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Investigation and verification of a bioluminescent biosensor for the quantitation of ara-CTP generation: A biomarker for cytosine arabinoside sensitivity in acute myeloid leukaemia

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ABSTRACT

A novel whole cell bacterial biosensor, which emits light in response to the active metabolite of cytosine arabinoside (ara-C, cytarabine), ara-CTP, has been investigated and verified. The biosensor has been formulated as an *ex vivo* assay, designed for peripheral blood or bone marrow cells, which can produce a clinical result within a working day. The nucleoside analogue ara-C is a key agent for treatment of acute myeloid leukaemia (AML); treatment decisions are made rapidly with AML, patients often receiving same-day commencement of chemotherapy. Currently no rapid predictive test is available to select appropriate therapy for patients prior to treatment. Experiments were designed to determine optimal assay conditions using leukaemic cell lines. We observed a significant increase ($\sim$15 fold) in bioluminescence signal compared to control after 8-h incubation of the biosensor with ara-C. This corresponded to a $>$2-log increase in light output per bacterial cell. Interestingly, bioluminescence conferred a survival advantage to the bacteria following ara-C treatment. The assay is sensitive (lower limit of quantitation of 0.05 μM), selective, accurate ($\leq$15% RE) and precise ($\leq$15% coefficient of variation) over a linear concentration range of ara-CTP (0.05–0.5 μM), and detection is independent of reaction volume. Recovery of added standard was tested using *ex vivo* patient leukaemic cells (n=5). Stability studies on lyophilized bacterial biosensor were performed to ensure maintenance of performance over 12 months. The biosensor assay could be invaluable to the clinician, assisting with treatment selection, and potentially mitigating the risks of resistance and toxicity observed with this drug.

1. Introduction

Cytosine arabinoside (ara-C), the mainstay of treatment of acute leukaemia for decades, and one of the most effective chemotherapeutic agents available, can be used as a single agent in different 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) and in combination with other chemotherapeutic agents such as fludarabine and the anthracycline daunorubicin (Robak and Wierzbowska, 2009). Acute myeloid leukaemia (AML) comprises a heterogeneous group of haematological disorders resulting from the malignant transformation of myeloid precursor cells, leading to the proliferation of immature cells in the bone marrow and blood with suppression of normal hemopoiesis (Hope et al., 2003). For the 260,000 people worldwide that are diagnosed with AML each year (Redaelli et al., 2004b), aggressive chemotherapy including ara-C is given at diagnosis (Redaelli et al., 2004a). However, up to 40% of patients fail to respond to the first course of chemotherapy treatment (Ferrara, 2004); lack of response is only seen after several weeks, when a patient may have suffered adverse side effects. Resistance to chemotherapy, including ara-C, is the main cause of treatment failure among AML patients (Galmarini et al., 2002), the risk of developing AML increases with age, being most common in individuals $\geq$60 years old (Redaelli et al., 2004b), for which the outlook is poor (Burnett et al., 2009; Löwenberg, 1996). For these

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elderly patients, where AML incidence and mortality rates are increasing, and where co-morbidities may rule out aggressive chemotherapy, a pre-screening test would be invaluable. A same-day predictive test could be beneficial to indicate the most effective chemotherapy regimen, particularly as treatment decisions have to be made within hours of diagnosis of this rapidly progressive disease (Redaelli et al., 2004a).

The cytotoxic effects of ara-C are mainly mediated *via* the tri-phosphorylated metabolite ara-CTP, through its incorporation into DNA strands leading to chain termination and synthesis arrest and interference with DNA polymerase, with a secondary effect on free radical generation and induction of apoptosis (Hu et al., 1995; Carter et al., 2003; Bezombes et al., 2003). The deoxycytidine analogue ara-C is transported into the cell *via* the specific nucleoside transporter (hENT1), and is rapidly phosphorylated *via* the enzyme 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 (Mancini, 1992). Ara-CTP has previously been proposed as a biomarker of clinical response to treatment with ara-C. Studies have suggested that reduced intracellular concentrations of ara-CTP are associated with clinical resistance to ara-C (Galmarini et al., 2002; Kufe et al., 1980), attributable to a number of factors including the rate of ara-CTP formation and its incorporation into DNA (Cai et al., 2008; Song et al., 2009). Moreover, intracellular ara-CTP levels have been shown to correlate with the cytotoxic effect of ara-C (Kufe et al., 1980) and with clinically observed therapeutic efficacy following treatment with high-dose ara-C (Liliemark et al., 1985; Plunkett et al., 1980).

This study used a whole-cell bioluminescent bacterial biosensor, rendered highly sensitive to ara-C through selection of a pyrimidine-requiring, bioluminescent strain of *Escherichia coli*, further mutated to be deficient in cytidine deaminase (*cdd* gene) and expressing human dCK (Alloush et al., 2010). Emission of light from bacteria rendered bioluminescent is due to 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 and acid with the emission of blue-green light (Meighen, 1993). The high metabolic rate of bacterial cells compared to mammalian cells is beneficial, allowing for fast, real-time toxicity testing (Alloush et al., 2003). Light output from bioluminescent biosensors can be accurately and reproducibly measured, with little background interference, using luminometers or low-light cameras.

Bioluminescent reporters have been described previously for a range of health applications (Billard and DuBow, 1998). Such reporters are commonly found to increase light output in response to toxic insult. However, to date the mechanism behind this phenomenon remains obscure. It has been shown that bioluminescence reduces susceptibility to UV damage (Cutter et al., 2007), a process that can be partially reversed with the addition of anti-oxidants (Szpilewska et al., 2003), which indicate that bioluminescence is a response to oxidative damage. Alternatively bioluminescence in bacteria may have evolved as a metabolic shunt to allow recycling of reactive intermediates (Galluzzi and Karp 2007).

The biosensor described herein has been developed and optimised into an assay for determination of uptake and phosphorylation of ara-C by human leukaemic cells. Application of the biosensor requires verification prior to use in clinical decision making, including determination of the quantitative power of the assay to measure the biomarker, and assessment of clinical utility by measuring the selectivity and sensitivity for the biomarker (Lee et al., 2002). Similarly proof of assay robustness and reproducibility are important, especially for use in a clinical setting. In the present study we describe for the first time, the optimisation of the assay and its technical verification for ara-C sensitivity using cultured leukaemic cell lines. Experiments were designed to evaluate assay sensitivity, precision, sample preparation, intra- and inter-assay accuracy, and sample storage conditions, demonstrating the suitability of the assay for application in future clinical trials. In addition the mechanism for the increase in bioluminescence observed has been investigated.

2. Materials and methods

2.1. Chemicals and reagents

Ara-C, Histopaque®-1077, cell culture-grade water and alkaline phosphatase (AP) derived from bovine intestinal mucosa were purchased from Sigma-Aldrich (UK). Ara-CTP solution (10 mM) was purchased from Jena Bioscience (Germany). Cell culture media and EDTA were purchased from Invitrogen (UK), foetal calf serum (FCS) was purchased from Biosera (UK). Bradford reagent and bovine serum albumin (BSA) standards were purchased from Bio-Rad (UK).

2.2. Cell lines and Blood samples

Cell lines (KG-1a, K562, CCRF-CEM and HEL) were supplied by the Health Protection Agency (HPA) or American Type Culture Collection (ATCC) and were maintained as recommended in RPMI 1640 medium containing L-glutamine (2 mM) and FCS (10%) at 37 °C with 5% CO_2. Cells were sub-cultured every 3 days to maintain exponential growth and testing was carried out between passages 5 and 15 from purchase. Cryopreserved cells from fresh AML peripheral blood samples (with blood blast burden $>80\%$), previously separated using Histopaque®-1077 by density gradient centrifugation to yield the mononuclear cell fraction (according to manufacturer's instructions), were thawed and re-suspended in RPMI 1640 medium before incubation with ara-C. Samples were collected from patients at Frimley Park Hospital, Surrey, UK, and Bristol Haematology and Oncology Centre, Bristol, UK, after obtaining informed consent.

2.3. Biosensor preparation

The biosensor was cultured overnight at 37 °C (16 h) in RPMI 1640 growth medium containing L-glutamine (2 mM) and FCS (10%). The optical density at 600 nm ($OD_{600\ nm}$) of the culture was measured using a Helios Alpha UV–vis spectrophotometer (Thermo Scientific, UK) and adjusted in RPMI 1640 (minus additives) to the required OD for each experiment. OD ranged from 0.01 to 1.0 absorbance units (AU). Prior to experiments, the culture was incubated at 37 °C with shaking (225 rpm) for 30 min. In all experiments, the assay was performed in black-sided 96-well microtiter plates (Greiner Bio-One Ltd, UK) with final well volume of 200 μL (unless otherwise stated), and bioluminescence from the biosensor was measured every 15 min for 15 h in a luminometer (Lumistar, BMG Labtech) to obtain the peak of bioluminescence.

2.4. Protein assessment

Lysates from cells (K562, HEL and CCRF-CEM) treated ± ara-C (25 μM) and calibration curve samples produced using BSA (0 to 0.2 mg/mL) were mixed with Bradford reagent (BioRad) 1:1 in 1 mL cuvettes. Samples were incubated for 5 min at room temperature before analysis of absorbance at 595 nm using a Helios Alpha UV–vis spectrophotometer (Thermo Scientific, UK) according to manufacturer's instructions.

2.5. Assay optimisation

Incubation time: The effect of ara-C (25 μM) was investigated using KG-1a cells (2×10^6 cells/mL) incubated with the drug for 0.5, 1 and 4 h prior to lysate preparation. Ara-C stock solution (10 mM) was prepared in sterile deionized water before dilution in RPMI 1640 medium to the working concentration (25 μM). Cells were collected at 300*g* for 5 min (at room temperature) and washed in twice the original volume of RPMI 1640 medium. Cells were centrifuged for a further 5 min at 300*g* and re-suspended in the original volume of RPMI 1640 medium. Cells were then lysed by the addition of saponin (0.1%) and EDTA (optimised as described below) to inhibit endogenous AP activity. Samples were vortex-mixed for 30 seconds, and cell debris was removed by centrifugation at 2800*g* for 5 min. Medium was maintained at 37 °C and centrifugation performed at room temperature throughout. Ara-C sensitivity was assayed in the presence of the biosensor ± IPTG and ± AP (conditions optimised as described below).

EDTA optimisation: Varying concentrations of EDTA (0–2.5 mM) were added to KG-1a cell suspensions previously treated with ara-C (25 μM) for 0.5 h, prior to the final centrifugation step (2800*g* for 5 min). The direct effect of EDTA on biosensor performance was assessed in RPMI 1640 medium containing the biosensor (OD 0.1 AU), IPTG (1 mM) and EDTA (0–100 mM).

OD and IPTG optimisation: Optimization of the bacterial OD and concentration of the *dCK*-inducer IPTG was performed using a checkerboard assay. The optimal levels were determined by considering signal/background ratio and the time to peak bioluminescence. The biosensor was prepared to varying OD (0.01–1.0 AU). Ara-C (25 μM) was added to all cultures and the concentration of IPTG was varied (0.1–2 mM). The reaction volume was 200 μL ($n=5$).

AP optimisation: Ara-CTP (0.5 μM) and IPTG (1 mM) were spiked into (i) RPMI 1640 medium and (ii) lysate from CCRF-CEM cells treated with ara-C (25 μM) for 0.5 h, and mixed with the biosensor (final OD 0.1 AU). AP was subsequently added across the range (0.1–2 Units/well). An AP negative sample was also evaluated. Ara-C sensitivity was assayed in the presence of biosensor (OD 0.1 AU) and ± IPTG (1 mM).

2.6. Assay verification

Freeze-thaw cycling: Cell lysates were prepared from KG-1a leukaemic cells and leukaemic blasts from patient sample (A002) treated with ara-C (25 μM) for 0.5 h. Lysates were aliquoted and analysed (i) immediately with the biosensor (OD 0.1 AU) ± IPTG (1 mM) and ± AP (1 Unit/well) and (ii) following two consecutive freeze-thaw cycles at −20 °C using the same assay conditions.

Bacterial viable counting: To investigate the nature of the effect of ara-C on the biosensor, viable counting and live/dead staining were performed. The biosensor (OD 0.1 AU) was cultured in RPMI 1640 medium containing ± IPTG (1 mM) and ± ara-C (25 μM) at 37 °C for 24 h with shaking (225 rpm). Aliquots (1 mL) were removed at hourly intervals and (i) plated on nutrient agar containing tetracycline (10 μg/mL), kanamycin (10 μg/mL) and ampicillin (100 μg/mL) respectively, or (ii) collected by centrifugation (2800*g* for 1 min), re-suspended in fresh RPMI 1640 medium and assayed using the LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, UK) according to manufacturer's instructions. Bioluminescence was monitored simultaneously. Agar plates were cultured overnight at 37 °C and colony-forming-units (cfu) counted the following day. This process was repeated for cultures of *lux*-expressing and *lux*-deficient versions of the biosensor strain, incubated in a darkroom to assess bacterial survival in the absence of bioluminescence or ambient light.

Preparation of calibration and quality control (QC) standards: Using a stock solution of ara-CTP (10 mM) a calibration curve ranging from 0.01 to 0.5 μM was prepared in RPMI 1640 medium. QC samples were produced using pooled lysate produced from untreated human leukaemic cell lines CCRF-CEM, HEL and K562. QC samples at three concentrations (denoted LQC [low], MQC [medium] and HQC [high]) were prepared by spiking the pooled matrix with appropriate amounts of ara-CTP to yield final concentrations of LQC=0.05 μM, MQC=0.1 μM and HQC=0.5 μM. This concentration range reflects the intended detection range in leukaemic cell lines and patient samples observed previously (Anderson et al., 2013). Analysis of each standard and QC sample was performed in triplicate using the biosensor (OD 0.1 AU) ± IPTG (1 mM) and ± AP (1 Unit/well).

Recovery of spiked standard: This was assessed using lysates produced from untreated patient leukaemic cells ($n=5$) of bone marrow and peripheral blood origin. Lysates were spiked with ara-CTP (0.1 μM) and analysed using the biosensor (OD 0.1 AU) ± IPTG (1 mM) and ± AP (1 Unit/well). Recovery was calculated as the percentage ratio between the measured and spiked concentrations, using a calibration curve (0 to 0.5 μM ara-CTP).

Dilution linearity and parallelism: Assessment of dilution linearity was performed using pooled untreated lysate from CCRF-CEM cells spiked with ara-CTP (0.1 μM) and subsequently diluted (2-, 4-, 10- and 20-fold). Samples were analysed with the biosensor (OD 0.1 AU) ± IPTG (1 mM) and ± AP (1 Unit/well). Parallelism was assessed using lysates produced from leukaemic cell lines (K562, HEL, CCRF-CEM) treated with ara-C (25 μM) for 0.5 h, subsequently diluted (2- and 4-fold) using untreated lysate. In both experiments results were back-calculated according to dilution.

Independence of volume: Independence of volume was assessed using lysate produced from KG-1a cells treated with ara-C (25 μM) for 0.5 h. The biosensor (OD 0.1 AU), IPTG (1 mM) and AP (1 Unit/well) concentrations were maintained, with lysates produced using either 8×10^6 cells (in 2 mL), or using a 50% scale-down with 4×10^6 cells (in 1 mL). Final well volumes were 200 or 100 μL respectively.

2.7. Lyophilization of the biosensor

The biosensor was lyophilized and the stability evaluated over a 12 month period using LQC and MQC samples. Briefly, a mixed streak of bacterial cells (from glycerol stocks of biosensor strain) grown overnight on agar containing kanamycin (10 μg/mL), tetracycline (15 μg/mL) and ampicillin (50 μg/mL) respectively was used to inoculate RPMI 1640 medium (50 mL) containing antibiotics, and incubated overnight at 37 °C with shaking at 220 rpm. The culture was diluted 1/10 with fresh RPMI medium without antibiotics (10 mL) and incubated at 37 °C with shaking until the cell density reached OD 0.250 AU. The culture was diluted (i) 1/10 in RPMI medium and incubated to OD 0.250 AU, and then (ii) 1/10 in RPMI medium and incubated at 37 °C to OD 0.5–0.6 AU. Exponential growth phase was determined by recording and plotting of the OD every 0.5 h. Bacterial suspensions were centrifuged at 12, 227*g* for 15 min (at room temperature) and the supernatant was removed and stored. The bacterial pellet was resuspended in RPMI medium containing 5% trehalose (w/v) to a final OD 4.0 AU. The suspension was transferred to a stirring unit and dispensed into vials (1 mL) and sealed. Cultures were freeze-dried using Lyophilizer 220 (Hull, USA) according to the cycle: 180 min at −45 °C with vacuum; slope of 0.1 °C per minute to −30 °C minus vacuum; 3600 min at −30 °C minus vacuum; 1200 min at −20 °C minus vacuum; 1440 min at 0 °C minus vacuum; 1440 min at 15 °C minus vacuum. Vials of lyophilized biosensor were stored at +4 °C prior to use.

2.8. Data evaluation

Significance ($p < 0.05$) was assessed using one-way ANOVA with Bonferroni's post-hoc test. The diagnostic discriminator was the maximal difference in light output between the plus AP and minus AP curves, which represents of the level of ara-CTP generated within the cells. This was expressed as a percentage sensitivity index (SI%) (Alloush et al., 2010; Anderson et al., 2013):

$$\text{Biosensor assay response (SI\%)} = [(+\text{AP}/-\text{AP})_{25\ \mu\text{M}} - (+\text{AP}/-\text{AP})_{0\ \mu\text{M}}] \times 100 \quad (1)$$

3. Results and discussion

3.1. Assay optimisation

The major focus of assay optimization was to maximize signal/background ratio whilst simultaneously minimising total assay time. Conditions requiring optimisation included: the incubation period of the AML cells with ara-C; the amount of biosensor; and the concentrations of assay components EDTA, IPTG and AP. Preserved assay conditions: (i) RPMI 1640 medium, (ii) 37 °C temperature, (iii) final assay volume 200 μL, (iv) stock solutions of IPTG (100 mM) and ara-C (10 mM) prepared in sterile water and the volume added to each well maintained across the concentrations of IPTG tested, (v) bioluminescence from the biosensor was monitored every 15 min to 15 h to capture the peak bioluminescence for all combinations.

Incubation time: An important requirement herein was to reduce the overall assay time to within a working day, allowing same-day results for patients. Ara-CTP concentration in leukaemic cells has been assessed previously and the peak concentration observed 4 h after exposure to ara-C (Estey et al., 1990). In a similar study (Muus et al., 1987) using leukaemic bone marrow cells from 69 patients with acute leukaemia, the formation of ara-CTP was found to peak following 0.5 h exposure to ara-C (125 pmol per 10^6 cells). As the time to extract leukaemic cells from whole blood requires extended centrifugation, and the time to reach maximum bioluminescence from the biosensor has been consistently observed at 5.25 h (Alloush et al., 2010), it would be impossible to include a 4-h incubation with ara-C and maintain same-day testing. To test the minimum incubation period with ara-C required without significant loss of signal an experiment was performed using KG-1a cells. Untreated and ara-C (25 μM) treated cells were incubated for 0.5–4 h prior to cell lysis. The concentration of ara-C was calculated from the *in vivo* dose, and validated in our previous study (Alloush et al., 2010). Fig. 1A shows the SI% values for each of the incubation times: 0.5 h (33%), 1 h (36%) and 4 h (49%). A significant increase in the SI% was observed after 4 h relative to 1 and 0.5 h ($p < 0.001$). However, this increase (SI = 13%) was considered insignificant relative to the benefits of same-day testing. No significant difference was observed between 1 and 0.5 h ($p > 0.05$), therefore the latter was included in the optimised protocol.

EDTA concentration: Ara-C is imported into mammalian cells and converted to ara-CTP within the cytosol (Hazra et al., 2009). Mammalian cells in continuous culture contain AP within secretory vesicles (Cox and Macleod, 1962). On lysis AP is released from these vesicles and could convert cytosolic ara-CTP to ara-C, invalidating any subsequent analysis using the biosensor. To prevent premature dephosphorylation of the active metabolite, EDTA was added prior to lysis to inhibit any endogenous AP (Whisnant and Gilman, 2002). The concentration of EDTA required to inhibit endogenous AP without causing detrimental suppression of bioluminescence was investigated. Firstly, the effect of EDTA (0–100 mM) on bioluminescence from the biosensor was assessed in RPMI 160 medium (Fig. 1B). In the absence of EDTA mean bioluminescence from the biosensor was 287,853 RLU, and addition of EDTA (0–100 mM) caused a dose-dependent decrease in this response, reducing the signal/background ratio. This experiment was repeated using lysate produced from KG-1a cells

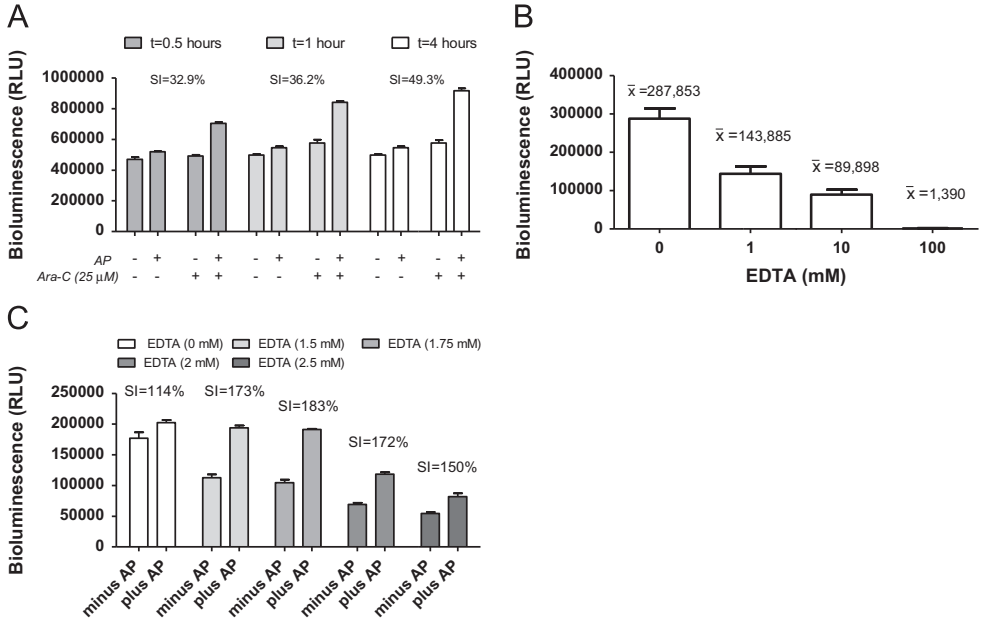

Fig. 1. Effect of ara-C (0 and 25 μM) incubation and EDTA on light output from the bacterial biosensor using lysate produced from KG-1a cells. The biosensor was cultured overnight in complete RPMI 1640 growth medium and the optical density adjusted to 0.1 AU in RPMI 1640 (minus additives). (A) Cells were incubated with Ara-C (0 or 25 μM) for 0.5, 1 and 4 h prior to assay. Samples represent peak bioluminescence from the biosensor ($t = 5.25$ h) in each case ($n = 5$). (B) EDTA (0–100 mM) and IPTG (1 mM) were added in a final volume of 200 μL per well and bioluminescence monitored every 15 min for 15 h with peak bioluminescence displayed ($t = 6.5$ h). (C) The biosensor was adjusted in lysate produced from KG-1a cells treated with ara-C (0 and 25 μM) in the presence of EDTA (0–2.5 mM) and IPTG (1 mM). Plus and minus AP (1 unit/well) samples represent peak light output ($t = 5.25$ h) in each case ($n = 5$). Error bars represent SD. RLU, relative light units. SI (%), sensitivity index as calculated using Eq. (1). Statistical significance was calculated using one-way ANOVA with Bonferroni's post-hoc test (***$p < 0.001$).

Table 1
Checkerboard assay for the optimization of biosensor OD and IPTG concentration displaying signal/background ratio and time to peak bioluminescence (hours). The biosensor was cultured overnight in complete RPMI 1640 growth medium and the optical density adjusted in RPMI 1640 (minus additives). Ara-C (25 μM) was added to all cultures with varying concentrations of IPTG (0.1–2 mM). The two numbers given for each combination represent (from top to bottom), (i) the signal/background ratio at the peak of light output and (ii) the time to peak of light output (hours) ($n=5$). Emboldened numbers represent the optimal combination.

OD (AU)	IPTG (mM)				
	0.1	0.5	1	1.5	2
0.01	3.0 15.00	2.4 15.00	12.5 15.00	12.4 15.00	2.4 15.00
0.05	1.8 10.75	2.0 10.75	13.1 10.75	12.1 10.75	2.1 10.75
0.1	2.1 8.00	11.5 8.00	**15.5** **8.00**	2.6 8.00	1.7 8.00
0.2	1.4 5.00	4.2 5.00	5.1 5.00	1.6 5.00	1.7 5.00
0.5	1.6 3.50	1.9 3.50	1.9 3.50	2.3 3.50	2.2 3.50
1.0	1.1 2.00	1.4 2.00	1.2 2.00	1.4 2.00	1.1 2.00

treated with ara-C (0 or 25 μM) in the presence of EDTA (0–2.5 mM), IPTG (1 mM) and ± AP (1 unit/well). Fig. 1C illustrates that absence of EDTA results in the ratio between ± AP being almost completely ablated (× 1.14), which was improved upon addition of EDTA: 1.5 mM (× 1.73), 1.75 mM (× 1.83), 2 mM (× 1.72) and 2.5 mM (× 1.5). The highest separation between ± AP (and subsequently SI%) was observed using 1.75 mM EDTA.

Optimisation of biosensor OD and IPTG concentrations: This was performed in a checkerboard experiment in which OD and IPTG concentration were varied simultaneously. Biosensor OD was varied from 0.01 to 1.0 AU and the concentration of IPTG from 0.1 to 2 mM (Table 1). Optimal conditions for OD and IPTG concentration were found to be 0.1 AU and 1 mM, respectively. The resulting signal/background ratio of 15.5 provided the desired assay sensitivity to discriminate between ara-C responsive and non-responsive samples. Under these conditions, the time to peak of bioluminescence in RPMI 1640 medium was 8 h, decreasing to 5.25 h when analysing cell lysate due to the increased nutrient abundance (Alloush et al., 2010). These conditions ensured that the time to peak of bioluminescence was suitable for a same-day assay.

Optimisation of AP concentration: This was performed in two stages. Firstly, the effect of AP concentration was tested in blank RPMI 1640 medium spiked with ara-CTP (0.5 μM) (Fig. S1A). This concentration of ara-CTP represents the levels measured in leukaemic cell lines known to be highly sensitive to ara-C (Anderson et al., 2013). In this experiment increasing the concentration of AP caused a significant increase in light output ($p<0.001$). The basal level of bioluminescence was ~70,000 RLU, which increased to ~280,000 RLU on addition of AP (0.1 U/well) (4-fold increase). Bioluminescence was further augmented when the concentration of AP was increased to 1 U/well, however increasing the concentration of AP > 1 U/well did not improve bioluminescence. Secondly, the experiment was repeated using lysate produced from CCRF-CEM cells treated with ara-C (25 μM) for 0.5 h (Fig. S1B). The CCRF-CEM cell line produces high levels of ara-CTP (~0.3 μM) following 0.5 h exposure to ara-C (25 μM) (Anderson et al., 2013). The maximal increase in bioluminescence from the biosensor in CCRF-CEM lysate was observed using 1 U/well. Fig. S1B also demonstrates the effect of cellular matrix on bioluminescence from the biosensor. The increase in nutrient availability in cell lysate over RPMI 1640 medium suppresses overall bioluminescence from the biosensor by increasing exogenous pyrimidine levels that compete with ara-CTP and reduce subsequent intercalation of the bacterial DNA (Alloush et al., 2010). Together the results from Fig. S1 indicate that an optimal concentration of 1 U/well AP is required.

3.2. Assay verification

Using optimised assay conditions for EDTA (1.75 mM), AP (1 Unit/well), IPTG (1 mM) and biosensor OD (0.1 AU), we investigated the effect of ara-C on biosensor viability, and assessed the limit of detection of the biosensor, the effect of the sample matrix on analyte recovery, assay linearity and parallelism, independence of volume, stability and intra- and inter-assay variation. The following conditions were maintained: (i) RPMI 1640 medium, (ii) 37 °C temperature, (iii) final assay volume 200 μL, (iv) bioluminescence from the biosensor monitored every 15 min to 15 h to capture the peak bioluminescence, (v) ara-C dosing at 25 μM to represent the *in vitro* equivalent of the *in vivo* clinical dose (200 mg/day/m^2), (vi) ara-CTP dosing at 0.05–0.5 μM to represent the range of concentrations of ara-CTP achievable following 0.5 h incubation with ara-C in both patient and immortalized leukaemic cell lines (Alloush et al., 2010; Anderson et al., 2013).

Biosensor viability: To verify whether under the optimized conditions ara-C was bactericidal to the biosensor, cell viability was assessed. Fig. 2 describes the relationship between bioluminescence from the biosensor in response to ara-C (25 μM) (Fig. 2A), and the resulting viable cell count (Fig. 2B). In support of the observed increase in bioluminescence under optimized conditions (Table 1), direct application of ara-C to the biosensor resulted in a 2.5 − log 10 increase in light out per colony-forming unit (cfu) (Fig. 2A). Concurrently the viable count shows a 2 − log 10 drop in viability (cfu/mL) at 6 h post-dosing. This would suggest that ara-C is bactericidal to the biosensor strain. Interestingly, at $t=2$ h post-dosing the signal to background ratio of bioluminescence starts to increase, which is mirrored by the reduction in growth of the biosensor. Fig. 2C shows the live/dead ratio of biosensor cells and replicates the results of the viable count experiment, with an observed change in live/dead ratio at $t=2$ h post-dosing compared to the control, an effect that reaches the maximum extent at $t=6$ h post-dosing. This indicates that the peak of bioluminescence from the biosensor reflects a concurrent reduction in viability in response to ara-C, replicating the response of mammalian cells to the drug; a phenomenon that is only visible in mammalian cells following extended incubation (48–72 h) (Anderson et al., 2013). To probe the mechanism for this impressive increase in bioluminescence, *lux*-expressing and -deficient strains of the biosensor were cultured ± ara-C, in the absence of ambient light. A significant improvement in bacterial survival was observed for the bioluminescent biosensor (Fig. 2D). The physiological role for bioluminescence is thought to be either stimulation of DNA repair *via* photoreactivation, or detoxification of reactive oxygen intermediates to reduce oxidative damage. It has previously been observed that ambient light and bioluminescence can rescue bacterial cells from UV-induced DNA damage (Cutter et al., 2007; Kozakiewicz et al., 2005). However, Lyzeń and Wegrzyn (2005) have shown poorer survival of dark mutants from a range of naturally bioluminescent bacteria following treatment with oxidants, an effect that could be partially reversed using antioxidants, suggesting that bioluminescence is a response to oxidative damage. The improvement in survival observed herein represents the first observation of this kind in response to ara-C, an effect that can be differentially controlled by induction of *dCK* expression using IPTG, and which supports the case for

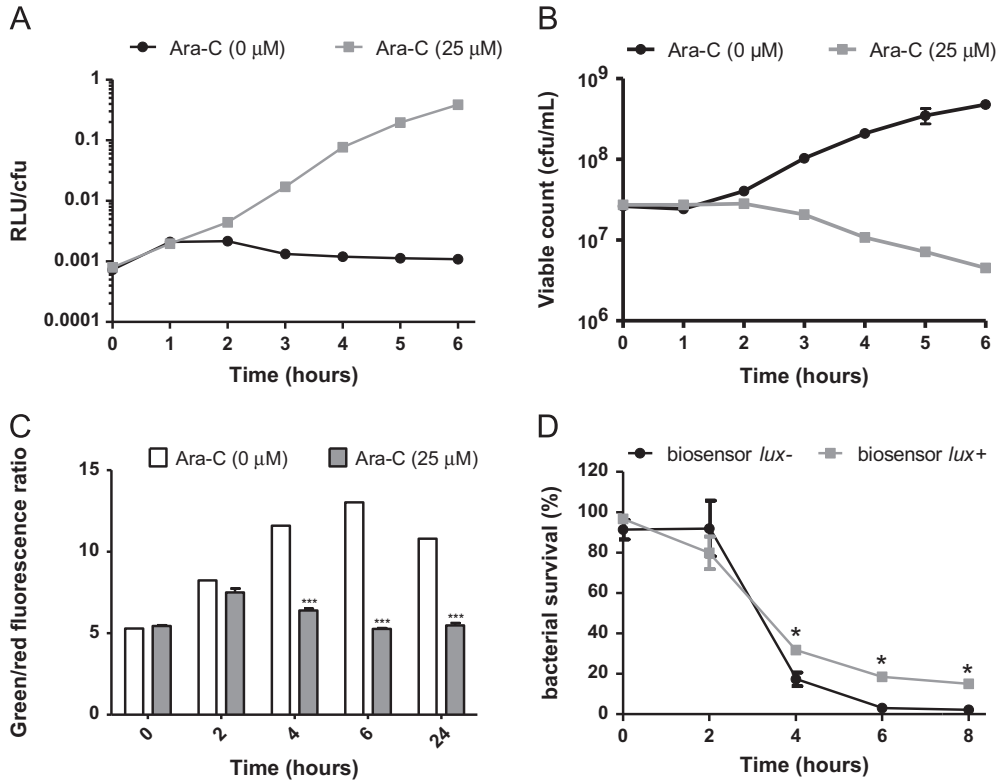

Fig. 2. Relationship between bioluminescence (A) and viable count of the biosensor (B) following treatment with ara-C (0 and 25 μM), (C) assessment of live/dead ratio and (D) influence of bioluminescence on bacterial survival following treatment with ara-C (25 μM). (A, B) The biosensor was cultured overnight in complete RPMI 1640 growth medium and the optical density adjusted to 0.1 AU in RPMI 1640 (minus additives). The culture was incubated at 37 °C for 6 h with shaking (225 rpm) ± ara-C in the presence of IPTG (1 mM). Aliquots were taken for assessment of bioluminescence and for plating on to nutrient agar at hourly intervals and viable colonies counted the following day. (C) Starter cultures + IPTG (1 mM) and ± ara-C (25 μM) were incubated for 24 h at 37 °C with shaking (225 rpm). Hourly aliquots (1 mL) were removed, washed by centrifugation (13,000 rpm for 1 min) and assayed using the LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, UK). (D) Starter cultures of *lux*+ and *lux*− strains of the biosensor were cultured for 8 h + IPTG (1 mM) and ± ara-C (25 μM) in total darkness. Aliquots were taken for assessment of bioluminescence and for plating on to nutrient agar at hourly intervals and viable colonies counted the following day. Error bars represent SD ($n=3$). Statistical significance was calculated using one-way ANOVA with Bonferroni's post-hoc test ($^{*}p<0.05$, $^{***}p<0.001$). RLU, relative light units, cfu, colony-forming units.

bioluminescence being a response to oxidative damage. Further investigation into the mechanism for this increase in bioluminescence is underway.

Limit of detection: Fig. 3A and B describes the raw data and resultant calibration curve produced using the concentration of spiked ara-CTP (0–0.5 μM) against bioluminescence ± exogenous AP results from the biosensor ($r^2=0.997$). The limit of detection of ara-CTP using the biosensor was found to be 0.025 μM, corroborating our previous results (Alloush et al., 2010). Specificity of the biosensor for ara-C has been demonstrated previously using purine analogues in current clinical use (Alloush et al., 2010). This indicates that the biosensor can specifically detect ara-CTP to a concentration typically observed in cell lines resistant to ara-C (Anderson et al., 2013), thus covering a suitable detection range to allow differentiation between ara-C responders and non-responders.

Dilution linearity and parallelism: Dilution linearity and parallelism were assessed independently using two separate methods (Fig. 3C and D respectively). Dilution linearity was tested by spiking ara-CTP (0.1 μM) into pooled untreated lysate prepared from CCRF-CEM cells, and diluting with the same untreated lysate to 2-, 4-, 10- and 20-fold, with five replicates ($n=5$) for each dilution level. Results were back-calculated using appropriate dilution factors and compared. The coefficient of variation (CV) between dilutions was 11.2% indicating that none of the assay components (such as protein content) were interfering in the detection of ara-CTP. For the parallelism study, three lysates prepared from cell lines previously treated with ara-C (25 μM) were diluted using untreated lysate to 2- and 4-fold, with five replicates ($n=5$) for each level. The three cell lines were chosen due to their relative sensitivities to ara-C, with CCRF-CEM and HEL being more sensitive than K562 cells (Takagaki et al., 2003). Results indicate that dilution of the lysate from human-derived cell lines was concentration and matrix-independent. Precision (CV) of the back-calculated results were 14.9% for the K562 cell line, 14.4% for the HEL cell line and 10.5% for the CCRF-CEM cell line, respectively.

Stability over transport: To evaluate transportation issues (such as transport between clinic and pathology laboratory), freeze-thaw cycling of lysates was performed. Fig. S2 describes KG-1a cell lysates (control and ara-C-treated) and a patient lysate (A002) produced from mononuclear cells (extracted from peripheral blood), which were analysed fresh and upon two subsequent cycles of freeze-thawing (-20 °C). KG-1a cell lysate produced a SI = 155% fresh, decreasing to 131% and 160% on cycles 1 and 2, respectively. For sample A002, fresh analysis produced a SI = 61% that reduced to 52% and 49% on cycles 1 and 2 respectively. No significant effect was observed as a result of freeze-thawing for 2 cycles in either sample type ($p>0.05$).

Protein assessment in cell lysates: A higher cellular density in the control or treated sample could result in unmatched matrix, affecting both bioluminescence from the biosensor and the SI% result for a given sample. To ensure that the optimised protocol produced an equal density of AML cells in untreated and treated samples, total protein was quantified in lysates from three leukaemic cell lines using the Bradford assay (Bradford, 1976). Fig. S3 shows the results compared to a standard curve using BSA (0–2 mg/mL). No significant difference in protein concentration

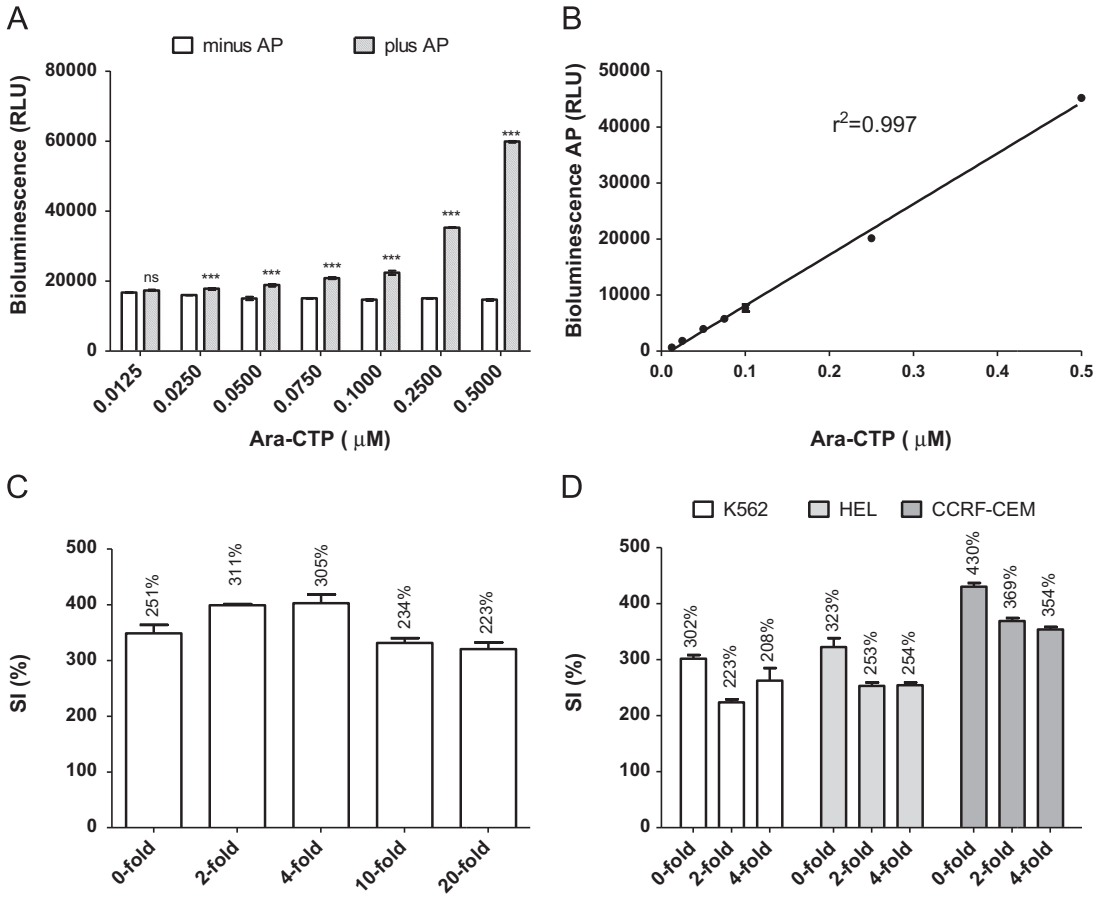

Fig. 3. Peak bioluminescence from the biosensor treated with ara-CTP (0–0.5 μM) ± alkaline phosphatase (A), resultant calibration curve (B) and assessment of dilution linearity (C) and parallelism (D) in leukaemic cell lines. (A) Peak bioluminescence demonstrating the relationship between light output from the biosensor in response to ara-CTP (0–0.5 μM) ± AP (1 U/well) and (B) calibration curve gernerated from ± AP. RLU, relative light units. (C) A pooled untreated lysate (CCRF-CEM) was spiked with ara-CTP (0.1 μM) and analysed ($n=5$) following 2-, 4-, 10- and 20-fold dilution using untreated lysate. (D) Lysates from three human leukaemic cell lines (K562, HEL, CCRF-CEM) of differing sensitivity to ara-C were analysed ($n=5$) following 2- and 4-fold dilution. Error bars represent SD. SI (%), sensitivity index as calculated using Eq. (1). Statistical significance was calculated using one-way ANOVA with Bonferroni's post-hoc test (ns, not significant, $^{***}p<0.001$).

was found between treated and untreated samples in each case, indicating that equal distribution of cellular material was achieved using the optimized protocol for lysate preparation.

Recovery of added standard: Selectivity of a method demonstrates the ability to determine the analyte in a number of independent sources of blank matrix in the presence of interference, such as degradation products. This was assessed using untreated lysate produced from leukaemic cells from five separate patients, including samples of both bone marrow and peripheral blood (Table 2). The recovery results ranged from 98.1% to 114.5%, with no significant difference observed, indicating that recovery was matrix independent. This suggests that the assay is sufficiently selective to negate the potentially detrimental effect of endogenous interfering components that may differ between subjects.

Independence of volume: With the potential view to future miniaturization of the assay, dependence on volume was tested. Miniaturisation is crucial as lowering the assay requirement for leukaemic cells would result in less sample per assay, allowing more doses and or drugs to be tested from limited starting material. Assessment of ara-C responsiveness can be at low-, standard- or high-dose, and the assay can similarly be used to assess the effect of potentiators of ara-CTP formation (such as fludarabine, clofarabine) (Anderson et al., 2013). The biosensor assay currently requires 4×10^6 cells per treatment. With white blood cell (WBC) counts typically varying from 0.5 to $\geq 300\times10^9$/L in patients with AML (How et al., 2012), a bone marrow aspirate of 1–2 mL can on average contain 20×10^6 cells prior to density gradient separation. Ideally a functional test of this nature would be carried out in addition to the cytogenetic analyses currently performed, and within this sample size. Fig. S4 describes the results for the optimized assay using 200 μL reaction volume containing 4×10^5 cells per well (prepared as 2 mL at 2×10^6 cells/mL) compared to a 50% reduction in all components (100 μL reaction volume containing 2×10^5 cells per well). The lysate used in this experiment was produced from KG-1a cells exposed to ara-C (0 or 25 μM). As expected the overall bioluminescence reduced by 50% as only half

Table 2
Assessment of ara-CTP recovery in five independent sources of matrix. Lysates produced from untreated patient leukaemic cells were spiked with ara-CTP (0.1 μM) and recovery was calculated as the percentage ratio between the measured and spiked concentrations. Calculation of measured concentrations was performed using a calibration curve (0–500 nM, $r^2=0.997$; Fig. 3A). Each sample was analysed in triplicate, with the mean result displayed.

Sample ID	Concentration of ara-CTP (μM)			Recovery (%)
	Basal	Measured	Expected	
A052	0	0.099	0.10	99.1
A018	0	0.100	0.10	100.4
A051	0	0.098	0.10	98.1
B044	0	0.115	0.10	114.5
A032	0	0.100	0.10	100.9

the number of biosensor cells per well were present in the scale-down assay. However, the SI% was preserved (133% *versus* 127%) with no significant change due to 50% scale-down of the assay. These results suggest that further miniaturization may be feasible, perhaps lowering the requirement to 4×10^4 cells/test.

Stability of lyophilized biosensor: For ease of use in a clinic laboratory setting, the biosensor was lyophilized and the stability verified over a 12 month period. Fig. S5 describes the results for (A) inter-batch (batches #1–5) and (B) intra-batch (batch 5) stability of the lyophilized biosensor. In all cases, lyophilized calibrators containing ara-CTP in blank sample matrix were used, termed low (0.05 μM ara-CTP) and medium (0.1 μM ara-CTP) calibrators. Batch-to-batch mean SI values for low and medium calibrators were 14.8% and 31.9%, with CV values of 10.9% and 6.2%, respectively. Run-to-run mean SI values for low and medium calibrators were 21.7% and 44.7% with run-to-run CV values of 15.4% and 14.9% respectively ($n=5$). These results conform to system suitability criteria for the ara-C biosensor (Table SI). Longer-term and accelerated stability studies are currently on-going to ensure maintenance of assay characteristics.

Intra- and inter-assay characteristics: Given the nature of chemotherapeutic agents such as ara-C, which are known to lead to secondary malignancies (Redaelli et al., 2004a, 2004b), it is beneficial to determine patients that will show clinical response to treatment. Possible sources of assay variability include the biological variability in the intracellular levels of factors affecting the analyte (for example endogenous alkaline phosphatase levels and the activity of dCK), and technical variability in relation to sample preparation and assay procedure. Biological variability in response to ara-C is known to be related to age, sex, disease classification and the cytogenetic profile of a patient (Redaelli et al., 2004b).

Accuracy and precision of the biosensor were determined in an appropriate matrix (Table 3). To evaluate assay precision, we measured the effect of three calibrators of known ara-CTP concentration (0.05–0.5 μM) each repeated three times on subsequent days ($n=5$/experiment). Standard deviation (SD) and CV were calculated for intra-day and inter-day assay results. An acceptable level of precision was defined as $\pm$ 15% CV, which was achieved for the LQC, MQC and HQC samples (0.05–0.5 μM). A further calibrator tested (0.01 μM ara-CTP) failed to produce an acceptable level of precision, and was therefore below the lower limit of quantitation (LLOQ) (data not shown).

To determine the accuracy of the assay, a 5-point calibration curve was employed in parallel for each run (0–1 μM), and SI% values were back-calculated to provide concentrations for the QC results. An acceptable % relative error (%RE) was pre-set at 15% for intra-day and inter-day results (Table S1). Intra-run accuracy levels were in the range −13.4% to 12.1% RE. Inter-run accuracy levels were in the range −10.2% to 3.5% RE with the highest deviation found at the HQC level. This indicates that the biosensor is most accurate at detecting lower concentrations of ara-CTP, and may suffer saturation effects at the higher concentrations ($\geq$ 0.5 μM ara-CTP).

Intra- and inter-run CV $<$ 15% were achieved for all QC levels across the three runs. Previous assessment of intracellular ara-CTP concentration in clinical material from AML patients ($n=24$) using the biosensor has indicated a wide separation in the response to ara-C: non-responders producing median SI=0% (range −7% to 6%); and responders producing median SI=55% (range 21–128%) (Alloush et al., 2010). Assay precision was highest at extremes of the detection range (LQC and HQC levels, 5.5% and 5.0% respectively), indicating that the assay should perform well in a clinical setting.

Calculation of the *Z* value was performed at each QC level, which represents a statistical parameter indicating the robustness of the assay (Zhang et al., 1999). Assays producing *Z* values $>$ 0.7–1.0 are considered highly robust. For the high QC sample (0.5 μM) the *Z* value was calculated as 0.73. This QC sample represents the level of ara-CTP found in ara-C sensitive cell lines such as CCRF-CEM and HEL (Anderson et al., 2013). This would suggest that the assay could be used for high-throughput screening of ara-C-treated samples, a concept which is already under development for cytogenetic profiling in AML (Dunlap et al., 2012).

Table 3
Assessment of assay precision using QC samples assayed in three independent batch runs. QC samples containing ara-CTP (0–0.5 μM) were assayed on 3 successive days and percentage sensitivity index (SI%) calculated as described in methods. Intra- and inter-day standard deviation (SD) and coefficient of variation (CV) are presented.

Run ID	Batch number	LQC (0.05 μM)	MQC (0.1 μM)	HQC (0.5 μM)
1	1	0.052	0.116	0.496
		0.048	0.115	0.463
		0.041	0.126	0.497
		0.048	0.105	0.399
		0.056	0.097	0.397
Intraday mean		0.049	0.112	0.451
Intraday SD		0.006	0.011	0.050
Intraday CV%		11.5	10.0	11.0
%RE		−2.0	11.8	−9.9
n		5	5	5
2	2	0.041	0.084	0.545
		0.047	0.101	0.442
		0.039	0.075	0.492
		0.044	0.076	0.441
		0.053	0.096	0.433
Intraday mean		0.045	0.087	0.471
Intraday SD		0.005	0.012	0.048
Intraday CV%		12.0	13.5	10.1
%RE		−10.2	−13.4	−5.8
n		5	5	5
3	3	0.058	0.10	0.41
		0.043	0.13	0.39
		0.052	0.09	0.45
		0.042	0.12	0.39
		0.054	0.12	0.48
Intraday mean		0.050	0.112	0.426
Intraday SD		0.007	0.015	0.037
Intraday CV%		14.1	13.4	8.8
%RE		−0.4	12.1	−14.8
n		5	5	5
Mean result		0.048	0.103	0.449
Interday SD		0.003	0.015	0.022
Interday CV%		5.5	14.2	5.0
%RE		−4.2	3.5	−10.2
n		15	15	15

4. Conclusions

The verification of a highly sensitive and specific biosensor for the active metabolite of ara-C, ara-CTP has been described, and the mechanism behind its mode of action has been investigated, using currently accepted industrial methodology. The assay measures bioluminescence as a precise and accurate determinant of ara-CTP sensitivity in cell lysate produced from leukaemic cells. Lysate preparation involves minimal sample manipulation using 8×10^6 leukaemic cells. The assay will evaluate patient samples and their potential response to ara-C, prior to treatment. It is intended to expand this study to include anthracycline and novel chemotherapeutic drugs used to treat AML.

5. Support and financial disclosure declaration

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

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.09.014.

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