statement

AM, MR and JL are employees of Randox Laboratories Ltd.; all other authors declare no competing financial interests.

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Contributors. EA, MAS and JGS wrote the paper; HA constructed the biosensor; EA, AM and MR performed experiments and analysed results; VS, MAS and JL designed the research; MC advised on HPLC; PM and JGS provided clinical expertise.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2013.02.012.

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