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 allowing 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® 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® 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.

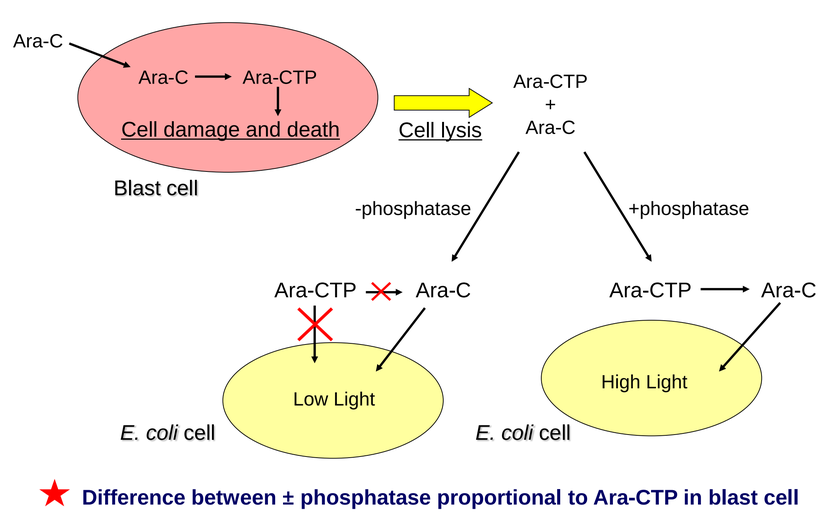
Patient blasts (4 x 10^6) were incubated for 30 minutes with Ara-C at a clinically relevant dose, the equivalent of 2g/m^2. Blasts were washed and lysed prior to exposure to the biosensor. Pre-incubation with fludarabine (5 μM) was for 4 hours. Fludarabine was prepared in DMSO so that cells were exposed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

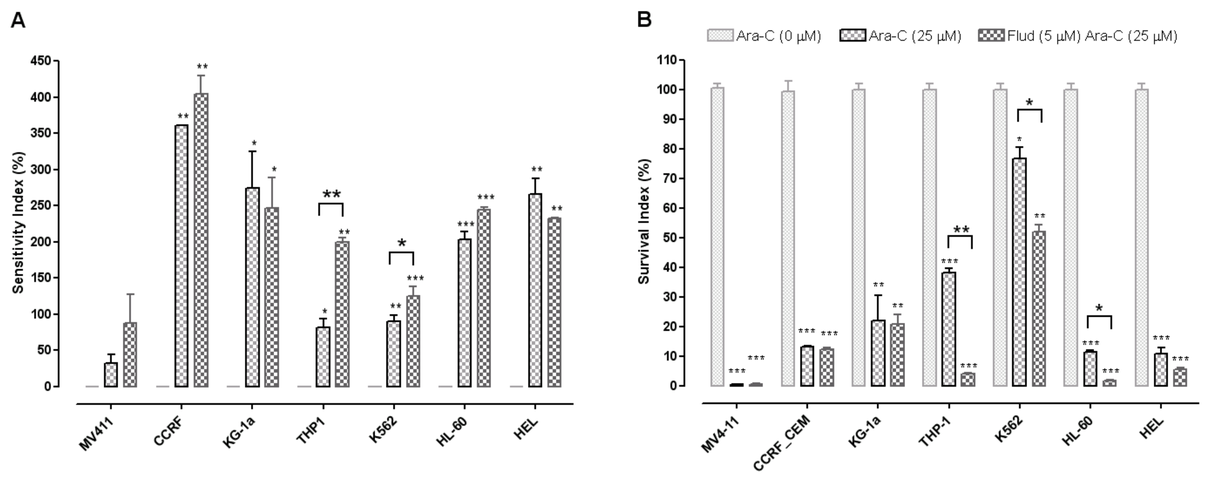
Results

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

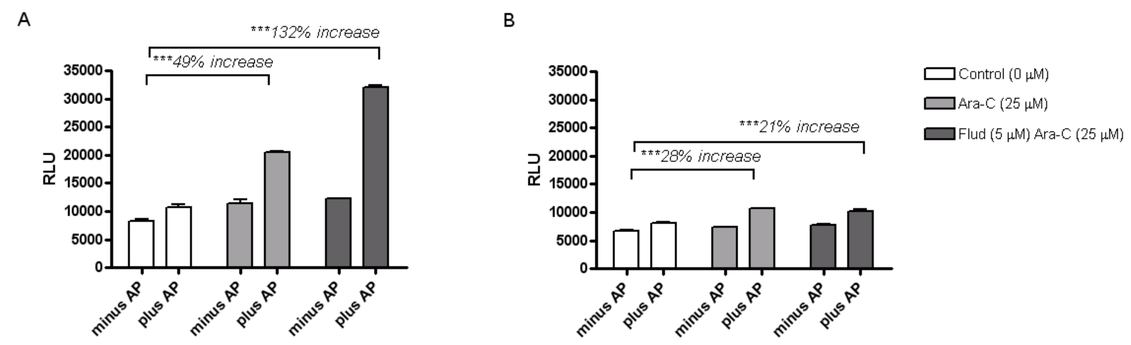


Table 1 shows results for the patient cohort. Patient ages ranged from 27 to 71 (median 53 years) and included four peripheral blood and three bone marrow samples. In four samples the 8-hour assay showed a significant improvement in blast cell sensitivity to combination treatment compared with Ara-C 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%)*

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® 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 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® 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.

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