

# Predictive value of an *in vitro* bioluminescent assay for rapid assessment of response to cytarabine and fludarabine in patients with acute leukaemia

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## 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.

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 allow tailoring of the dose and/or selection of combination therapy.

Currently there is no rapid, inexpensive test to assess patient sensitivity to Ara-C prior to treatment. We have previously reported a bioluminescent 8-hour assay which assesses Ara-CTP levels in leukaemic cell lines and patient samples independently of the cause of patient resistance (Anderson *et al.*, *Blood* 2009; **114**(22): p643). In theory any agent capable of potentiating generation of Ara-CTP from Ara-C can also be tested with this assay system. Here we present results using the 8-hour assay for combination therapy screening, as tested on seven ANLL cell lines and an initial cohort of seven patients with ANLL, dosed with Ara-C alone or in conjunction with the purine analogue fludarabine.

## Methods

### Cell lines (assay validation)

This assay was validated using CCRF-CEM (ALL), HL-60 (APL), HEL (erythroleukaemia), THP-1 (M5 AML), KG-1a (M0 AML), K562 (CML), MV4-11 (biphenotypic B myelomonocytic leukemia) cell lines and compared with the commercially available 3-day cytotoxicity Cell Titer-Glo<sup>®</sup> assay (Promega).

### Patients samples

Patients samples - peripheral blood (57%) or bone marrow (43%) from patients at presentation with ANLL (n = 7). Patient ages ranged from 27 to 71 (median 53 years), FAB sub-type distribution M4 (30%), M2 (14%), secondary AML (14%), M0 (14%), biphenotypic AML (14%) and Ph+ ALL (14%). Samples were provided blind and the bioluminescent 8-hour assay was performed in two separate centres for confirmatory purposes. Test results were verified using the commercially available 3-day Cell Titer-Glo<sup>®</sup> assay (Promega) and compared with clinical outcome where known.

### 8-hour assay principle

The biosensor used in the assay 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.

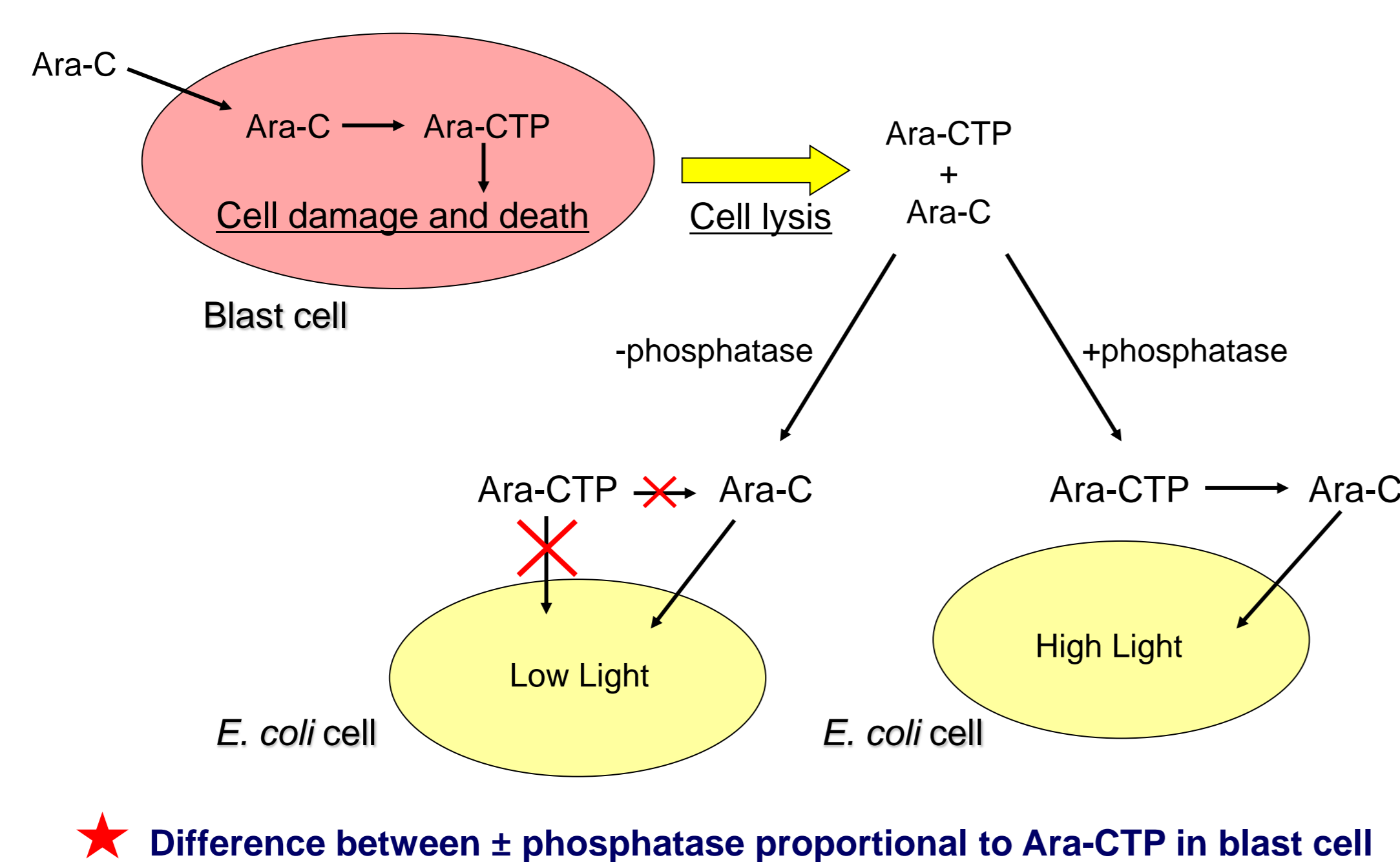
Patient blasts (4 x 10<sup>6</sup>) were incubated for 30 minutes with Ara-C at a clinically relevant dose, the equivalent of 2g/m<sup>2</sup>. Blasts were washed and lysed prior to exposure to the biosensor. Pre-incubation with fludarabine (5 μM) was for 4 hours. Fludarabine was prepared in DMSO so that cells were exposed to a final concentration of 0.1% DMSO.

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.

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



➤ 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.

## Conclusions

➤ Assay produces a result within 8 hours of sampling allowing same-day indication of patient sensitivity to Ara-C. Testing with combination therapy requires 6 or 24 hour pre-incubation.

➤ Assay is simple to perform, without the requirement for cell culture equipment - necessary for the 3-day Cell Titer-Glo<sup>®</sup> 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<sup>™</sup>).

➤ Proof of principle analysis for Ara-C testing has shown 94% correlation with clinical outcome in ANLL patient samples (n=32) and 100% with the 3-day Cell Titer-Glo<sup>®</sup> assay (n=47). Combination testing has shown 100% correlation with clinical outcome to date (n=2).

➤ This assay may be useful in predicting the beneficial effect of using compounds such as fludarabine in association with Ara-C to maximise the generation of Ara-CTP, as validated in Thp-1 and K562 cell lines, and patient samples.

## Acknowledgments

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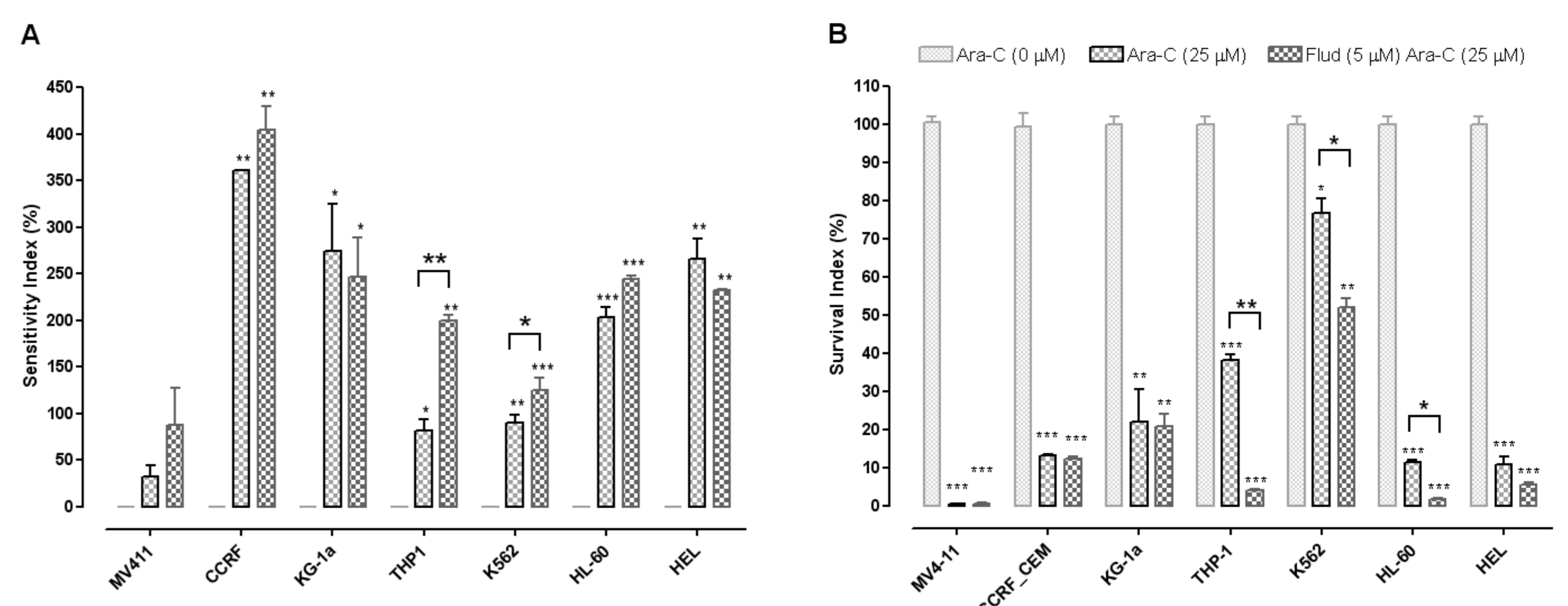
## Results

### Cell lines

Validation studies were performed using leukaemic cell lines exposed to fludarabine and Ara-C and analysed using the 8-hour assay (Figure 2A) and the 3-day cytotoxicity assay (Figure 2B). A high degree of reproducibility was achieved from replicate assays (n=10). The Thp1 and K562 cell lines showed marked improvement in sensitivity index with combination therapy over Ara-C alone.

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: Response of cell lines to Ara-C versus FLA regime using (A) the 8-hour assay and (B) 3-day cytotoxicity assay



### Patient samples

Figure 3 shows typical 8-hour assay results from two patients using lysate produced following *in vitro* treatment of samples with Ara-C alone or fludarabine (5 μM) pre-treatment for 4 hours followed by Ara-C (FLA regime). The sensitivity index (%) is shown. Figure 3A shows an increased sensitivity index in response to the combination therapy over Ara-C alone (132% versus 49%). 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 3B shows a patient for whom combination showed no improvement in sensitivity index (28% versus 21%) over Ara-C alone (p>0.05).

Figure 3: Comparison of treatment with Ara-C versus FLA regime in (A) sensitive and (B) resistant patient samples

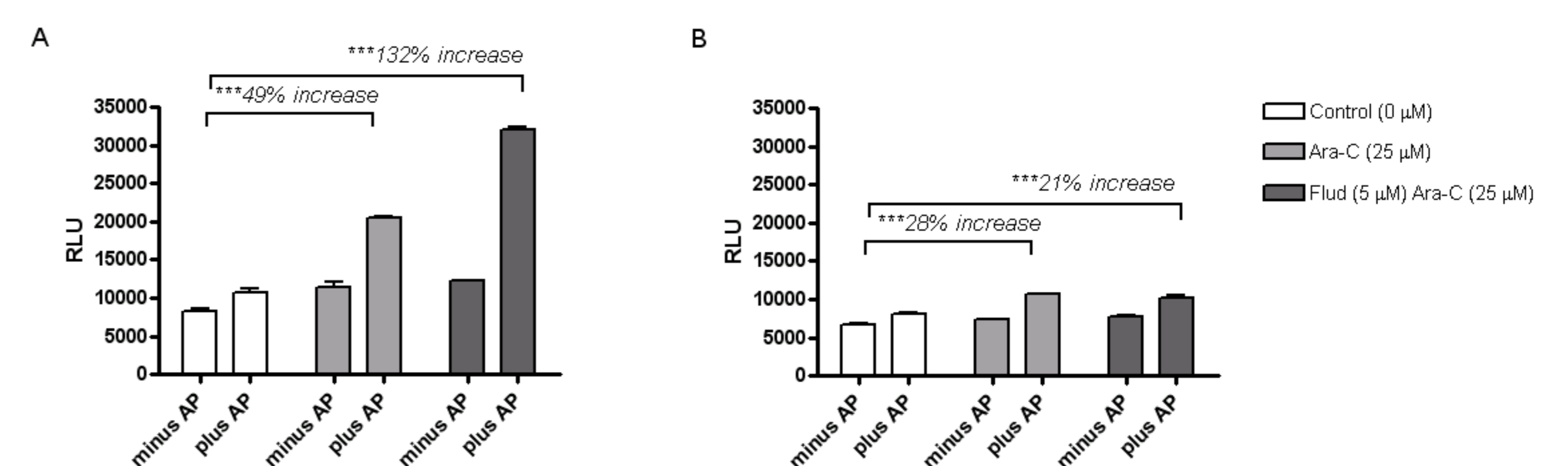


Table 1 shows results for the patient cohort. Patient ages ranged from 27 to 71 (median 53 years) and included four peripheral blood and three bone marrow samples. In four samples the 8-hour assay showed a significant improvement in blast cell sensitivity to combination treatment compared with Ara-C alone (p<0.05 in all cases\*). Confirmation of the predictive capacity of the 8-hour assay was observed in two of these patients: one was initially resistant to daunorubicin/Ara-C, and achieved remission with FLAG-Idarubicin; the second also successfully achieved remission with FLAG-Idarubicin.

Table 1: Patient samples analysed using the 8-hour assay treated with Ara-C versus FLA regime

Patient	Diagnosis	Outcome	8-hour assay result (Ara-C)	8-hour assay result (FLA)
A006	M0 AML	NR with DA, CR with FLAG-Ida	Resistant (6%)	Sensitive (58%)*
A008	Secondary AML	Deceased	Resistant (3%)	Sensitive (15%)*
A021	M0 AML	NR with DA, CR with FLAG-Ida	Resistant (7%)	Sensitive (14%)*
A032	Not known	Currently in treatment	Sensitive (28%)	Sensitive (21%)
B007	M4 AML	CR	Partially sensitive (13%)	Partially sensitive (12%)
B027	T-cell AML	CR on cycle 1 (DA)	Sensitive (28%)	Sensitive (36%)
B034	Not known	CR on cycle 1 (DA)	Sensitive (49%)	Sensitive (132%)*