Rapid In-vitro Testing for Chemotherapy Sensitivity in Leukaemia Patients

Elizabeth Anderson and Vyv Salisbury

Abstract Bioluminescent bacterial biosensors can be used in a rapid in vitro assay to predict sensitivity to commonly used chemotherapy drugs in acute myeloid leukemia (AML). The nucleoside analog cytarabine (ara-C) is the key agent for treating AML; however, up to 30 % of patients fail to respond to treatment. Screening of patient blood samples to determine drug response before commencement of treatment is needed. To achieve this aim, a self-bioluminescent reporter strain of *Escherichia coli* has been constructed and evaluated for use as an ara-C biosensor and an in vitro assay has been designed to predict ara-C response in clinical samples. Transposition mutagenesis was used to create a cytidine deaminase (cdd)-deficient mutant of *E. coli* MG1655 that responded to ara-C. The strain was transformed with the *lux*CDABE operon and used as a whole-cell biosensor for development an 8-h assay to determine ara-C uptake and phosphorylation by leukemic cells. Intracellular concentrations of 0.025 μmol/L phosphorylated ara-C were detected by significantly increased light output ($P < 0.05$) from the bacterial biosensor. Results using AML cell lines with known response to ara-C showed close correlation between the 8-h assay and a 3-day cytotoxicity test for ara-C cell killing. In retrospective tests with 24 clinical samples of bone marrow or peripheral blood, the biosensor-based assay predicted leukemic cell response to ara-C within 8 h. The biosensor-based assay may offer a predictor for evaluating the sensitivity of leukemic cells to ara-C before patients undergo chemotherapy and allow customized treatment of drug-sensitive patients with reduced ara-C dose levels. The 8-h assay monitors intracellular ara-CTP (cytosine arabinoside triphosphate) levels and, if fully validated, may be suitable for use in clinical settings.

Keywords Bioluminescent · Biosensor · Cytarabine · Leukaemia chemotherapy

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G. Thouand and R. Marks (eds.), *Bioluminescence: Fundamentals and Applications in Biotechnology–Volume 2*, Advances in Biochemical Engineering/Biotechnology 145,
DOI: 10.1007/978-3-662-43619-6_6,

Abbreviations

AP	Alkaline phosphatase
AML	Acute myeloid leukemia
ara-C	Cytarabine, cytosine arabinoside
ara-CMP	Cytosine arabinoside monophosphate
ara-CTP	Cytosine arabinoside triphosphate
cdd	Cytidine deaminase
CLA	Cladarabine/cytarabine
DNR	Daunorubicin
dCK	Deoxycytidine kinase
FLA	Fludarabine/cytarabine
hENT1	Human equilibrative nucleoside transporter
IPTG	Isopropyl β-D-1-thiogalactopyranoside
NPM1	Nucleophosmin-1 gene
pyrE	Orotate phospho-ribosyltransferase gene

Contents

1 Introduction: Bioluminescent Biosensors for Drug Sensitivity

The *luxCDABE* gene cassette from the terrestrial bacterium *Photorhabdus luminescens* has facilitated the wide range of clinical applications using bioluminescent reporters. In contrast to the marine bacteria *Aliivibrio fischeri* and *Vibrio harveii* where *lux* gene expression has a limited temperature range, the *lux* cassette from *P. luminescens* is fully functional at clinically relevant temperatures up to 45 °C [1] and can be inserted into Gram negative pathogens to enable rapid testing for antibiotic sensitivity and real-time monitoring of antimicrobial pharmacodynamics [2]. All the *lux* genes essential for luminescence are arranged in the single gene cassette; *luxCDE* encode a fatty acid reductase complex involved in synthesis of the fatty aldehyde substrate for the luminescence reaction catalysed by the luciferase *LuxAB* subunits [3] Two of the substrates for the bioluminescent reaction, reduced flavin mononucleotide ($FMNH_2$) and molecular oxygen, are both readily available in aerobic bacteria and expression of the complete *lux* cassette gives light output without the need for any exogenous substrate [1] The *P. luminescens lux* gene cassette has also been modified to give expression in Gram positive bacteria [4, 5] allowing extended clinical applications.

1.1 Bioluminescent Reporter Technology

Bioluminescent bacterial biosensors produce light as a direct indicator of the physiological status of the bacteria in real time. The high metabolic rate of bacterial cells, compared to mammalian cells, means that these reporters are ideal for fast, accurate, real time in situ testing. Response to drugs, toxic chemicals or other environmental insults can be measured in a non-destructive fashion with a high level of sensitivity and they are biosensors of choice for comparing the efficacies of fast acting biocides, within milliseconds of challenge [6]. Light output from the biosensor can be very accurately measured, with no background interference, using either luminometers or low light cameras.

There are some limitations to the employment of this technology; the need for molecular oxygen restricts the use of bioluminescent biosensors in anaerobic environments, although it has been reported that 10 nM oxygen is sufficient for luminescence detection [7]. Also the light produced by bioluminescent biosensors at a wavelength of 490 nM, is absorbed by mammalian cells and particularly by haemoglobin and melanin, with an estimated 10 fold reduction of light output for every cm of mammalian tissue [8]. However it is possible to image light output through skin muscle and bone—making non-invasive monitoring of disease processes possible using small mammals such as mice [9, 10]

1.2 Real-Time Monitoring of Anti-microbial Effects

The pharmacodynamics of antimicrobial agents are usually studied by observing changes in viable counts of bacteria over time for a range of drug concentrations, demonstrating the ability of the agent to prevent bacterial cell replication [11]. Although this method accurately demonstrates antimicrobial effects on bacterial cell replication, it is indirect and requires significant incubation time to produce countable bacterial colonies. Introduction of real-time monitoring using bioluminescent constructs of bacterial pathogens, where the *lux* genes are regulated by a constitutive promoter, has enabled direct, real time testing of antimicrobial effects on bacterial metabolism both in vitro and in vivo, using the neutropenic-mouse thigh model of infection [12].

A *Streptococcus pneumoniae* biosensor expressing the modified *P. luminescens luxABCDE* operon has been used to test novel antimicrobial agents including linezolid and gemifloxacin [4, 13]. Bioluminescence point readings and recovery counts were performed hourly over 12 h and showed that bioluminescence gave an earlier indicator of cellular recovery than counting techniques. Other fluoroquinolones, including moxifloxacin and ciprofloxacin, have been used to challenge a bioluminescent *E. coli* biosensor [2] and a bioluminescent biosensor strain of *Pseudomonas aeruginosa* [14], the latter in a continuous perfusion model of biofilm growth. Bioluminescent bacterial biosensors have also been used as an in vitro wound model for testing antimicrobial wound dressings [15] and a bioluminescent *E. coli* biosensor has demonstrated the antimicrobial properties of fresh human and bovine milk [16].

All of these bioluminescent bacterial biosensors were found to be sensitive, real-time reporters of antimicrobial efficacy. Similar biosensors have been used to assess susceptibility to fast acting biocides including phenol, Virkon and chlorhexidine digluconate [17], ethanol [18] and electrochemically activated solutions [6]. Bioluminescence may be used to monitor real-time, almost instant effects and distinguish the different mechanism of action of biocides.

1.3 Intracellular Anti-microbial Effects

Bioluminescent constructs of pathogenic bacteria have been successfully employed to monitor intracellular bacterial survival and antibiotic susceptibility inside mammalian cells [5]. Bioluminescent reporter bacteria are incubated with mammalian cells to allow for uptake. External bacteria are then removed with a bactericidal agent that is not taken up by mammalian cells (such as colistin or lysostaphin) and light output from an intracellular bioluminescent reporter can be used to monitor intracellular bacterial survival and drug penetration into cells.

Following on from this work—the possibility of using a bioluminescent bacterial biosensor for chemotherapy monitoring in leukaemic cells was considered.

The rapid, real-time monitoring of light output presented a simple and direct method of demonstrating drug uptake and conversion to an active form by leukaemic cells.

2 The Clinical Need in Leukaemia

Acute myeloid leukaemia (AML) is a heterogeneous group of haematological disorders resulting from the malignant transformation of myeloid precursor cells. The acute leukaemias are aggressive disorders in which one or more transformations occur in a haematopoietic stem cell or progenitor cell. This can lead to the development of a clonal malignancy via either increased proliferation, reduced cell death (apoptosis) or a block in the differentiation and maturation process. This leads to the proliferation of immature and undifferentiated cells in the blood and bone marrow and suppression of normal haemopoiesis [19]. The presenting symptoms are linked to bone marrow failure caused by accumulation of these malignant blast cells in the bone marrow, including frequent infections due to neutropaenia, anaemia and thrombocytopaenia (reduced clotting). In addition, infiltration of blasts cells may occur in the gums, skin and potentially central nervous system (CNS). If left untreated, the acute leukaemias are rapidly fatal, with patients succumbing within weeks or months. The disease is diagnosed by morphological examination of the blood and bone marrow, defined as the presence of >20 % blasts in the bone marrow at clinical presentation. Immunophenotyping is used to define the lineage of the leukaemic blasts and classify the disease according to the WHO classification of AML (2008). Accurate classification is very important as treatment selection and patient prognosis are intimately linked to the subtype of AML. In addition, much research has been dedicated to the investigation of key cytogenetic and molecular markers that are linked to prognosis and classification of the disease (Fig. 1). There is a clear correlation between cytogenetic scoring, categorised as favourable, intermediate or adverse and overall survival of AML patients [20]. One key mutation is the internal tandem duplication (ITD) of the *FLT3* gene, a receptor-type tyrosine kinase detected in approximately 20 % of AML patients and associated with a poor prognosis [21]. This effect can be negated by the presence of a point mutation in the nucleophosmin-1 gene *NPM1*, which reverses the poor prognosis for patients with *FLT3*-ITD [22] (Fig. 2).

One of the main issues for the patient is the speed of progress of the disease. When a patient is diagnosed with AML, they will often be required to commence treatment same-day. The decision to treat and the outcome for an individual patient will be dependent on a number of factors, with the single largest determinant being age of the patient at diagnosis. This is linked to the inability of elderly patients (>70 years) to tolerate the intensive chemotherapy regimens that have led to vast improvement in remission rates for younger patients (Fig. 3).

Given that the UK median age for diagnosis of AML is 65 years, this is a very important consideration. Other factors include sex of the patient, white blood cell

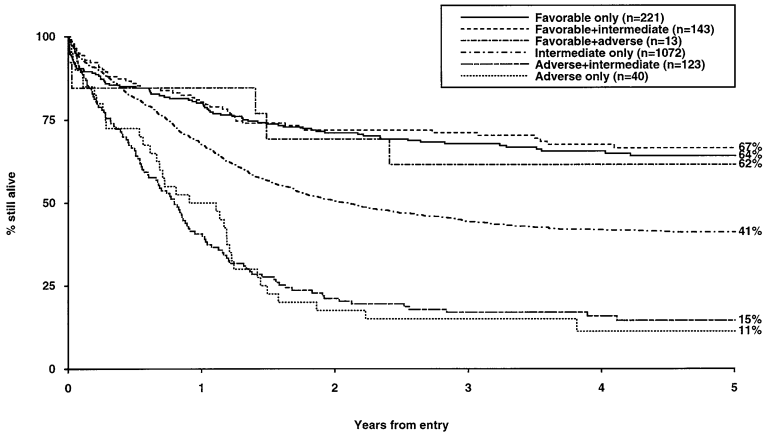

Fig. 1 Influence of cytogenetic abnormalities on overall survival in AML [2]

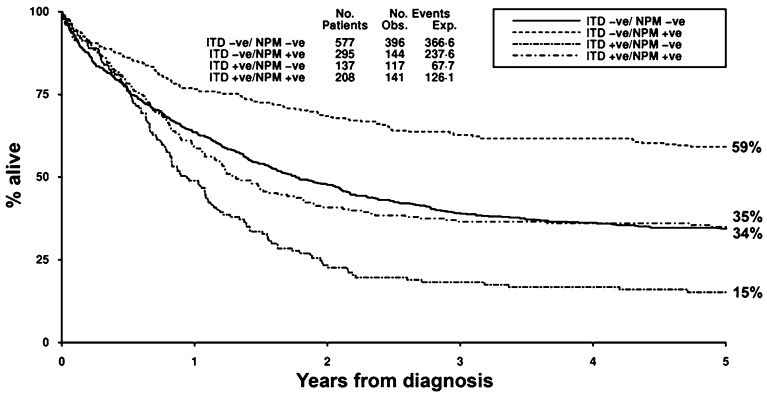

Fig. 2 Outcome stratified according to FLT3/ITD and NPM1 mutant status in total cohort [4]

(WBC) count at presentation, secondary disease status, and the specific genetic abnormalities associated with the patient's disease. Figure 4 illustrates patient progress following diagnosis. Treatment involves induction and consolidation phases to induce remission and to maintain low levels of any residual disease. Remission is defined as a reduction in the circulating blast burden by 95 % within the first 6 weeks post-commencement of treatment. The main chemotherapeutic agents used in induction and consolidation treatment of presenting patients are the anthracycline daunorubicin (DNR) and the nucleoside analogue cytosine arabinoside (cytarabine; ara-C).

Effective treatment of AML patients is still challenging and the clinical outcome is often unpredictable. Only 70 % of newly diagnosed patients receiving standard regimens including ara-C respond to treatment. Furthermore, a large

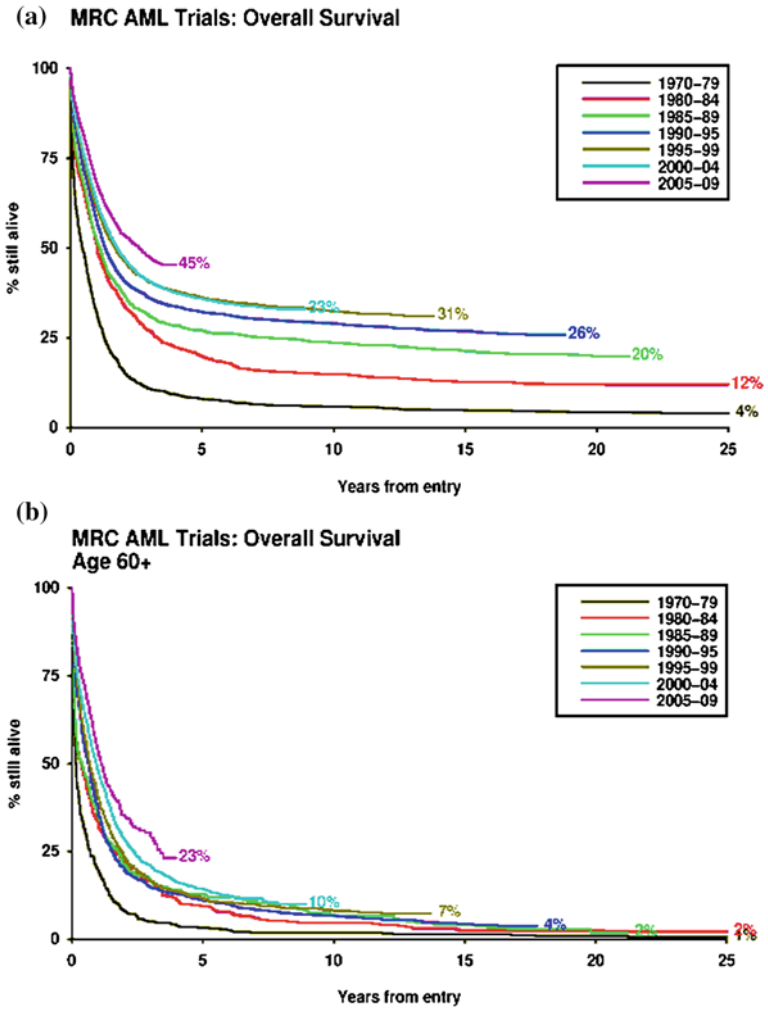

Fig. 3 Survival of adult patients with AML treated in UK trials over the past four decades. **a** Patients over 60 years and, **b** Aged 16–59 years

proportion of these patients fail to achieve long-term remission and also develop resistance to subsequent therapy [23].

Ara-C is one of the most active single anticancer agents and has been the mainstay treatment of AML for over three decades. It is used as in different doses at presentation (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 relapse where it is combined with other cytotoxic agents such as the ribonucleotide reductase inhibitor fludarabine [5]. Resistance to chemotherapy, including cytarabine, is a major reason for treatment failure among patients with AML [24]. A study from the Cancer and Leukaemia Group B

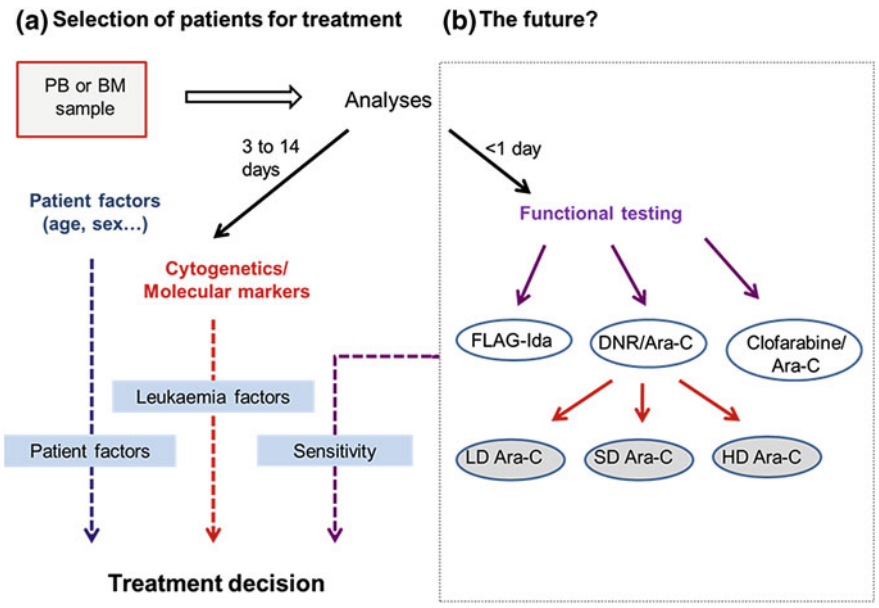

Fig. 4 Flow diagram illustrating **a** the current selection procedure for AML patients suitable for treatment with intensive chemotherapy and **b** the future including identification of the patient's individual chemosensitivity profile

examined 474 patients younger than 60 years and demonstrated a 34 % 5 year overall survival rate [25]. The outlook for older patients is even worse [26]. This is largely due to poor tolerability of intensive chemotherapy regimens in patients over 70 years, and the presence of primary disease resistance. Treatment-related mortality is a larger factor in the elderly, with death due to infection, haemorrhage and organ failure more common. Clinicians currently judge fitness of the individual patient, and if intensive treatment is deemed unsuitable then supportive care is provided with or without inclusion of low-dose single agent chemotherapy. Low-dose ara-C (20 mg/m^2/day) is used in this situation and can prolong survival without the morbidity and mortality associated with standard or high dose therapy in this age group.

In vivo, ara-C is transported into the cell via the specific human equilibrative nucleoside transporter (hENT1), and is rapidly phosphorylated by deoxycytidine kinase (dCK) to its monophosphate form. Ara-CMP is further phosphorylated by nucleoside kinases into its active tri-phosphorylated form, ara-CTP. Drug inactivation can result from ara-C conversion into ara-uracil (ara-U) by cytidine deaminase or from dephosphorylation of ara-CMP by cytoplasmic nucleotidase [27]. The antiproliferative and cytotoxic effects of ara-CTP are due to its ability to interfere with DNA polymerase and to incorporate into DNA strands leading to chain termination and DNA synthesis arrest. High-dose ara-C can also cause accumulation of cytochrome c in the cytosol, loss of mitochondrial membrane

potential and an increase in reactive oxygen species [24, 25]. Ara-C has recently been found to induce apoptosis via the death receptor pathway, involving signalling through sphingomyelin enriched plasma membrane lipid rafts [26]. Chemoresistance to ara-C can arise from a number of factors influencing the rate of ara-CTP formation and incorporation into DNA including low drug uptake via reduced expression of the transporter hENT1, increased conversion into ara-U by cytidine deaminase, reduced level or activity of the enzyme dCK, or increased dephosphorylation of the active metabolite by cytoplasmic nucleotidase [28].

AML is treated at presentation with a combination of DNR and ara-C. DNR inhibits topoisomerase II and subsequent uncoiling of DNA prior to DNA replication, and is also used in combined chemotherapy regimens to treat acute lymphoid leukaemia (ALL) and non-Hodgkin's lymphoma. DNR is a chemotherapeutic drug with serious adverse effects including cardiotoxicity that impacts on the usefulness of the drug. This cardiotoxicity is cumulative and so treatment with DNR at presentation precludes use of the drug after induction failure. For those patients that fail to achieve remission or who subsequently relapse a second cocktail of chemotherapeutic agents is used. The nature of AML means that the relapsed clone is often immunophenotypically distinct from the presenting clone and will require an alternative treatment regimen. At relapse the most common regimen is fludarabine, high dose ara-C, the anthracycline idarubicin and granulocyte-macrophage colony stimulating factor (GM-CSF). Fludarabine, and more recently clofarabine, are purine analogues that inhibit ribonucleoside reductase in the AML cell, reducing the ability of the cell to produce endogenous nucleotides. This acts to potentiate the intercalation of ara-CTP into the DNA, and can be very useful in treating cells that do not respond well to ara-C alone.

It is important to emphasise that currently no pre-treatment assessment of chemotherapy efficacy is performed for individual patients prior to treatment. In the research setting in vitro assessment of ara-C efficacy has traditionally involved measurement of (a) cell death (b) reduction in S-phase activity or (c) use of AML clonogenic assays for leukaemic cells exposed to ara-C [29]. However, these methods are non-standardised, time consuming, expensive and are not suitable for routine screening. Therefore, patients are treated with regimens including ara-C regardless of their sensitivity to the drug and can suffer the serious side-effects such as myelosupression, chemical conjunctivitis, cerebellar dysfunction and the development of drug resistant secondary cancers [30].

The use of bioluminescent biosensor technology to develop ara-C/DNR and ara-C/fludarabine biosensors has great potential to make a difference to patients with AML. A biosensor to predict response to anthracyclines (daunorubicin, doxorubicin, idarubicin) would have particular benefits because they cause dose-dependent cardiotoxicity that can lead to clinical heart failure. For example, if the biosensor predicts a high level of anthracycline sensitivity, treatment with reduced dose lessens risk of heart damage. Prediction of resistance may lead a clinician to avoid this cardiac-associated risk altogether in favour of an alternative combination therapy.

Patients with AML and their healthcare teams, including clinicians, laboratory workers and Acute Healthcare Trusts could benefit directly and immediately.

Patients could benefit from receiving tailored treatment that will specifically target their cancer, providing the clinician with a 'drug response profile' to combinations and doses of chemotherapeutics, to inform patient treatment decision making. Patients with highly responsive cancer cells (especially those over 70 years) could be given effective low dose chemotherapy with subsequent reduced side effects, including reduction in hospitalisation, time off work and risk of secondary malignancies. The rising numbers of elderly AML patients will be key beneficiaries. These patients may have health conditions that rule out aggressive chemotherapy; test results that indicate effectiveness of low dose chemotherapy will be vital for their treatment. Those with ara-C resistant cells could be given effective combined chemotherapy to negate the risks of single agent failure.

Patients will also benefit from receiving test results in the first 24 h of diagnosis, a time of maximum anxiety for patients and their families. Rapid results that indicate the most effective chemotherapy will help with difficult decisions about treatment that have to be made within hours of diagnosis for this rapidly progressive disease.

Healthcare providers will benefit from targeted chemotherapy leading to reduced hospitalisation of patients with adverse side effects and possible reduction in additional courses of chemotherapy. In the long term, the bioluminescent biosensor technology could be used to predict response to chemotherapy in a range of haematological malignancies, including the chronic leukeamias, lymphomas and myelomas and also in solid tumours, where minor adjustments of the assay protocol would allow rapid testing of biopsy material for response to an array of relevant drugs. This is a platform technology; the biosensor is capable of response to a number of cytotoxic nucleoside analogues and could also be used for therapeutic drug monitoring. The technology has the potential to contribute to the health of patients diagnosed with haematological and solid tumour cancers by rapidly predicting response to different doses and combinations of chemotherapy.

3 Construction of a Bacterial Biosensor for Chemotherapy Sensitivity Testing

3.1 Mode of Action of Target Drug

Cytarabine (ara-C) is an anti-cancer agent widely used in chemotherapy for over three decades. In vivo, ara-C is transported into the cell via the specific nucleoside transporter (hENT1), and is rapidly phosphorylated by deoxycytidine kinase (dCK) to its monophosphate form. Ara-CMP is further phosphorylated by nucleoside kinases into its tri-phosphorylated active form ara-CTP [31]. Drug inactivation can result from ara-C conversion into ara-uracil by cytidine deaminase or from dephosphorylation of ara-CMP by cytoplasmic nucleotidase [29]. The antiproliferative and cytotoxic effects of ara-CTP are due to its ability to interfere with

DNA polymerase and to incorporate into DNA strands leading to chain termination and DNA synthesis arrest [32]. Resistance to ara-C results from factors that influence the rate of ara-CTP formation and incorporation into DNA; a biosensor that measures ara-CTP levels within leukaemic cells will predict sensitivity or resistance to the drug [31].

In vitro assessment of ara-C efficacy has traditionally involved measurement of leukaemic cell death or S phase activity, following exposure to the drug, or the use of clonogenic proliferative assays [29]. These methods are cumbersome, time consuming, expensive and unsuitable for routine screening. Therefore patients are treated with regimens including ara-C regardless of their sensitivity to the drug. [31].

3.2 Construction of Cytarabine Sensitive E. Coli *Host*

Ara-C has no effect on *E. coli* as the bacteria lack deoxycytidine kinase (dCK) and deaminate ara-C into ara-uracil through the activity of deoxycytidine deaminase (cdd). Alloush et al. [31] constructed a suitable cytarabine sensitive strain by starting with *E. coli* MG1655, an auxotrophic derivative of wild-type *E. coli* K-12 that requires pyrimidine in order to grow in minimal medium, due to suboptimal expression of the enzyme orotate phospho-ribosyltransferase coded for by the orotate phosphoribosyltransferase (*pyrE*) gene [34]. This strain was rendered cdd-deficient using random transposition mutagenesis with P1phage carrying a Tn10 transposon, which can transpose from the phage into the *E. coli* chromosome. Mutants carrying the transposon were selected on Luria Bertani agar plates containing 10 mg/L tetracycline. The *cdd* mutants were selected after 24-h incubation by growth at 37 °C on plates containing10 mg/L of the analog 5-fluoro-2_ deoxycytidine, which is toxic to *E. coli* expressing *cdd* activity [31]. The resulting *pyrE* deficient, *cdd* deficient strain was transformed with plasmid pTrcHUMdCK, expressing the human deoxycitidine kinase cDNA under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter [35]. The transformed strain gave IPTG inducible expression of dCK resulting in growth inhibition by 100 μM ara-C, as long as IPTG was present (Fig. 5b). This constituted a suitable host bacterial strain [31], with increased uptake of ara-CTP, rather than de novo dCTP synthesis (due to low level of *pyrE* expression), reduced conversion of ara-C to ara-U (due to *cdd* transposon knockout) and increased conversion of ara-C to ara-CTP (due to IPTG inducible dCK expression).

3.3 Bioluminescent Biosensor Development

The broad host range vector plasmid pBBR1MCS-2 [36] carrying the *luxCDABE* cassette from *P. luminescens* as an *Eco*R1 PCR fragment, was used by Alloush et al. [31] to transform the cytarabine sensitive host *E. coli* strain to give the self

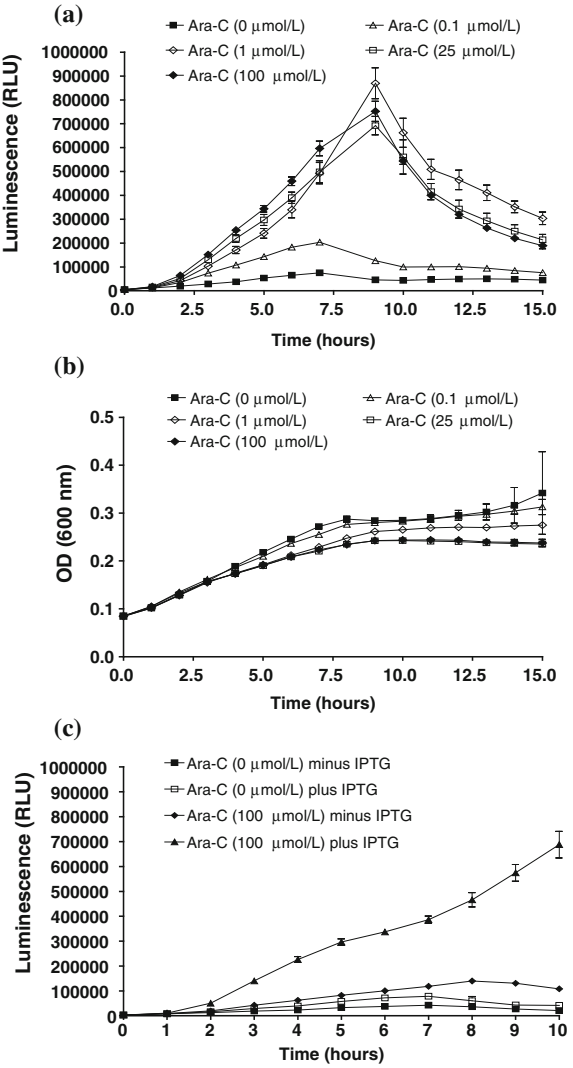

Fig. 5 Biosensor response. The effect of ara-C concentrations (0.1, 1, 25, and 100 µmol/L) on luminescence (**a**) and growth (**b**) of *E. coli* HA1 in the presence of IPTG; the effect of IPTG induction of dCK in the presence of ara-C (**c**) is also shown. Growth was monitored by measuring 600 A; luminescence is shown as relative light units (RLU); n = 10 and error bars represent SD

bioluminescent bacteria biosensor *E. coli* HA1. When challenged in LB broth or RPMI 1640 medium with ara-C in the presence of IPTG, there was a significant increase of light output from the biosensor (Fig. 5a) with concentrations as low as 0.1 µmol/L. This significantly increased light output was only observed during treatment with the pyrimidine analog ara-C and only in the presence of IPTG-activated dCK (Fig. 5c). The specificity of the biosensor to ara-C was indicated by a lack of biosensor response in a control assay with the purine analog fludarabine. To increase the specificity of the biosensor, direct effects of the active intracellular drug derivative, ara-CTP, on the bacterial biosensor were monitored (Fig. 6). The

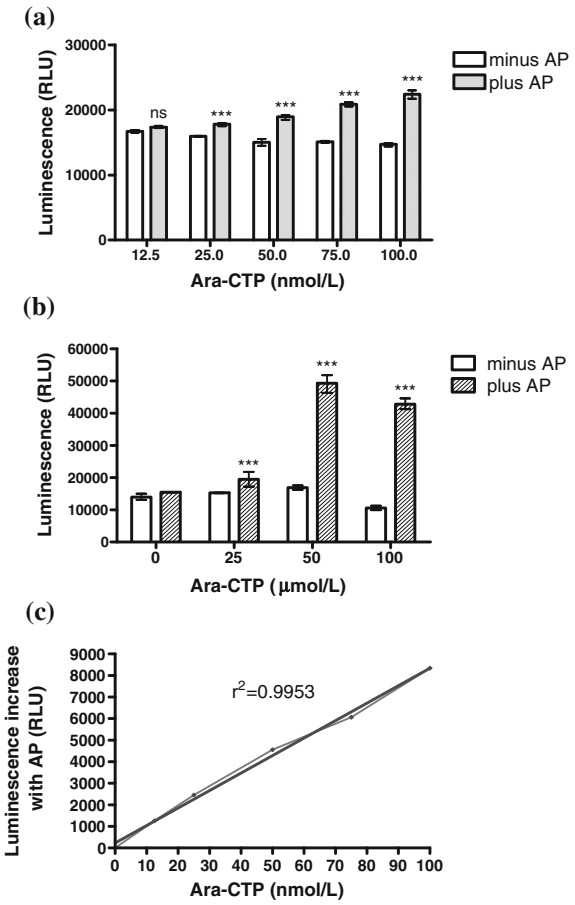

Fig. 6 The effect of ara-CTP on the biosensor. *Peak* light emission, measured as relative light units (RLU), from *E. coli* HA1 in RPMI at 37 °C treated with ara-CTP at 12.5, 25, 50, 75, and 100 nmol/L (**a**) and 0, 25, 50, and 100 µmol/L (**b**) in the absence and presence of AP (mean of n = 3 and error *bars* show range). Correlation between ara-CTP and light emission ±AP (**c**)

results indicate that ara-CTP does not enter the reporter bacteria, showing no increase in light output compared with the untreated control, unless alkaline phosphatase is added at the start of the assay. The increase in peak light output in the AP-treated samples (Fig. 6a, b) is similar to that observed with ara-C [31].

The bioluminescence increase brought about by ara-C in the biosensor is similar to previous reports of enhanced light emission in luciferase-based biosensors brought about by impairment of the bacterial intracellular equilibrium, leading to NADPH accumulation [37], or by DNA damage [38]. It is reported that bioluminescence stimulates DNA repair in bacteria, possibly by providing photons for bacterial photolyase activity [39] and that lux genes are regulated by the bacterial SOS stress response [40], which may explain the increase in bioluminescence in the presence of ara-C.

The bioluminescent bacterial biosensor developed by Alloush et al. [31] can be maintained stably by selection on nutrient agar supplemented with 10 mg/L

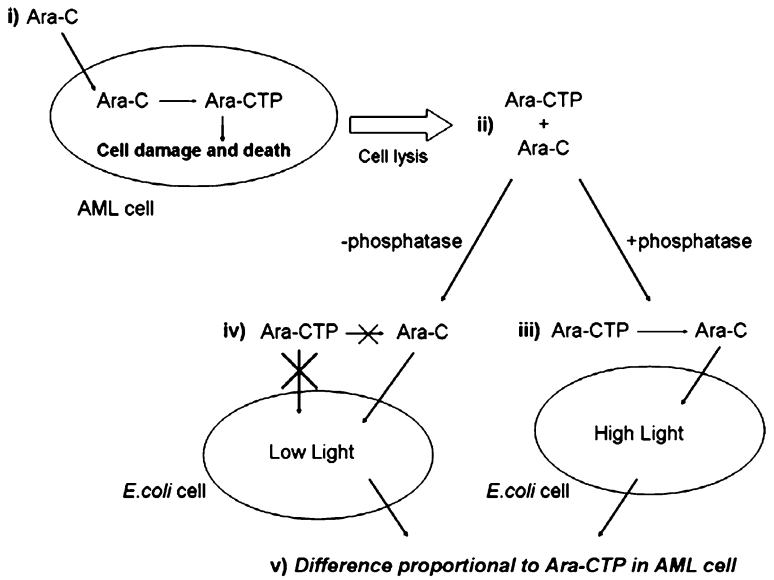

Fig. 7 Biosensor assay procedure. *(i)* AML cells from patients with AML exposed to *ara-C* are washed and lysed in the presence of EDTA and saponin. *(ii)* Subsequent cell lysate (containing both *ara-C* and *ara-CTP*) are exposed to the bacterial biosensor in the presence or absence of alkaline phosphatase (*AP*). *(iii)* In the presence of *AP*, *ara-CTP* is converted to *ara-C*, which on entering the bacterium allows the generation of bioluminescence. *(iv)* In the absence of *AP ara-CTP* remains intact and cannot enter the bacterium. *(v)* The ratio between ±AP is directly proportional to the concentration of *ara-CTP* in the patient blasts, which is representative of the patient's ability to convert *ara-C* to *ara-CTP*

tetracycline, 100 mg/L ampicillin and 25 mg/L kanamycin (for maintenance of Tn10, pTrcHUMdCK and pBBR1MCS-2lux+, respectively). It can be lyophilized for use in a routine in vitro assay for cytarabine sensitivity of clinical samples of leukaemia patient bone marrow or peripheral blood.

4 Validation of the Biosensor Assay EA

Following design and construction of a biosensor with enhanced sensitivity to ara-C, we developed the technology into a rapid assay to determine ara-C uptake and phosphorylation by human leukaemic cells (Fig. 7). The assay principle involves exposure of AML blast cells to ara-C (25 μM) for 30 min, during which a proportion of ara-C is transported into the cell via the transporter hENT1, and metabolised by dCK to ara-CTP. Following exposure to ara-C, blasts are washed and lysed by addition of saponin (which does not affect the integrity of the bacterial biosensor) in the presence of EDTA to inhibit endogenous alkaline

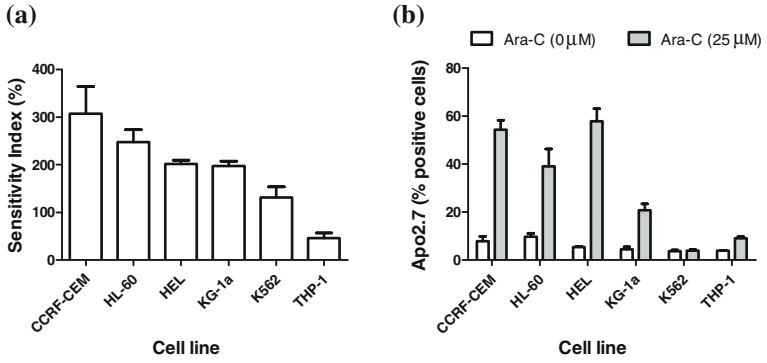

Fig. 8 Comparison of **a** accumulation of ara-CTP in leukaemic cells as measured by the biosensor assay after 30 min incubation with ara-C and **b** expression of the apoptotic marker APO2.7 in cells treated for 48 h with ara-C. APO2.7 detects a mitochondrial protein, 7A6 that is expressed in early apoptosis

phosphatases (AP). The addition of exogenous AP results in conversion of ara-CTP to ara-C that can enter the biosensor and induce bioluminescence. Without addition of AP only ara-C can enter the biosensor, and the remaining pool of ara-CTP remains excluded. The resultant difference in bioluminescence is proportional to the level of ara-CTP in the lysate.

4.1 Validation of Ara-CTP Measurement

Validation of this biosensor assay was carried out using immortalised leukaemic cell lines with known and differing sensitivities to ara-C [41]. Cell lines were treated in vitro with ara-C at a range of in vivo relevant concentrations, and the effect assessed using a range of research techniques. Results from the biosensor assay 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.

Across the cell lines treated with cytarabine (25 μM) there was a significant correlation between ara-CTP levels measured using HPLC and the sensitivity index values from the biosensor assay ($R = 0.9722$, $p = 0.0028$) indicating that the biosensor is accurately measuring intracellular ara-CTP. In addition, there was a correlation in the majority of the cell lines between ara-CTP accumulation and cell death as measured by 3-day cytotoxicity assay, and expression of the mitochondrial protein 7A6, expressed in early apoptosis (Fig. 8). This indicates that the biosensor assay is capable of determining relative sensitivity to ara-C.

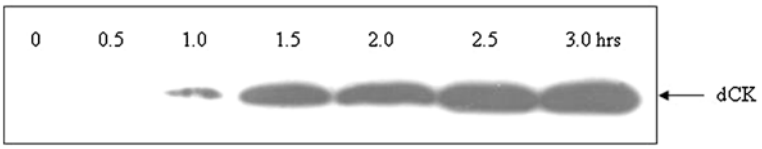

Fig. 9 Representative Western blot for time course of IPTG-induced expression of dCK protein between 0 and 3 h

4.2 Assay Sensitivity and Specificity

Figure 9 shows expression of the human *dCK* gene within the biosensor. This is selectively controlled by addition of IPTG to the biosensor, which causes a time-dependent increase in the level of dCK as measured by Western Blotting using a specific antibody. This corroborated by a time-dependent increase in light output from the biosensor in response to ara-C (Fig. 10a) that is specific to ara-C and not the purine analogue fludarabine (F-ara-A) (Fig. 10b).

Figure 11 illustrates the inverse relationship between light output from the biosensor and toxicity of ara-C to the biosensor strain. On addition of ara-C the biosensor demonstrates an impressive 2.5-log increase in light output per bacterial colony-forming unit (cfu) (Fig. 10a). This is emphasised by the concurrent decrease in bacterial viability in response to ara-C (Fig. 10b).

Figure 12a, b describe 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 and a linear relationship was observed up to 0.5 μM. This concentration range represents a suitable detection range to allow differentiation between ara-C responsive and non-responsive leukaemic cell lines.

4.3 Assay Reproducibility and Stability

To evaluate assay precision [42], 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) (Table 1). Standard deviation (SD) and coefficient of variation (CV) were calculated for intra-day and inter-day assay results. An acceptable level of precision was defined as ±15 % CV, which was achieved for the LQC, MQC and HQC samples (0.05–0.5 μM). 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. Intra- and inter-run CV <15 % were achieved for all QC levels across the three runs.

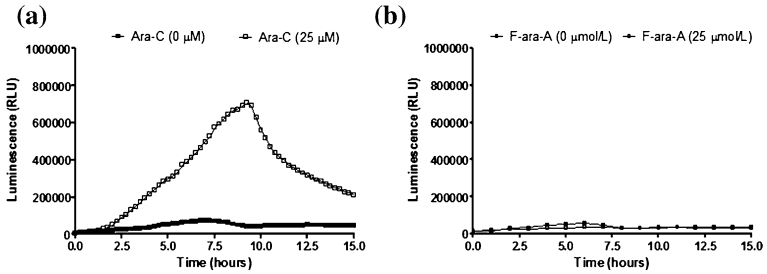

Fig. 10 The effect of IPTG-induced expression of dCK on light emission from the biosensor following exposure to the pyrimidine analogue ara-C (**a**) but not the purine analogue fludarabine (F-ara-A) (**b**). RLU, relative light units

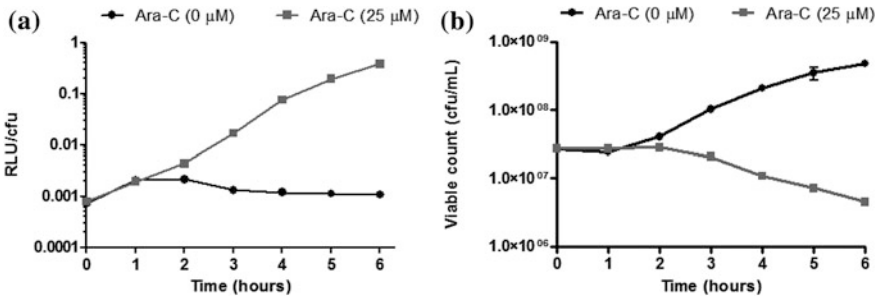

Fig. 11 The relationship between light output and toxicity of ara-C to the biosensor strain. **a** Light output from the biosensor per colony-forming unit (*cfu*) in response to ara-C, and **b** the effect of ara-C on growth of the biosensor. *RLU* relative light units

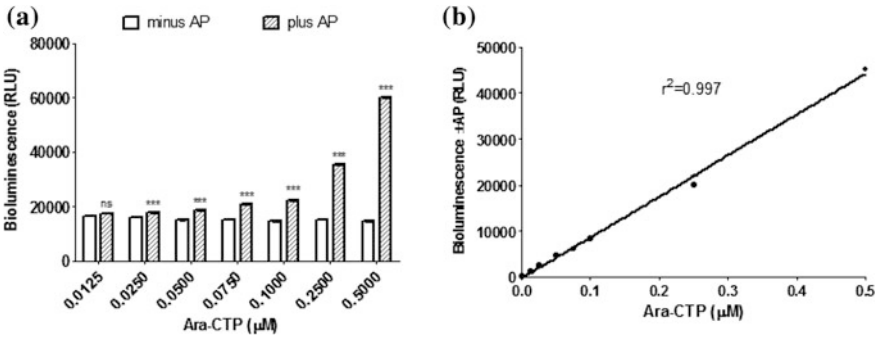

Fig. 12 *Peak* bioluminescence from the biosensor treated with ara-CTP (0–0.5 µM) ± alkaline phosphatase (**a**), and the resultant calibration *curve* (**b**)

For convenience and ease of use in a clinic laboratory setting, the biosensor was lyophilized and the stability verified over a 12 month period. Figure 13 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

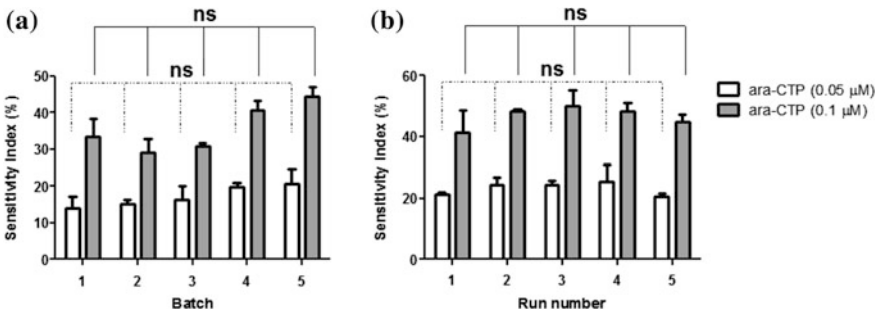

Fig. 13 Inter- and intra-batch stability testing for the lyophilized biosensor. **a** Inter-batch assessment was performed using 5 batches of lyophilized biosensor produced over a period of 12 months. **b** Intra-batch assessment was performed using batch 5 of the lyophilized biosensor over the period of 12 months (n = 5)

Table 1 Assessment of assay precision using QC samples assayed in three independent batch runs

Run id	LQC (0.05 μM)	MQC (0.1 μM)	HQC (0.5 μM)
1			
Intraday mean	0.049	0.112	0.451
Intraday SD	0.006	0.011	0.050
Intraday CV %	11.5	10.0	11.0
2			
Intraday mean	0.045	0.087	0.471
Intraday SD	0.005	0.012	0.048
Intraday CV %	12.0	13.5	10.1
3			
Intraday mean	0.050	0.112	0.426
Intraday SD	0.007	0.015	0.037
Intraday CV %	14.1	13.4	8.8
Mean result	0.048	0.103	0.449
Interday SD	0.003	0.015	0.022
Interday CV %	5.5	14.2	5.0
n	15	15	15

Three levels of QC sample were produced covering a range of ara-CTP concentrations of clinical relevance (0.05–0.5 μM). A 5-point calibration curve was used to back-calculate the concentration of ara-CTP in each sample and the mean calculated within run (n = 5). Intra-day and inter-day standard deviation (*SD*) and coefficient of variation (*CV* %) were calculated

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 from the biosensor for low and medium calibrators were 14.8 and 31.9 %, with acceptable CV % values (<15 %) for both batch-to-batch and run-to-run analysis (n = 5). Longer-term and accelerated stability studies are currently on-going to ensure maintenance of assay characteristics.

5 Clinical Testing

Initial clinical testing of the biosensor assay used fresh peripheral blood and cryopreserved bone marrow samples. Mononuclear cells from fresh AML peripheral blood samples (collected at Frimley Park Hospital, Surrey, UK from patients following informed consent) were isolated by density gradient centrifugation and re-suspended in RPMI 1640 medium. Cryopreserved samples, previously separated by density gradient centrifugation to yield the mononuclear cell fraction, were thawed and re-suspended in RPMI 1640 medium prior to incubation with ara-C. In all cases, samples were obtained from patients at presentation with blast burdens >80 % for whom clinical outcome following induction therapy with ara-C was known. In vitro dosing was performed with ara-C at 25 μM, which represents the equivalent of the standard in vivo dose of 200 mg/m^2/day (based on an 80 kg individual).

Preliminary testing of these blood and bone marrow samples taken at presentation from 12 known responding and 12 known non-responding ara-C–treated patients gave biosensor assay responses between 21 and 128 % (median 55 %) for responding patients and between −7 and 6 % (median 0 %) for non-responding patients (Fig. 14). Assay precision is highest at extremes of the detection range (Table 1), indicating that the assay should perform well in a clinical setting. A typical example of each response type is shown in Fig. 15; patient sample CR (with clinical outcome of complete remission) showed a significant difference in the peak light output ($P < 0.001$) in the presence and absence of AP, indicating response to a drug (Fig. 15a), whereas sample NR (nonresponsive clinical outcome) exhibited no significant difference ($P > 0.05$) in light output between the (±) curves, indicating a low concentration of ara-CTP in the cells (Fig. 15b).

In theory any agent capable of potentiating generation of ara-CTP from ara-C can also be tested on the biosensor system. Figure 16 shows results using the assay for combination therapy screening in a patient that received induction therapy with DNR and ara-C but who subsequently relapsed and was treated with a regimen containing fludarabine/ara-C (FLA). The biosensor assay was performed on this sample with ara-C alone or in conjunction with the purine analogue fludarabine. Figure 16 shows an increase in light from the ara-C-treated sample +AP, however pre-treatment with fludarabine (highlighted in red) induced a significant increase in light, indicating that fludarabine had potentiated generation of ara-CTP in this patient.

The biosensor assay was optimised for this combination therapy using a range of pre-incubation periods based on the protocol for in vitro dosing proposed by Ahlman et al. [43]. The optimal dosing schedule required 4 h pre-incubation with fludarabine (5 μM) followed by the optimised protocol with ara-C dosing. This biosensor assay system offers a valuable tool in predicting response in patients receiving ara-C or fludarabine/ara-C. This may be of importance in patients demonstrating chemoresistance, at presentation and at relapse.

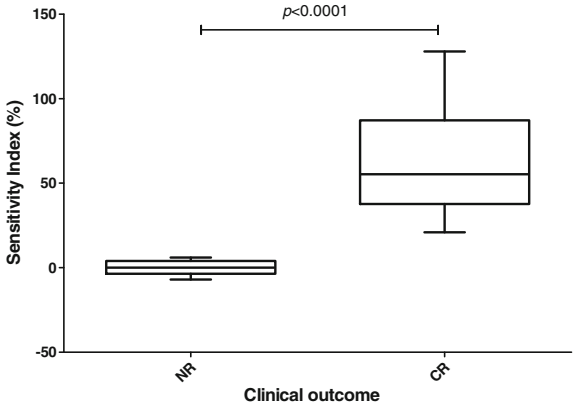

Fig. 14 Box-Whisker plot of patient data from responders (n = 12) and non-responder (n = 12) to ara-C treatment. Median results were 55 and 0 % respectively. *NR* non-remission, *CR* complete remission

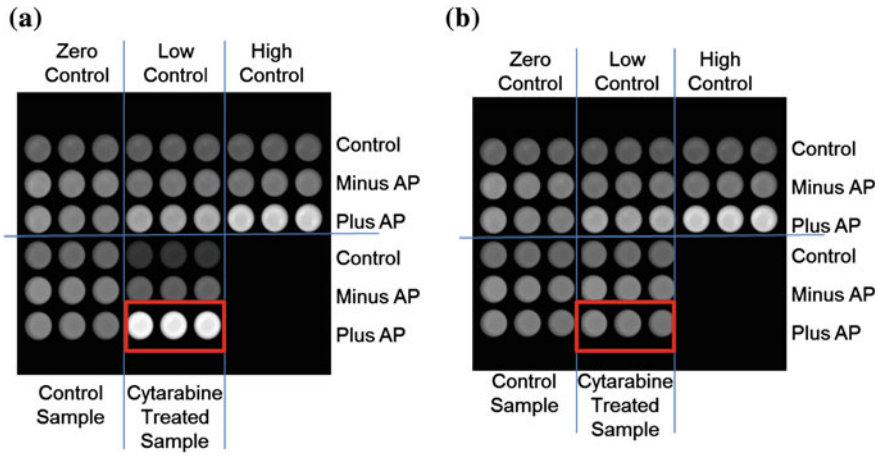

Fig. 15 Comparison of results from the biosensor for (**a**) a patient that responded to treatment with a regimen including ara-C after one cycle of chemotherapy, and (**b**) a patient that failed to respond to ara-C. Areas highlighted in *red* represent ara-C treated samples incubated with exogenous alkaline phosphatase (*AP*) and show increased light in (**a**) but not in (**b**) indicating that patient **a** was capable of producing sufficient ara-CTP to induce remission, whereas patient **b** was not. Controls with known quantities of ara-CTP (zero, low and high) are included to quality control the results. **a** Sensitive patient (remission after 1st cycle). **b** Resistant patient (no remission)

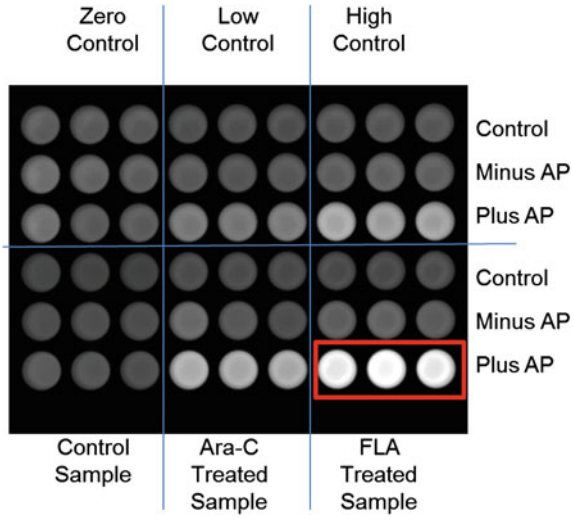

Fig. 16 Comparison of results from the biosensor for a patient that failed to respond to DNR/ara-C at presentation but who responded to treatment with a regimen including fludarabine and ara-C (FLA) at relapse. Light from the biosensor increased dramatically following pre-treatment with fludarabine (*red box*) compared to ara-C alone, indicating that fludarabine was capable of potentiating the production of sufficient ara-CTP to induce a response. *Controls* with known quantities of ara-CTP (zero, low and high) are included to quality control the results

6 Future Perspectives

The construction of a bioluminescent bacterial biosensor for chemotherapy sensitivity testing has paved the way for a rapid assay platform that can be used in a clinical setting, enabling determination of the degree of response before patients undergo chemotherapy.

A single drug predictive test has been designed and validated, for use before AML patients start ara-C chemotherapy. This test has shown close correlation with (i) a 3-day commercially available cytotoxicity test (Promega CellTiterGlo®) and (ii) patient clinical outcomes to therapy. The same blood samples could be concurrently tested for response to combined chemotherapy. Using this technology to develop ara-C/DNR biosensors has great potential. This would be invaluable for patients with AML that frequently require same-day diagnosis and commencement of treatment. At present there is no simple, same-day technology on the market for screening AML patient samples before starting a course of single or combined chemotherapy and no test showing development of resistance during treatment. Also, for patients with very responsive cancer, there is no predictive test to indicate effective minimally toxic treatment with low dose chemotherapy.

6.1 Multi Drug Testing

A biosensor to predict response to anthracyclines (daunorubicin, doxorubicin, idarubicin) would have particular benefits because they cause dose-dependent cardiotoxicity that can lead to clinical heart failure. If the multi-drug test device predicts a high level of anthracycline sensitivity, treatment with reduced dose lessens risk of heart damage. Prediction of resistance may lead a clinician to avoid this cardiac-associated risk altogether in favour of an alternative combination therapy. A multi-drug test device may also have use after the first round of chemotherapy, if patients develop resistance to ara-C. Initial testing with fludarabine/ara-C (Fig. 5.3) and a range of analogues of fludarabine has shown that the biosensor could be used to predict the response to treatment for an individual with relapsed AML.

Current research focuses on miniaturisation of the test to enable more drugs and doses thereof to be tested using a single peripheral blood sample or bone aspirate. The further projected scale of the market for this chemotherapy theranostic biosensor is substantial, because it has the potential to be developed to screen for sensitivity to a wide range of anti-cancer drugs and also, with modification, could be extended to solid tumour biopsy material.

A quick and simple multi-drug test device is under development, based on blood or bone marrow samples before the start of treatment, to show response to combined drugs used for treatment. The results of the multi-drug test will ensure that a patient receives the right combination of chemotherapy drugs, in the correct dose, to meet their needs and so prevent any delay in effective treatment. The test device will be particularly useful to patients whose leukaemic cells are sensitive to low doses of chemotherapy, allowing them to be treated with minimum drug doses, with less side effects. For elderly patients, where AML incidence and mortality rates are increasing, and where other health conditions may rule out aggressive chemotherapy, this screening test could be particularly timely.

6.2 Cytarabine Biosensor

The current bacterial biosensor relies on gene expression from two plasmids, *pTrcHUMdCK*, expressing the human deoxycytidine kinase cDNA under the control of an IPTG-inducible promoter and *pBBR1MCS-2lux+*, expression the *lux* genes for self bioluminescence. Although this biosensor is relatively stable provided that antibiotic selection for the plasmids is maintained during growth and lyophilisation, it could be an advantage to insert the human *dCK* gene and the *luxCDABE* genes into the chromosome of the *E. coli* host.

6.3 Applications with Other Drugs

Preliminary screening indicates that the *E. coli* HA1 biosensor strain can be used to determine resistance or sensitivity to deoxyribonucleoside kinase-dependent drugs, i.e. nucleoside analogue drugs that utilise a deoxyribonucleoside kinase (dNK) pathway. This includes cladribine, used in treatment for hairy cell leukaemia and multiple sclerosis; gemcitabine, used in pancreatic, breast and non-small cell lung cancers; nelarabine, used for T-cell acute lymphoblastic leukaemia; zalcitabine, lamivudine and zidovudine used in HIV treatment and acyclovir used for Herpes virus. The biosensor has potential use both for sensitivity screening and therapeutic drug monitoring of these drugs.

6.4 Additional Chemotherapy Biosensors

Development of the biosensor and assay protocol has opened up the field for additional bioluminescent bacterial biosensors that respond to widely used chemotherapeutic drugs.

Anthracyclins, including daunorubicin, doxorubicin and idarubicin, remain an important class of chemotherapeutic agents. However, their efficacy in treating cancer is limited by a cumulative dose-dependent cardiotoxicity, which can cause irreversible heart failure [44]. Construction and evaluation of a bioluminescent biosensor to rapidly monitor drug sensitivity of cancer cells in clinical samples will be of considerable use to inform dosing strategies with these drugs.

6.5 Rapid Testing in Other Cancers

The need for rapid testing in haematological malignancies such as AML is clearly established. However the technology could be extended to solid tumours, where biopsy samples could be treated for disaggregation before undergoing rapid assay with bioluminescent biosensors. It could be of use in brain tumours where chemotherapy may be the only course of treatment and also in breast and other tumours where treatment with chemotherapy is used to reduce tumour size before removal.

It is clear that in the field of predicting response of cancer cells to chemotherapy, there have been great advances in genotypic testing and there many rapid tests for marker genes available to clinicians. But some malignancies, including AML are known to have heterogeneous origins and the possibility of employing bioluminescent bacterial biosensors for rapid testing of the phenotypic response to cancer cells to chemotherapeutic drugs has been welcomed by clinicians. It is hoped that it will be a contributory factor in a movement towards stratified treatment for individual patients, dependent on in vitro response of their tumour cells.

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