Clinical evaluation of an *in vitro* assay for rapid assessment of Cytosine Arabinoside response in patients with Acute Leukaemia and CLL

E. Anderson[1], V.C. Salisbury[1], M.A. Smith[2], M. Ruddock[3], A. Martin[3], J. Lamont[3], H.M. Alloush[1], K.S. Suh[4], J.G. Smith[5]

[1]Centre for Research in Biomedicine, University of the West of England, Bristol, UK. [2]Royal Marsden NHS Foundation Trust, Surrey, UK.
[3]Randox Laboratories Ltd, Belfast, UK. [4]Tumor Bank & Genomics Program, Hackensack University Medical Center, NJ, USA.
[5]Frimley Park NHS Foundation Trust, Surrey, UK. Email: elizabeth3.anderson@uwe.ac.uk

Centre for Research in Biomedicine — CRIB

Introduction

The nucleoside analogue cytosine arabinoside (Ara-C) remains the mainstay treatment of acute non-lymphoblastic leukaemia (ANLL) even although up to 30-percent of patients fail to respond. Furthermore a large proportion of patients fail to achieve long-term remission and develop resistance to subsequent therapy. Currently there is no rapid, inexpensive test to assess patient sensitivity to Ara-C prior to treatment. Here we present a clinical evaluation of a bioluminescent 8-hour assay which assesses intracellular Ara-CTP levels. This is an update of preliminary work in ANLL cell lines (Smith *et al.*, *Blood* 2005; **106**(11): 695a).

In vivo, Ara-C is transported into cells via the specific nucleoside transporter (hENT1) and is rapidly phosphorylated by deoxycytidine kinase (dCK) to the active tri-phosphorylated form, Ara-CTP, which is able to interfere with DNA polymerase and is incorporated into DNA strands leading to chain termination and DNA synthesis arrest. Ara-C can also induce apoptosis via release of mitochondrial cytochrome c, with an increase in reactive oxygen species, and via the death receptor pathway involving signalling through sphinomyelin enriched plasma membrane lipid rafts.

Resistance to treatment is multi-factorial, including increased export of the parent compound from cells, insufficient conversion of Ara-C to the active metabolite Ara-CTP, and increased deamination of Ara-C to the inactive Ara-uracil (Ara-U). There is a requirement for a test to identify the extent of resistance, independent of cause, which in combination with cytogenetic screening could allowing tailoring of the dose and/or selection of combinatorial therapy.

Methods

Cell lines (assay validation)

This assay was validated using CCRF-CEM, HL-60, HEL, THP-1 (M5 AML), KG-1a (M0 AML), K562, MV4-11 cell lines and compared with the commercially available 3-day cytotoxicity Cell Titer-Glo® assay (Promega).

Patients samples

Patients samples - peripheral blood (53%) or bone marrow (47%) from patients at presentation with ANLL (n = 45), ALL (n=2) or CLL (n =12). Patient ages ranged from 24 to 94 (median age 67.5 years), FAB sub-type distribution M4 (26%), M2 (20%), secondary AML (20%), M0 (14%), M1 (6%), M3 (6%), JAK2+ (3%), and Ph+ ALL (5%). Samples were provided blind and biosensor testing performed in two separate centres for confirmatory purposes. Test results were verified using the commercially available 3-day Cell Titer-Glo® assay (Promega) and compared with clinical outcome where known.

8-hour assay principle

The biosensor is a non-pathogenic strain of *E.coli,* genetically modified to express human dCK for conversion of Ara-C to the active metabolite Ara-CTP, inducible using isopropyl β-D-1-thiogalactopyranoside (IPTG). The biosensor also contains a *lux*-expressing plasmid and produces increased light output in response to Ara-C. The biosensor is lyophilised for ease of use and storage.
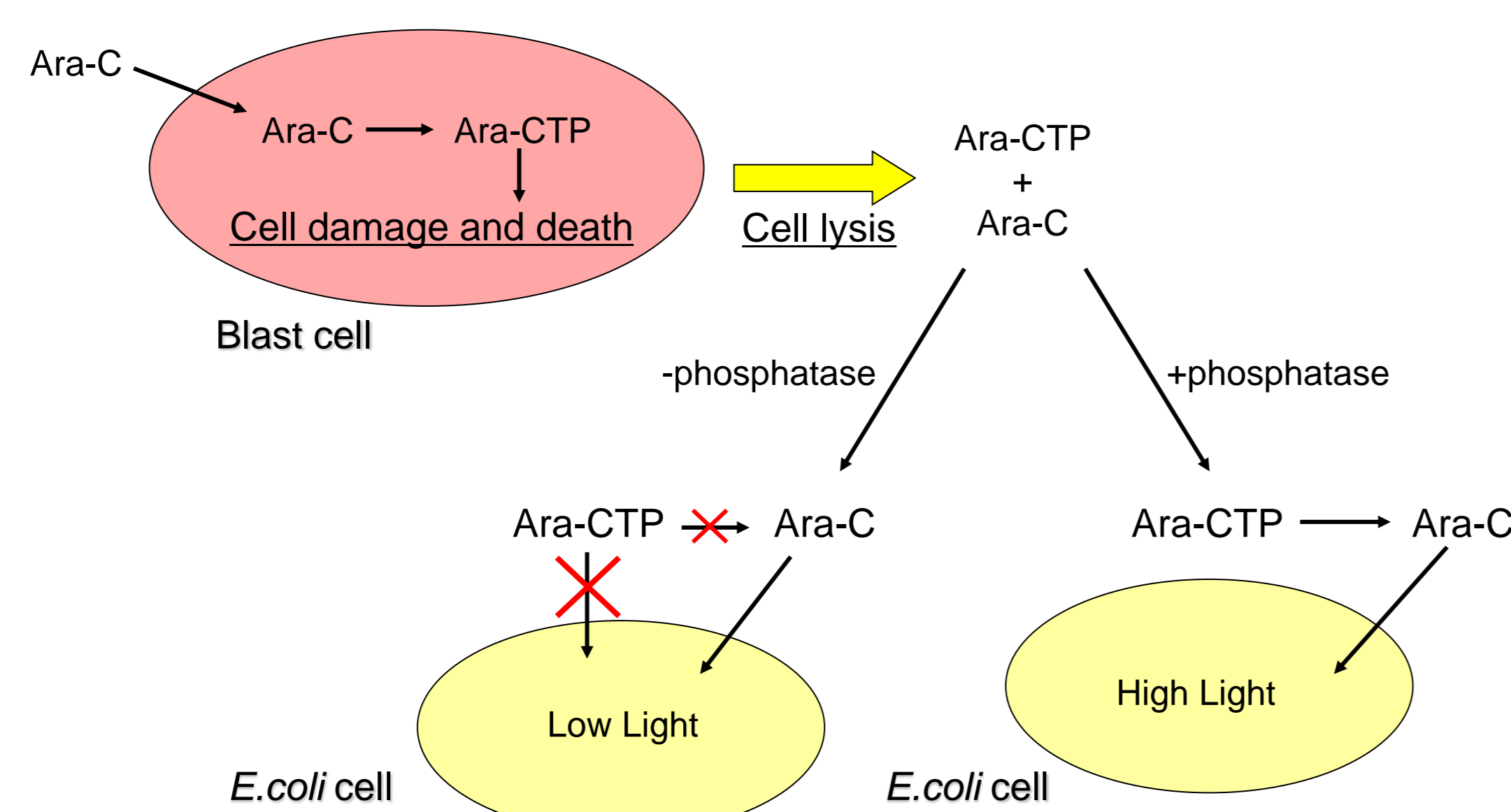
Patient blasts (4 x 10⁶) were incubated for 30 minutes with Ara-C at a clinically relevant dose, the equivalent of 2g/m². Blasts were washed and lysed prior to exposure to the biosensor.

Blasts from sensitive patients produced high light output, whereas those from resistant patients produced low light output (Figure 1).

Statistics

One-way ANOVA with Bonferroni's Post-Hoc test was used to assess significance for cell line and patient samples. Correlation with clinical outcome was assessed by ROC curve analysis.

Figure 1: Schematic of the biosensor system showing cellular responses to Ara-C

Ara-C
Ara-C → Ara-CTP
Cell damage and death
Blast cell
Cell lysis
Ara-CTP + Ara-C
-phosphatase / +phosphatase
Ara-CTP ✗→ Ara-C / Ara-CTP → Ara-C
Low Light / High Light
E.coli cell / *E.coli* cell

★ Difference between ± phosphatase proportional to Ara-CTP in blast cell

➢Ara-CTP itself cannot enter the bacterial cell and must first be converted to Ara-C. This is accomplished by adding alkaline phosphatase (AP) to the sample.

➢Comparison between ± AP indicates the proportion of Ara-C converted from Ara-CTP, and thus the patient's ability to import and metabolise the drug to the active metabolite.
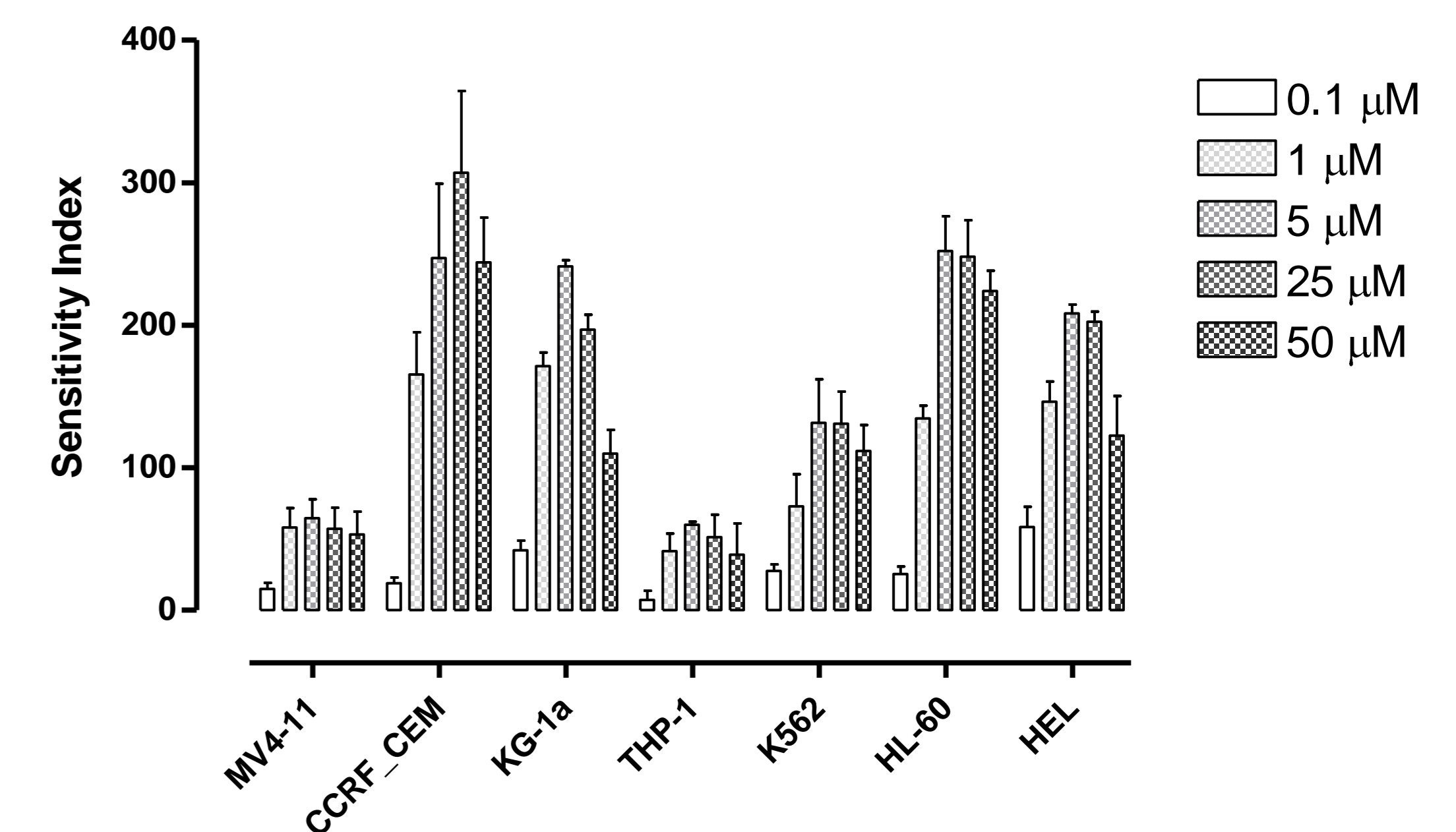
Results

Cell lines

Validation studies were performed using leukaemic cell lines exposed to various doses of Ara-C and analysed using the 8-hour assay. A high degree of reproducibility was achieved from replicate assays (n=10).

The sensitivity index is calculated from +AP/-AP results for treated and untreated samples. It is a measure of the level of Ara-C to Ara-CTP conversion achieved by the cell type. Immortalised cell lines achieve high values due to their homogeneous nature.

Figure 2: Dose response of cell lines to Ara-C treatment using the 8-hour assay

Legend: 0.1 µM, 1 µM, 5 µM, 25 µM, 50 µM. Y-axis: Sensitivity Index. X-axis: MV4-11, CCRF_CEM, KG-1a, THP-1, K562, HL-60, HEL.

Patient samples

Figures 3A and B show typical 8-hour assay results using lysates produced from patients known to be sensitive to Ara-C. Raw data is presented in relative light units (RLU) and the calculated sensitivity index values are shown. Addition of AP to the lysate produced a significant increase in light output from the biosensor compared to the untreated control (Figure 3A p=0.0032; Figure 3B p=0.0097). Figures 3C and D show results using lysates produced from resistant patients. No increase in light output was observed on addition of AP to the Ara-C treated sample indicating that these blasts were incapable of generating and sustaining Ara-CTP levels (Figure 3C p=0.0655; Figure 3D p=0.1432).

Figure 3: Assay results from patient samples with known clinical outcomes

A peripheral blood — Ara-C (0 µM), Ara-C (25 µM), ** 128%
B bone marrow — Ara-C (0 µM), Ara-C (25 µM), ** 118%
C peripheral blood — Ara-C (0 µM), Ara-C (25 µM), 3%
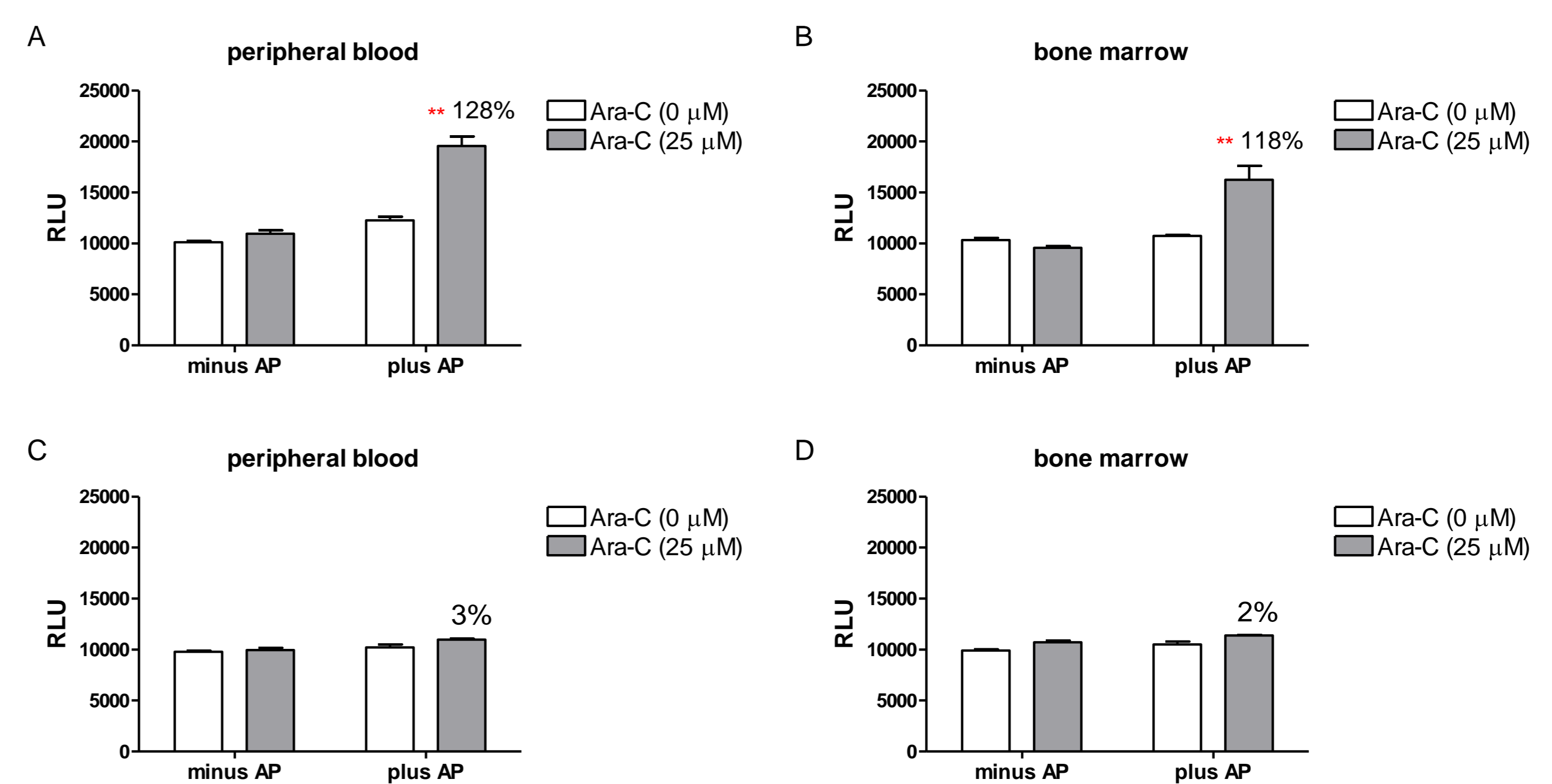D bone marrow — Ara-C (0 µM), Ara-C (25 µM), 2%

Figure 4 shows typical 8-hour assay results using patient lysate produced following *in vitro* treatment of samples with Ara-C alone (Figure 4A) or fludarabine (5 µM) pre-treatment for 4 hours followed by Ara-C (FLA regime) (Figure 4B). The sensitivity index (%) is shown. This indicates a significant improvement in *in vitro* sensitivity to Ara-C following pre-treatment with fludarabine (p<0.0001) versus Ara-C alone (p=0.0068).

Figure 4: Comparison of a patient sample treated with Ara-C versus FLA regime

A — Ara-C (0 µM), Ara-C (25 µM), ** 27%
B — Ara-C (0 µM), Fluid (5 µM) + Ara-C (25 µM), *** 127%
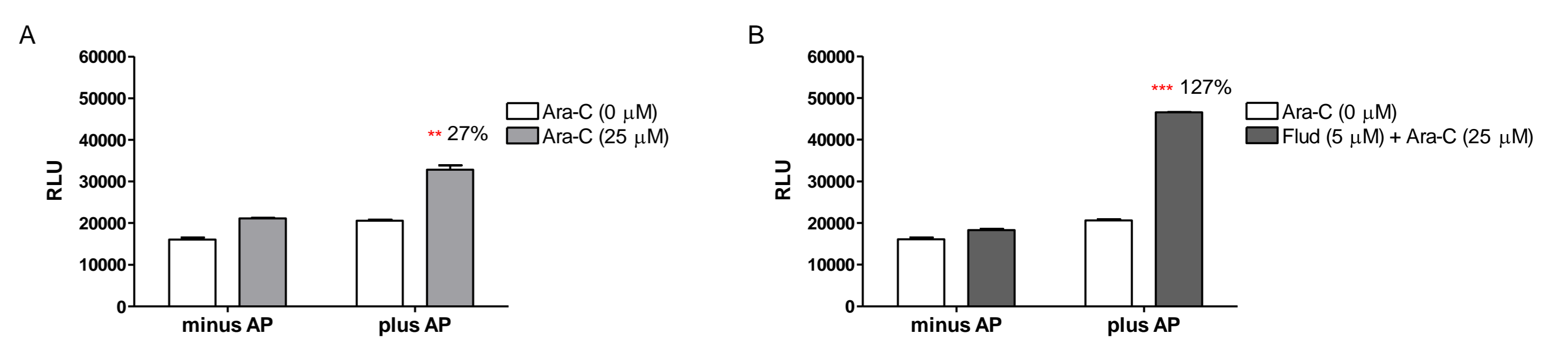
➢The 8-hour assay showed 51% and 49% of samples to be Ara-C sensitive and resistant respectively (n=47).

➢100% correlation with Cell Titer-Glo® assay.

➢Of the 32 clinical outcomes available at present, 30 correlate with the 8-hour assay (p=0.052). In these two patients the assay predicted sensitivity whereas clinical outcome indicated Ara-C resistance. This may raise the possibility of Ara-CTP intercalation being overcome *in vivo* due to altered expression of low fidelity Y-Family polymerases.

➢A patient (M0 ANLL), with clinical resistance to Ara-C entered remission with FLAG-Ida. The 8-hour assay predicted correctly increased response to Ara-C in combination with fludarabine (Figure 4A and B).

➢2 patients with Ph+ ALL have been tested to date (data not shown). The 8-hour assay predicted sensitivity to Ara-C in both cases, which was confirmed using the Cell Titer-Glo® assay.

➢Interestingly, in the 12 patients with B-CLL tested, the 8-hour assay suggested Ara-CTP generation in all cases. This may indicate the potential novel use of Ara-C in this patient group.

Conclusions

➢Assay produces a result within 8 hours of sampling allowing same-day indication of patient sensitivity to Ara-C.

➢Assay is simple to perform, without the requirement for cell culture equipment - necessary for the 3-day Cell Titer-Glo® assay.

➢Assay can determine patient resistance independent of the specific cause of resistance, unlike other kits specific to one resistance mechanism, for example assessment of drug efflux (MultiDrugQuant™).

➢Proof of principle analysis has shown 94% correlation with clinical outcome in ANLL patient samples (n=32) and 100% with the 3-day Cell Titer-Glo® assay (n=47).

➢This assay may be useful in predicting the beneficial effect of using Fludarabine in association with Ara-C to maximise the generation of Ara-CTP.