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(54) Title: METHOD FOR DETERMINING THE AMOUNT OF CYTIDINE ANALOGUES

(57) Abstract: The present application describes a method for determining the amount of a cytidine analogue in a mixture, the cytidine analogue having an optionally substituted unsaturated 6-membered heterocyclyl ring comprising 3 ring carbon atoms and 3 ring nitrogen atoms, the method comprising treating the mixture to reduce the 6-membered heterocyclyl ring of the cytidine analogue and then determining the amount of the reduced cytidine analogue. In particular, the method is useful for determining the amount of 5-azacitidine (AZA) incorporated into the RNA and/or 5-aza-2'-deoxycytidine (DAC) incorporated into the DNA. The mixture is taken from a cellular extract, cytoplasmic extract and/or a nucleic acid extract.



## METHOD FOR DETERMINING THE AMOUNT OF CYTIDINE ANALOGUES

### FIELD OF THE INVENTION

The present invention relates to a method for determining the amount  
5 of an analyte in a mixture. More particularly, the present invention  
relates to a method for determining the amount of a cytidine  
analogue in a mixture.

### BACKGROUND

10 There exist various circumstances in which it would be beneficial to  
know the amount of an analyte present in a mixture.

For instance, to study or treat a disease state it may be beneficial  
to determine the amount of an analyte in a mixture (e.g. the amount  
of a drug, or a metabolite of a drug, in a biological sample, e.g.  
15 from a patient treated with the drug). In some disease states it may  
be difficult to gain a proper understanding of the disease, or of  
suitable treatments thereof, without knowing such details.

Various methods and techniques have been developed to determine the  
amount of an analyte present in a mixture, however, such methods and  
20 techniques may not always be applicable to certain analytes or  
mixtures or may not provide the desired sensitivity. For example,  
there are diseases that are poorly understood due to a poor  
understanding of the pharmacokinetics of the drugs used to treat  
those diseases. A subsequent result is that for some diseases,  
25 treatment strategies remain suboptimal. An example is the use of 5-  
azacitidine (AZA) to treat the haematological neoplasms  
Myelodysplastic Syndrome (MDS) and Chronic Myelomonocytic Leukemia  
(CMML). Despite being the primary pharmacological agent currently  
used for the treatment of MDS and CMML, only ~50% of treated  
30 patients will respond to AZA and the drivers of AZA resistance *in*  
*vivo* are poorly understood.

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The study of MDS and CMML (including the development of effective treatments thereof) has been hampered, at least in part, by a lack of understanding of the role of AZA in treating the progression of these diseases. A contributing factor is that currently available  
5 methods for determining the amount AZA in a mixture (e.g. a sample obtained from a subject treated with AZA) are largely inadequate.

Although the efficacy of AZA compared to supportive care alone has been shown in MDS and CMML, only approximately half of AZA treated MDS or CMML patients respond to treatment. Response to AZA treatment  
10 is associated with improved survival outcomes and decreased likelihood of leukemic transformation. Although some clinical parameters and genetic mutations have weak correlations with favorable AZA response, the molecular mechanisms underlying primary AZA resistance are poorly understood. Furthermore, AZA response is  
15 rarely sustained and a significant fraction of patients who initially respond will eventually relapse within a two-year period, with very poor subsequent prognosis thereupon.

The current understanding of the intracellular dynamics of AZA is that following cellular uptake, AZA is metabolized and incorporated  
20 into DNA and RNA. DNA incorporated-AZA, in its deoxyribonucleic form 5-aza-2'-deoxycytidine (DAC), mediates DNA demethylation via the covalent trapping of DNA methyltransferases, which eventually leads to the degradation of the methyltransferases through a proteasomal mechanism. However, it is unclear whether AZA-induced DNA  
25 demethylation is responsible for its efficacy in MDS and CMML. While AZA therapy causes DNA hypomethylation in patients, hypomethylation is not predictive of clinical response. In addition, studies have failed to find any correlation between AZA-mediated DNA  
30 demethylation and subsequent gene re-expression, suggesting additional molecular mechanisms might be at play *in vivo* to explain AZA's efficacy. Furthermore, recent data has implicated immune response as a result of double stranded RNA production from  
endogenous retroviral elements as one mode of AZA's efficacy. Some of the mechanisms proposed for primary AZA resistance have included  
35 insufficient intracellular concentration of AZA triphosphates, either due to insufficient intake through membrane transporters,

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deoxycytidine kinase deficiency, excessive deamination by cytidine deaminase (CDA), or high intracellular nucleotide pools, which limits DAC incorporation into DNA. Alternatively, increased cell cycle quiescence of hematopoietic cells, limiting the DNA replication-dependent incorporation of DAC, has also been proposed as a marker of AZA resistance. While most studies have focused on the DNA demethylation effect resulting from the conversion of AZA into DAC, metabolic labelling studies have suggested that the majority of intracellular AZA (80~90%) is in fact incorporated into RNA. Furthermore, comparative studies of AZA and DAC have shown that they display different effects on cell viability and gene expression, suggesting that AZA incorporation into RNA might have distinct consequences. AZA incorporation into RNA leads to the covalent trapping of RNA methyltransferases and demethylation of some RNAs, as well as the destabilisation of other transcripts. However, the overall biological consequences of AZA incorporation into RNA are poorly understood, as is any potential role in mediating therapeutic response.

A fuller understanding of the intracellular dynamics of AZA upon *in vivo* treatment would greatly improve knowledge of the mechanisms underlying AZA resistance and thus allow clinicians to more quickly and accurately identify those patients likely to respond to AZA treatment. The development of analytical methods to measure AZA dynamics *in vivo* has been constrained by the chemical instability of AZA in aqueous solutions, which can greatly decrease the abundance of intracellular AZA and require technologies with very high detection sensitivity. Additionally, the small molecular weight difference (1 Da) between AZA or DAC and endogenous cytidine (C) or deoxycytidine (dC) respectively requires technologies with high resolution in order to accurately quantify AZA or DAC levels intracellularly. While radiolabelled AZA followed by scintillation based quantification of sub-cellular components has been applied to samples *ex vivo*, this technology is not readily amenable to studying intracellular AZA dynamics *in vivo*. Mass spectrometry has been applied to study AZA or DAC triphosphate levels in samples from patients undergoing treatment, but these methods have been unable to assess AZA or DAC incorporation into DNA or RNA simultaneously

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within the same samples, limiting an overall picture of AZA's intracellular dynamics from being painted.

For at least these reasons, it would be advantageous if at least preferred embodiments of the present invention were to provide an alternative method for determining the amount of a cytidine analogue  
5 in a mixture.

#### SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a method for  
10 determining the amount of a cytidine analogue in a mixture, the cytidine analogue having an optionally substituted unsaturated 6-membered heterocyclyl ring comprising 3 ring carbon atoms and 3 ring nitrogen atoms, the method comprising treating the mixture to reduce the 6-membered heterocyclyl ring of the cytidine analogue and then  
15 determining the amount of the reduced cytidine analogue.

In an embodiment, the amount of the reduced cytidine analogue is determined by a quantification technique selected from mass spectroscopy, liquid chromatography, LC-MS and NMR.

In an embodiment, the amount of the reduced cytidine analogue is  
20 determined by mass spectroscopy.

In an embodiment, the mixture is a cellular extract.

In an embodiment, the cellular extract is an aqueous cellular extract.

In an embodiment, the cellular extract is a cytoplasmic extract.

25 In an embodiment, the cellular extract is a nucleic acid extract.

In an embodiment, the nucleic acid extract is a DNA extract.

In an embodiment, the nucleic acid extract is an RNA extract.

In an embodiment, the method further comprises:

a) after treating the nucleic acid extract to reduce the 6-  
30 membered heterocyclyl ring of the cytidine analogue, depolymerising

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the nucleic acid extract to afford the constituent deoxyribonucleosides and ribonucleosides; and

- b) determining the amount of the reduced cytidine analogue in the nucleic acid extract to thereby determine the amount of the cytidine analogue that had been in the nucleic acid extract.

In an embodiment, the method further comprises:

a) after treating the DNA extract to reduce the 6-membered heterocyclyl ring of the cytidine analogue, depolymerising the DNA to afford the constituent deoxyribonucleosides; and

- b) determining the amount of the reduced cytidine analogue in the extract to thereby determine the amount of the cytidine analogue that had been in the DNA.

In an embodiment, the method further comprises:

a) after treating the RNA extract to reduce the 6-membered heterocyclyl ring of the cytidine analogue, depolymerising the RNA to afford the constituent ribonucleosides; and

b) determining the amount of the reduced cytidine analogue in the extract to thereby determine the amount of the cytidine analogue that had been in the RNA.

- In an embodiment, the depolymerisation is performed by an enzymatic process.

In an embodiment, the enzymatic process comprises the use of a phosphatase, phosphodiesterase or both a phosphatase and a phosphodiesterase.

- In an embodiment, the enzymatic process is performed at a pH of between about 7 and 9.

In an embodiment, the depolymerisation is performed in less than about 1 hour.

- In an embodiment, the depolymerisation is performed at less than about 40 °C.

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In an embodiment, the mixture is reduced by contacting the mixture with a reducing agent.

In an embodiment, the reducing agent is  $\text{NaBH}_4$ .

5 In an embodiment, the amount of the reduced cytidine analogue is determined using a mass spectrometer having a resolving power of at least 229,000 full width at half maximum (FWHM).

10 In an embodiment, the step of determining the amount of the reduced cytidine analogue comprises subjecting the reduced cytidine analogue to liquid chromatography, mass spectroscopy or LC-MS in a solution comprising less than about  $0.01 \text{ mol L}^{-1}$  ammonium formate buffer.

In an embodiment, the method further comprises determining the amount of an analyte other than the reduced cytidine analogue.

15 In an embodiment, the amount of the reduced cytidine analogue and the amount of the analyte other than the reduced cytidine analogue are determined by liquid chromatography, mass spectroscopy or LC-MS, especially mass spectroscopy.

20 In an embodiment, the amount of the reduced cytidine analogue and the amount of the analyte other than the reduced cytidine analogue are determined simultaneously by liquid chromatography, mass spectroscopy or LC-MS, especially mass spectroscopy.

In a second aspect, the present invention provides a method for determining the amount of 5-aza-2'-deoxycytidine (DAC) incorporated into the DNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

25 a) obtaining cells from the subject;

b) extracting nucleic acid from the cells to afford a nucleic acid extract;

30 c) treating the nucleic acid extract to reduce the 6-membered heterocyclyl ring of the 5-aza-2'-deoxycytidine (DAC) incorporated into the DNA;

d) treating the nucleic acid extract to depolymerise the DNA;  
and

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e) determining the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) in the product of step d) to thereby determine the amount of the 5-aza-2'-deoxycytidine (DAC) that had been incorporated into the DNA of the subject.

5 In an embodiment, the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) is determined by liquid chromatography, mass spectroscopy or LC-MS, especially mass spectroscopy.

In a third aspect, the present invention provides a method for determining the amount of 5-azacitidine (AZA) incorporated into the  
10 RNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

a) obtaining cells from the subject;

b) extracting nucleic acid from the cells to afford a nucleic acid extract;

15 c) treating the nucleic acid extract to reduce the 6-membered heterocyclyl ring of the 5-azacitidine (AZA) incorporated into the RNA;

d) treating the nucleic acid extract to depolymerise the RNA;  
and

20 e) determining the amount of the reduced 5-azacitidine (reduced AZA) in the product of step d) to thereby determine the amount of the 5-azacitidine (AZA) that had been incorporated into the RNA of the subject.

In an embodiment, the amount of the reduced 5-azacitidine (reduced  
25 AZA) is determined by liquid chromatography, mass spectroscopy or LC-MS, especially mass spectroscopy.

In a fourth aspect, the present invention provides a method for determining the amount of 5-aza-2'-deoxycytidine (DAC) that had not  
30 been incorporated into the DNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

a) obtaining cells from the subject;

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b) extracting the cytoplasm from the cells to afford a cytoplasmic extract;

c) treating the cytoplasmic extract to reduce the 6-membered heterocyclyl ring of the 5-aza-2'-deoxycytidine (DAC) in the  
5 extract; and

d) determining the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) in the extract to thereby determine the amount of the 5-aza-2'-deoxycytidine (DAC) in the cytoplasm of the cells.

10 In an embodiment, the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) is determined by liquid chromatography, mass spectroscopy or LC-MS, especially mass spectroscopy.

In a fifth aspect, the present invention provides a method for determining the amount of 5-azacitidine (AZA) that had not been  
15 incorporated into the RNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

a) obtaining cells from the subject;

b) extracting the cytoplasm from the cells to afford a cytoplasmic extract;

20 c) treating the cytoplasmic extract to reduce the 6-membered heterocyclyl ring of the 5-azacitidine (AZA) in the extract; and

d) determining the amount of the reduced 5-azacitidine (reduced AZA) in the product of step c) to thereby determine the amount of the 5-azacitidine (AZA) in the cytoplasm of the cells.

25 In an embodiment, the amount of the reduced 5-azacitidine (reduced AZA) is determined by liquid chromatography, mass spectroscopy or LC-MS, especially mass spectroscopy.

In an embodiment, the method further comprises determining the amount of 1, 2, 3 or 4 analytes selected from deoxycytidine (dC), 5-  
30 methyldeoxycytidine (mdC), cytidine (C) and 5-methylcytidine (mC).

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In an embodiment, the amount of the 1, 2, 3 or 4 analytes is determined by liquid chromatography, mass spectroscopy or LC-MS, especially mass spectroscopy.

In a sixth aspect, the present invention provides a method for  
5 determining the amount of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) incorporated into the DNA and RNA, respectively, of a subject that has been administered 5-azacitidine (AZA), the method comprising:

- a) obtaining cells from the subject;
- 10 b) extracting nucleic acid from the cells to afford a nucleic acid extract;
- c) treating the nucleic acid extract to reduce the 6-membered heterocyclyl ring of the 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) incorporated into the DNA and RNA;
- 15 d) treating the extract to depolymerise the DNA and RNA; and
- e) determining the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA) in the product of step d) to thereby determine the amount of the 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) that had been  
20 incorporated into the DNA and RNA of the subject.

In an embodiment, the amounts of the reduced 5-aza-2'-deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA) are determined by liquid chromatography, mass spectroscopy or LC-MS, especially mass spectroscopy.

25 In a seventh aspect, the present invention provides a method for determining the amount of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) that had not been incorporated into the DNA or RNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

- 30 a) obtaining cells from the subject;
- b) extracting the cytoplasm from the cells to afford a cytoplasmic extract;

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c) treating the cytoplasmic extract to reduce the 6-membered heterocyclyl ring of the 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) in the extract; and

d) determining the amount of the reduced 5-aza-2'-  
5 deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA) in the product of step c) to thereby determine the amount of the 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) in the cytoplasm of the cells.

In an embodiment, the amounts of the reduced 5-aza-2'-deoxycytidine  
10 (reduced DAC) and reduced 5-azacitidine (reduced AZA) are determined by liquid chromatography, mass spectroscopy or LC-MS, especially mass spectroscopy.

In an eighth aspect, the present invention provides a method for determining the amount of 5-aza-2'-deoxycytidine (DAC) and 5-  
15 azacitidine (AZA) that i) has and ii) has not been incorporated into the DNA or RNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

a) obtaining cells from the subject;

b) taking a portion of the cells obtained in step a) and  
20 extracting nucleic acid from the cells to afford a nucleic acid extract, treating the nucleic acid extract to reduce the 6-membered heterocyclyl rings of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) incorporated into the DNA and RNA, and treating the extract to depolymerise the DNA and RNA to afford a first sample;

25 c) taking a portion of the cells obtained in step a) and extracting the cytoplasm from the cells to afford a cytoplasmic extract, and treating the cytoplasmic extract to reduce the 6-membered heterocyclyl rings of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) to afford a second sample; and

30 d) determining the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA) in the first sample and the second sample to thereby determine the amount of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) that

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had and had not been incorporated into the DNA and RNA of the subject.

In an embodiment, the amounts of the reduced 5-aza-2'-deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA) are determined  
5 by liquid chromatography, mass spectroscopy or LC-MS, especially mass spectroscopy.

In a ninth aspect, the present invention provides a method for identifying a subject suffering from haematological neoplasms who will respond to treatment with AZA, the method comprising:

- 10 a) administering AZA to the subject;
  - b) obtaining cells from the subject after administration of AZA;
  - c) determining the amount of DAC incorporated into the DNA of the subject by the method of the second aspect of the present  
15 invention; and
  - d) identifying the subject as a subject likely to respond to AZA treatment when DAC is incorporated into the DNA of the subject and is accompanied by the induction of double stranded RNA intracellularly and increased levels of pro-inflammatory cytokines.
- 20 Double stranded RNA are produced from endogenous retroviral elements, which are transcriptionally activated by the loss of DNA CpG methylation that results from the incorporation of DAC into DNA. The double stranded RNA thus produced can lead to the induction of pro-inflammatory cytokines through interferon pathways in a process  
25 termed "viral mimicry". The double stranded RNA can be detected by 1.) quantitative PCR or 2.) through the use of double-stranded RNA specific antibodies (such as the J2 antibody, Scicons), followed by microscopy (example, assays such as immunofluorescence) or by using flow cytometry. Cytokines can be detected using cytokine specific  
30 antibodies, through ELISA assays, by flow cytometry-based assays (example Luminex assays, Luminex) or by mass spectrometry.

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In a tenth aspect, the present invention provides a method for identifying and treating subjects suffering from haematological neoplasms which will respond to AZA, the method comprising:

- a) administering AZA to the subject;
- 5        b) obtaining cells from the subject after administration of AZA;
- c) determining the amount of DAC incorporated into the DNA of the subject by the method of the second aspect of the present invention;
- 10       d) identifying the subject as a subject likely to respond to AZA treatment when DAC is incorporated into the DNA of the subject and is accompanied by the induction of double stranded RNA intracellularly and increased levels of pro-inflammatory cytokines; and
- 15       e) administering an effective amount of AZA to the identified subject.

Typically, the subject is a human.

The above aspects are collectively referred to herein as "methods of the present invention".

20

#### BRIEF DESCRIPTION OF THE FIGURES

Preferred embodiments of the present invention are described below, by way of example only, with reference to the accompanying drawings in which:

- 25       **Figure 1(A)** shows chemical structures of cytidine (C), deoxycytidine (dC), 5-azacitidine (AZA) and decitabine (DAC).

**Figure 1(B)** is a schematic illustration depicting intracellular metabolism of AZA. Following cellular uptake, ~80% of AZA gets incorporated into RNA by RNA polymerases. The remaining fraction is  
30       converted into DAC by ribonucleotide reductase and incorporated into DNA by DNA polymerases.

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**Figure 1(C)** shows selected reaction monitoring chromatograms from liquid chromatography-triple quadrupole mass spectrometry (TQMS) for buffer spiked with 1  $\mu$ M DAC only (left) or 1  $\mu$ M dC only (right) as described in Example 1. The m/z for dC (top chromatograms, left and right panels) and DAC (bottom chromatograms, left and right panels) are indicated, as are the retention times (RT) for the molecules.

**Figure 1(D)** shows a representative mass spectrum of TQMS at RT of 1.34 min indicating that TQMS cannot separate signals from DAC (black peak, i.e. peak at 228) and the naturally occurring isotopes of dC (red peak, i.e. peak at 229) due to poor mass resolution, as described in Example 1.

**Figure 1(E)** shows representative high resolution Orbitrap mass spectra at a retention time (RT) of 0.98 min, showing clear baseline separation between DAC,  $^{15}\text{N}$ -dC, and  $^{13}\text{C}$ -dC (left, with respective m/z values) despite their identical chromatographic retention times (right), as described in Example 1.

**Figure 2(A)** is a scatter plot depicting quantification of DAC standards of different concentrations (0.5-1000 nM DAC), showing the effect of 37°C incubation on DAC signal intensity, as described in Example 1. Two identical sets of standards were analysed, one prepared fresh without any incubation prior to MS ("No incubation", dashed line, circles), and the other incubated for six hours at 37°C prior to MS ("With incubation", solid line, squares). Data from three independent experiments is shown, with whiskers corresponding to standard deviation.

**Figure 2(B)** is a gel electrophoresis image showing the fragmentation efficiency for different combinations of DNA amount and incubation time, as described in Example 1. Four incubation times were examined (0, 1, 2, and 6 hours) for each of the three DNA masses (1, 2, and 5  $\mu$ g).

**Figure 2(C)** is a scatter plot showing the improvement in DAC LC-MS signal due to  $\text{NaBH}_4$  reduction, as described in Example 1. Two identical sets of standards were analysed, one without any reduction ("No reduction", dashed line, squares), and the other with reduction ("With reduction", solid line, circles). Data from three independent

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experiments is shown, with whiskers corresponding to standard deviation.

**Figure 2(D)** is a scatter plot showing the improvement in DAC LC-MS signal due to omission of ammonium formate, as described in Example 1. Two identical sets of standards were analysed, one with ammonium formate (circles), and the other without (squares). Data from three independent experiments is shown, with whiskers corresponding to standard deviation.

**Figure 2(E)** is a schematic illustration depicting the modified sample preparation method described in Example 1, incorporating decrease of fragmentation time to 1h and NaBH<sub>4</sub> reduction steps, and improvements in LC-MS.

**Figure 3(A)** is a graph showing signal intensities of DNA-incorporated DAC measured from different input quantities of DNA (0-1250 ng, x-axis) extracted from RKO cells treated with different concentrations of AZA (100nM - 1250 nM) for three consecutive days in a two-factor experiment to determine minimum input amount of DNA required to reliably detect DNA-incorporated DAC (as described in Example 1). 500 ng (highlighted with a shaded box) was determined as the minimum amount of DNA to reproducibly detect good signal across all the tested AZA treatment dosages. Points represent mean of three independent experiments, and whiskers represent standard deviation.

**Figure 3(B)** is a graph showing signal intensities of RNA-incorporated AZA measured from different input quantities of RNA (0-1250 ng, x-axis) extracted from RKO cells treated with different concentrations of AZA (100nM - 1250 nM) for three consecutive days in a two-factor experiment to determine minimum input amount of RNA required to reliably detect RNA-incorporated AZA (as described in Example 1). 500 ng (highlighted with a shaded box) was determined as the minimum amount of RNA to reproducibly detect good signal across all the tested AZA treatment dosages. Points represent mean of three independent experiments, and whiskers represent standard deviation.

**Figure 3(C)** is a schematic illustration showing the AZA-MS assay, illustrating the separation of the various sub-cellular components

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(cytoplasmic nucleotides, RNA, and DNA) from the same sample prior to LC-MS.

**Figure 3(D)** shows results of allelic bisulfite sequencing of the MLH1 locus in RKO cells, as described in Example 1. Top panel shows the schematic of the gene, with a CpG island (upper box) and the region assayed for CpG methylation (lower box). Lollipop visualizations of the methylation status in control, DMSO treated cells ("untreated", left panel) and RKO cells treated for three days 1.25 $\mu$ M AZA (right panel) are shown. Equivalent bar-graph quantification of the data is presented on the right. Whiskers represent standard deviation. \*, p value < 0.05, student's t-test.

**Figure 3(E)** is a graph showing q-RT-PCR of MLH1 expression levels in RKO cells treated with different doses of AZA (0 - 1250 nM) for three days, as described in Example 1. The results show robust re-expression after treatment with 1250 nM AZA.

**Figure 3(F)** includes a bar graph (lower panel) depicting AZA quantification in cytoplasm and RNA from RKO cells, treated either with 1.25  $\mu$ M of AZA (bars on right hand side, 11.46 and 1.32 pmol) or control (DMSO, bars on left hand side, 0 and 0 pmol) for three days, as described in Example 1. The calibration curve used for AZA quantification is shown, along with AZA chemical structure and the R squared value (upper panel). Abundance measurements for AZA in the cytoplasm (left bar graph) and incorporated into RNA (right bar graph) are shown. Data is from triplicate experiments, with standard deviation depicted by whiskers. \*\*\*, p value < 0.001, student's t-test.

**Figure 3(G)** includes a bar graph (lower panel) depicting DAC quantification in cytoplasm and RNA from RKO cells, treated either with 1.25  $\mu$ M of AZA (bars on right hand side, 9.59 and 5.65 pmol) or control (DMSO, bars on left hand side, 0 and 0 pmol) for three days, as described in Example 1. The calibration curve used for DAC quantification is shown, along with DAC chemical structure and the R squared value (upper panel). Abundance measurements for DAC in the cytoplasm (left bar graph) and incorporated into DNA (right bar graph) are shown. Data is from triplicate experiments, with standard

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deviation depicted by whiskers. \*\*\*, p value < 0.001 , student's t-test.

**Figure 4(A)** is a scatter plot showing a linear positive correlation between increasing AZA treatment dosage (x-axis) and increased DAC incorporation into DNA (y-axis) in RKO cells treated for three days, as described in Example 1. Points represent mean from triplicate experiments, with whiskers representing standard deviation.

**Figure 4(B)** is a scatter plot showing a linear negative trend between increasing AZA treatment dosage (x-axis) and decreased DNA cytosine methylation (y-axis) in RKO cells treated for three days, as described in Example 1. Points represent mean from triplicate experiments, with whiskers representing standard deviation.

**Figure 4(C)** is a scatter plot showing a linear positive correlation between increasing AZA treatment dosage (x-axis) and increased AZA incorporation into RNA (y-axis) in RKO cells treated for three days, as described in Example 1. Points represent mean from triplicate experiments, with whiskers representing standard deviation.

**Figure 4(D)** is a scatter plot showing no correlation between increasing AZA treatment dosage (x-axis) and RNA cytosine methylation (y-axis) in AZA treated RKO cells, as described in Example 1. Points represent mean from triplicate experiments, with whiskers representing standard deviation.

**Figure 5(A)** is a schematic illustration showing the standard cycle of seven consecutive days of AZA treatment (black vertical bars) for subjects. The three longitudinal time points for collection of bone marrow samples from each patient are also shown (Pre-treatment, cycle 1 day 8 (C1d8) and cycle 1 day 28 (C1d28)).

**Figure 5(B)** is a table of patient characteristics (of the patients described in Example 1) based on published guidelines: WHO classification (Vardiman, J.W. et al. (2002) The World Health Organization (WHO) classification of the myeloid neoplasms *Blood*, **100**, 2292-2302), IPSS-R scoring (Greenberg, P.L. et al. (2012) Revised international prognostic scoring system for myelodysplastic syndromes *Blood*, **120**, 2454-2465) and AZA response (Cheson, B.D. et

al. (2006) Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia *Blood*, **108**, 419-425).

**Figure 5(C)** is a bar graph depicting the mean DAC abundance in DNA of bone marrow CD34<sup>+</sup> cells at each of the three time points in AZA responders (n=4, green, i.e. bars on left) and non-responders (n=4, orange, i.e. bars on right), as described in Example 1. Whiskers show standard deviation. \*, p value < 0.05, student's t-test.

**Figure 5(D)** is a bar graph depicting the mean cytosine methylation levels in DNA of bone marrow CD34<sup>+</sup> cells at each of the three time points, in AZA responders (n=4, dark grey, i.e. bars on left) and non-responders (n=4, light grey, i.e. bars on right), as described in Example 1. Whiskers show standard deviation. \*, p value < 0.05, student's t-test.

**Figure 5(E)** is a pair of graphs showing DAC abundance (left panel) and DNA methylation levels (right panel) in bone marrow CD34<sup>+</sup> cells of each of the four AZA responders (R1 - R4) shown longitudinally over the course of AZA treatment, as described in Example 1.

**Figure 5(F)** is a pair of graphs showing DAC abundance (left panel) and DNA methylation levels (right panel) of bone marrow CD34<sup>+</sup> cells in each of the four AZA non-responders (N1 - N4) shown longitudinally over the course of AZA treatment, as described in Example 1.

**Figure 6(A)** (left) is a bar graph showing mean abundance measurements of unincorporated AZA in the cytoplasm of bone marrow CD34<sup>+</sup> cells of AZA responders (n=4, dark grey, i.e. bars on left) and non-responders (n=3, light grey, i.e. bars on right) at different time points during AZA therapy, as described in Example 1. Whiskers represent standard deviation, and \*, p value < 0.05, student's t-test. Individual patient measurements are also shown in a graphical illustration (right).

**Figure 6(B)** (left) is a bar graph showing mean abundance measurements of unincorporated DAC in the cytoplasm of bone marrow CD34<sup>+</sup> cells of AZA responders (n=4, dark grey, i.e. bars on left)

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and non-responders (n=3, light grey, i.e. bars on right) at different time points during AZA therapy, as described in Example 1. Whiskers represent standard deviation, and \*, p value <0.05, student's t-test. Individual patient measurements are shown in a graphical illustration (right).

**Figure 6(C)** (left) is a bar graph showing mean abundance measurements of RNA-incorporated AZA in bone marrow CD34<sup>+</sup> cells of AZA responders (n=4, dark grey, i.e. bars on left) and non-responders (n=4, light grey, i.e. bars on right) at different time points during AZA therapy, as described in Example 1. Whiskers represent standard deviation, and \*, p value <0.05, student's t-test. Individual patient measurements are also shown in a graphical illustration (right).

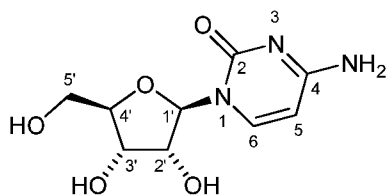
**Figure 7** is a bar graph showing the results of the experiment performed in Example 2 - comparing the reducing agents sodium borohydride (NaBH<sub>4</sub>) and sodium triacetoxyborohydride (NaBH(OAc)<sub>3</sub>) in the reduction of both AZA and DAC. Y-axis shows relative signal intensity compared to NaBH<sub>4</sub> reduction of 1000nM AZA or 1000nM DAC, respectively.

#### DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

In a first aspect, the present invention provides a method for determining the amount of a cytidine analogue in a mixture, the cytidine analogue having an optionally substituted unsaturated 6-membered heterocyclyl ring comprising 3 ring carbon atoms and 3 ring nitrogen atoms, the method comprising treating the mixture to reduce the 6-membered heterocyclyl ring of the cytidine analogue and then determining the amount of the reduced cytidine analogue in the mixture. The cytidine analogue may be present in the mixture as a separate compound or may be incorporated into a nucleic acid, such as DNA or RNA.

Cytidine comprises a ribose moiety covalently bound to a pyrimidine moiety. Cytidine has the structure:

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As used herein, "cytidine analogue having an optionally substituted unsaturated 6-membered heterocyclyl ring comprising 3 ring carbon atoms and 3 ring nitrogen atoms" refers to a compound having a structure similar to cytidine but having an optionally substituted unsaturated 6-membered heterocyclyl ring comprising 3 ring carbon atoms and 3 ring nitrogen atoms in place of the pyrimidine ring of cytidine.

In some embodiments, the 6-membered heterocyclyl ring comprises the nitrogen atoms in the 1-, 2- and 3-; 1-, 2- and 4-; 1-, 2- and 5-; 1-, 2- and 6-; 1-, 3- and 4-; 1-, 3- and 5-; 1-, 3- and 6-; 1-, 4- and 5-; 1-, 4- and 6-; 1-, 5- and 6-; 2-, 3- and 4-; 2-, 3- and 5-; 2-, 3- and 6-; 2-, 4- and 5-; 2-, 4- and 6-; 2-, 5- and 6-; 3-, 4- and 5-; 3-, 4- and 6-; 3-, 5- and 6-; or 4-, 5- and 6- positions.

For this purpose, the numbering priority of the 6-membered heterocyclyl ring (e.g. "1-, 3- and 5-" positions) follows the numbering priority used in cytidine as shown above (i.e. covalently bound by the nitrogen ring atom at the 1-position). In some embodiments, the 6-membered heterocyclyl ring comprises the nitrogen atoms in the 1-, 2- and 3-positions. In some embodiments, the 6-membered heterocyclyl ring comprises the nitrogen atoms in the 1-, 2- and 4-positions. In some embodiments, the 6-membered heterocyclyl ring comprises the nitrogen atoms in the 1-, 3- and 5-positions.

In some embodiments, the 6-membered heterocyclyl ring of the cytidine analogue is a triazinone.

In some particular embodiments, the 6-membered heterocyclyl ring of the cytidine analogue comprises ring nitrogen atoms at the 1-, 3- and 5-positions. In some embodiments, the 6-membered heterocyclyl ring comprising ring nitrogen atoms at the 1-, 3- and 5-positions is a triazinone (1,3,5-triazinone). In some embodiments, the 1,3,5-triazinone is a 1,3,5-triazin-2-one.

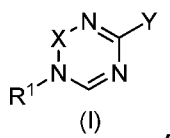
- 20 -

In an embodiment, the cytidine analogue is a pharmaceutically active compound.

In an embodiment, the cytidine analogue is 5-azacitidine (AZA).

In an embodiment, the cytidine analogue is 5-aza-2'-deoxycytidine  
5 (DAC).

In an embodiment, the cytidine analogue is a compound of Formula (I)



wherein:

R<sup>1</sup> is a saccharide moiety which is optionally substituted with a  
10 substituent selected from phosphate, phosphate ester, C<sub>1-6</sub>-alkyl and C(O)C<sub>1-6</sub>-alkyl;

X is selected from CH<sub>2</sub>, CHOH, CHOR<sup>2</sup>, CHNH<sub>2</sub>, CHNHR<sup>2</sup>, CHNR<sup>2</sup>R<sup>2</sup>, C=O, C=NH and C=NR<sup>2</sup>;

Y is selected from H, OH, OR<sup>2</sup>, NH<sub>2</sub>, NHR<sup>2</sup> and NR<sup>2</sup>R<sup>2</sup>; and

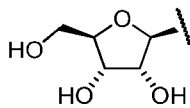
15 each R<sup>2</sup> is independently selected from C<sub>1-6</sub>-alkyl;

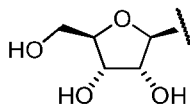
or tautomers thereof.

In an embodiment, X is C=O.

In an embodiment, Y is NH<sub>2</sub>.

In some embodiments, the cytidine analogue comprises a ribose moiety

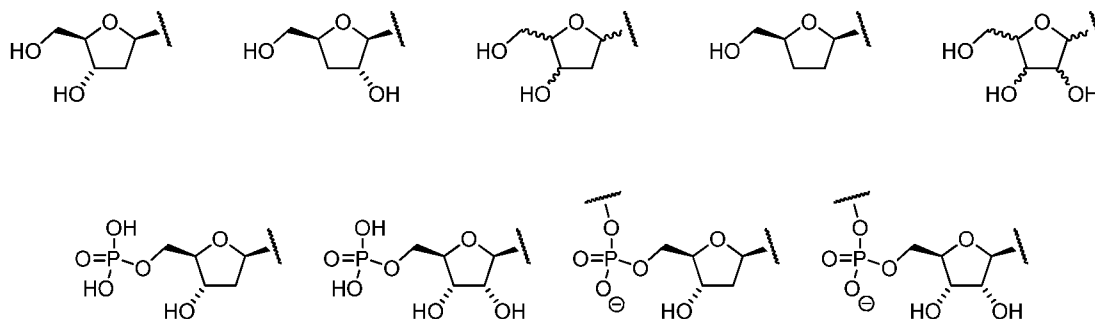


20 (i.e. a moiety of the formula ). In other embodiments, the cytidine analogue comprises a saccharide moiety in place of the ribose moiety of cytidine. In some embodiments, the ribose moiety or saccharide moiety is incorporated into a nucleic acid (e.g. DNA or RNA).

25 As used herein, "saccharide moiety" refers to a moiety having a structure corresponding to a saccharide or saccharide analogue (e.g.

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reduced analogues thereof, oxidised analogues thereof and deoxy analogues thereof) having a single H or OH group removed to form a radical. The saccharide moiety may have the structure of a monosaccharide (e.g. ribose, glucose), a disaccharide or  
 5 oligosaccharide. An oligosaccharide is an oligomer of saccharides comprising from 3 to 9 monosaccharides. The saccharide moiety may also be optionally substituted with a substituent selected from phosphate, phosphate ester, C<sub>1-6</sub>-alkyl and C(O)C<sub>1-6</sub>-alkyl. Examples of saccharide moieties include:

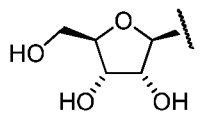


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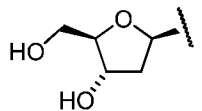
The term "C<sub>1-6</sub>-alkyl" refers to a linear or branched alkyl group having 1 to 6 carbon atoms. Examples of C<sub>1-6</sub>alkyl include methyl (Me), ethyl (Et), propyl (Pr), isopropyl (i-Pr), butyl (Bu), isobutyl (i-Bu), sec-butyl (s-Bu), tert-butyl (t-Bu), pentyl,  
 15 neopentyl, hexyl and the like.

In an embodiment, the saccharide moiety is a monosaccharide. In an embodiment, the saccharide moiety is a ribose moiety.

In an embodiment, the saccharide moiety has the structure:



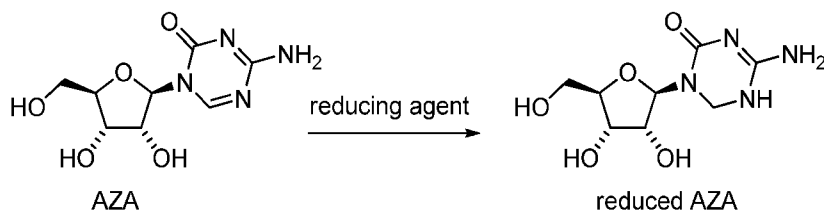
20 In an embodiment, the saccharide moiety has the structure:



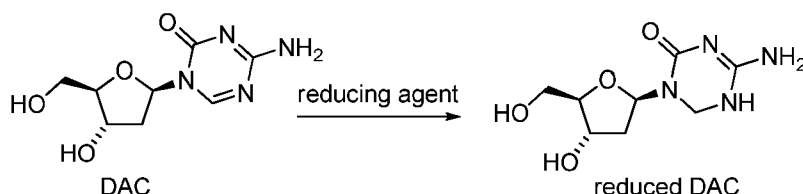
In the methods of the present invention, the mixture is treated to reduce the 6-membered heterocyclyl ring of the cytidine analogue.

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For example, when AZA is reduced, it forms the corresponding reduced AZA, as shown below:



Similarly, when DAC is reduced, it forms the corresponding reduced DAC, as shown below:



Loss due to degradation (e.g. hydrolysis or solvolysis) is a significant problem with quantifying many cytidine analogues having an unsaturated 6-membered heterocyclyl ring comprising 3 ring carbon atoms and 3 ring nitrogen atoms, including cytidine analogues comprising a 1,3,5-triazinone moiety, as these compounds can spontaneously degrade in aqueous solutions, making reliable quantification very difficult if not impossible. For example, AZA and DAC, including AZA or DAC incorporated into RNA or DNA, readily degrade in aqueous solutions making reliable quantification of AZA and/or DAC very difficult. It is believed that reducing the 6-membered heterocyclyl ring results in a reduced counterpart (i.e. reduced cytidine analogue) which exhibits greater stability in aqueous solutions than the parent unreduced compound (i.e. the cytidine analogue). The greater stability of the reduced cytidine analogue permits subsequent manipulations/operations without appreciable loss due to degradation. Accordingly, by treating the mixture to reduce the 6-membered heterocyclyl ring of the cytidine analogue, the ring is reduced to afford a more stable reduced cytidine analogue that can be subjected to further manipulations/operations, to thereby accurately determine the amount of the reduced cytidine analogue and, thus, the amount of the cytidine analogue in the mixture. As will be appreciated, where

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there is no appreciable loss of the reduced cytidine analogue due to degradation, determining the amount of the reduced cytidine analogue allows accurate determination of the amount of the cytidine analogue originally present in the mixture.

5 In the methods of the present invention, the amount of the reduced cytidine analogue is determined. This determination may be made by any technique which can accurately distinguish and measure the amount of the reduced cytidine analogue. For example, determining the amount of the reduced cytidine analogue may make use of  
10 instruments/techniques such as liquid chromatography (e.g. normal or reverse phase high pressure liquid chromatography (HPLC) or fast protein liquid chromatography (FPLC)), NMR (e.g.  $^1\text{H}$  NMR), mass spectroscopy (MS) or combinations thereof (e.g. LC-MS).

In some embodiments, the amount of the reduced cytidine analogue is  
15 determined by mass spectroscopy. Mass spectroscopy (also referred to as mass spectrometry and often abbreviated "MS") is a well-known analytical technique that ionizes chemical species and distinguishes the ions based on their mass-to-charge ratio ( $m/Q$  or  $m/z$ ). In a typical MS procedure, a sample is ionized (e.g. by electron  
20 bombardment/impact (EI) or electrospray ionization (ESI)) to cause bond disassociation, thereby creating charged fragments (ions). The charged fragments (ions) are then separated according to their mass-to-charge ratio, typically by accelerating the charged fragments (ions) and subjecting them to an electric or magnetic field where  
25 they are deflected and distinguished on the basis of their mass-to-charge ratio. The ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. Results are typically displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio. The  
30 components in a sample can be identified by correlating the known or predicted mass-to-charge ratio to the identified masses or through a characteristic fragmentation pattern.

As a person skilled in the art will appreciate, there are many parameters that can be manipulated to obtain meaningful data from a  
35 mass spectrometer. A person skilled in the art will be able to

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determine appropriate parameters based on the analyte to be determined.

As a person skilled in the art will also appreciate, there are many types of mass spectrometers (e.g. tandem quadrupole mass spectrometer, triple quadrupole mass spectrometer, orbitrap or Fourier transform ion cyclotron resonance mass spectrometer). In view of their high precision, orbitrap mass spectrometers are particularly useful in the methods of the present invention. The precision of a mass spectrometer may be measured according to its "resolving power". The "resolving power" of a mass spectrometer as defined by the 2013 IUPAC recommendations is a measure of the ability of a mass spectrometer to distinguish between two peaks of different mass to charge ratios. It is represented by the equation  $m/dm$ , where  $m$  is the mass and  $dm$  the peak width required for separation at mass of  $m$ . In the methods of the present invention, a resolving power of at least 200,000 full width at half maximum (FWHM) may be particularly useful (for example, at least about 229,000 FWHM, at least about 230,000 FWHM, at least about 250,000 FWHM, at least about 280,000 FWHM or at least about 300,000 FWHM. It is estimated that a resolving power of at least about 229,000 FWHM (such as is possible using an Orbitrap mass spectrometer (280,000 FWHM; available from Q Exactive Plus, ThermoFisher)) is sufficiently capable of resolving ions having about 1 Da separation (e.g. to separate DAC from the different isotopes of dC). Accordingly, a mass spectrometer having a resolving power of at least 229,000 FWHM may be particularly useful in the methods of the present invention.

Mass spectrometry is typically performed by injecting into the spectrometer a solution containing the analyte. Mass spectrometers are commonly interfaced to a liquid chromatograph to form an LC-MS. In such a setup, the output from the liquid chromatograph is fed into the input of the mass spectrometer. The solution injected into the LC-MS or MS may be any suitable solution. The solution is typically buffered with standard buffers (e.g. ammonium formate, phosphate, citrate, tris, ammonium bicarbonate, 0.1% trifluoroacetic acid or 0.1% formic acid). Buffers are typically used at a range of concentrations. For example, ammonium phosphate buffer is typically

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used in mass spectroscopy applications at about 0.1 mol L<sup>-1</sup>. It is believed that buffers such as ammonium phosphate buffer may degrade the cytidine analogue and/or the reduced cytidine analogue.

Accordingly, in some embodiments less than about 0.1 mol L<sup>-1</sup> (e.g.

5 less than 0.05 mol L<sup>-1</sup>, less than about 0.01 mol L<sup>-1</sup>, less than about 0.005 mol L<sup>-1</sup>) ammonium phosphate buffer is used for performing the liquid chromatography, mass spectroscopy or LC-MS.

In some embodiments, the mass spectrograph is interfaced to a liquid chromatograph, thereby forming an LC-MS.

10 The methods of the present invention involve determining the amount of the cytidine analogue in a mixture. The mixture may be any mixture of compounds or a solution comprising one or more compounds. The mixture may be any mixture that an operator wishes to determine the amount of a cytidine analogue therein. Examples include  
15 solutions comprising, or suspected of comprising, one or more cytidine analogues. Alternatively, the method may be used to establish that a mixture contains no detectable amount of a cytidine analogue.

In some embodiments, the mixture is a cellular extract. A cellular  
20 extract is a mixture derived from the cells of an animal or plant, especially an animal, more especially a human. The cellular extract may be obtained or derived from cells by methods known to those skilled in the art, for example by lysis of cells in hypotonic buffers, including Tris-buffered or Phosphate-buffered solutions,  
25 which may contain detergents (such as Sodium Dodecyl Sulfate (SDS), Triton-X, Tween 20, sodium deoxycholate or NP-40), or may contain no detergents. Alternative buffers may include chaotropic agents such as guanidine hydrochloride or urea. Cell extracts may also be prepared by mechanical lysis of cells (such as sonication, high  
30 shear mixing, use of a mortar and pestle or grinder) or through multiple freeze-thawing of cells. In some embodiments, the cellular extract is an aqueous cellular extract.

In some embodiments, the mixture is a cytoplasmic extract. A cytoplasmic extract is a mixture derived from the cytoplasm of cells  
35 of an animal or plant, especially an animal, more especially a

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human. The cytoplasmic extract may be obtained or derived from the cytoplasm of cells by methods known to those skilled in the art, for example by freezing cell pellets at -80 °C followed by addition of a methanol:aqueous buffer in the ratio of 2:1 together with vortexing.

- 5 Alternative methods for preparing cytoplasmic extracts include using gentle hypotonic lysis in buffers containing mild detergents (such as NP-40) or no detergents, followed by a gentle, low speed centrifugation to pellet intact nuclei. In such methods, the supernatant collected is or comprises the cytoplasmic extract.
- 10 Alternatively, commercially available kits are available (e.g. the NE-PER™ Nuclear and Cytoplasmic Extraction kit from ThermoFisher Scientific) for separately collecting cytoplasmic extracts.

- In some embodiments, the mixture is a nucleic acid extract. A nucleic acid extract is a mixture comprising nucleic acids derived from the nucleus of cells of an animal or plant, especially an animal, more especially a human. The nucleic acid extract may be obtained or derived from the nucleus of cells by methods known to those skilled in the art, for example by using commercially available kits (e.g. AllPrep DNA/RNA/Protein kit from Qiagen) which
- 15 rely on solid matrices (such as silica) to bind nucleic acids, enabling purification from cell extracts prepared (as listed above in the cellular extraction step). Alternatively, phenol:chloroform extraction may also be used to isolate DNA and RNA.
- 20

- In some embodiments, the nucleic acid extract is a DNA extract. The DNA extract may be obtained or derived from the nucleus of cells by methods known to those skilled in the art, for example by using commercially available kits (e.g. AllPrep DNA/RNA/Protein kit from Qiagen) which rely on solid matrices (such as silica) to bind DNA specifically, enabling purification from cell extracts prepared (as
- 25 listed above in the cellular extraction step). Alternatively, buffered phenol:chloroform extraction may also be used to isolate DNA specifically.
- 30

- In some embodiments, the nucleic acid extract is an RNA extract. The RNA extract may be obtained or derived from the nucleus of cells by methods known to those skilled in the art, for example by using commercially available kits (e.g. AllPrep DNA/RNA/Protein kit from
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Qiagen) which rely on solid matrices (such as silica) to bind RNA specifically, enabling purification from cell extracts prepared (as listed above in the cellular extraction step). Alternatively, acid phenol:chloroform or unbuffered phenol:chloroform extraction may  
5 also be used to isolate RNA specifically.

In some embodiments, the mixture is reduced by contacting the mixture with a reducing agent. A reducing agent is an element or compound that donates an electron to another chemical species in a redox chemical reaction. The reducing agents described in the  
10 Examples are sodium borohydride ( $\text{NaBH}_4$ ) and sodium triacetoxymborohydride ( $\text{NaBH}(\text{OAc})_3$  or  $\text{Na}(\text{CH}_3\text{COO})_3\text{BH}$ ).  $\text{NaBH}_4$  is a common and readily available reducing agent. Other reducing agents may be used in the methods of the present invention. Examples of reducing agents that may be used in the methods of the present invention  
15 include sodium cyanoborohydride ( $\text{NaCNBH}_3$ ), borane ( $\text{BH}_3$ ) and borane adducts such as 2-methylpyridine borane (picoline-borane),  $\text{BH}_3 \cdot \text{THF}$ , lithium aluminium hydride ( $\text{LiAlH}_4$ ), lithium borohydride ( $\text{LiBH}_4$ ), potassium borohydride ( $\text{KBH}_4$ ). The mixture may be contacted with the reducing agent by any means which brings the mixture and reducing  
20 agent into contact with one another. For example, the reducing agent may be added to the mixture or the mixture may be added to the reducing agent. One or both of the mixture and the reducing agent may be in solution (i.e. dissolved or at least partially dissolved in a solvent, e.g.  $\text{NaBH}_4$  in water) to facilitate the contact between  
25 the mixture and the reducing agent.

In some embodiments, the method further comprises determining the amount of an analyte other than the reduced cytidine analogue. The analyte other than the reduced cytidine analogue may be any analyte capable of being measured. Particular examples of analytes other  
30 than the reduced cytidine analogue include deoxycytidine (dC), 5-methyldeoxycytidine (mdC), cytidine (C) and 5-methylcytidine (mC).

The determination of the amount of analyte other than the reduced cytidine analogue may be performed simultaneously (i.e. at the same time as determining the amount of the reduced cytidine analogue) or  
35 may be performed at a time before or after the determination of the reduced cytidine analogue. The determination may be performed on the

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same sample, or may be performed on different portions of the same sample (that may have been subjected to more or less sample preparation steps). In some particular embodiments, the amount of one or more of deoxycytidine (dC), 5-methyldeoxycytidine (mdC),  
5 cytidine (C) and 5-methylcytidine (mC) are determined simultaneously via mass spectroscopy with the determination of the amount of the reduced cytidine analogue (e.g. reduced 5-aza-2'-deoxycytidine (reduced DAC) and/or reduced 5-azacitidine (reduced AZA)).

In some embodiments, nucleic acid (e.g. DNA and/or RNA, for example  
10 in a nucleic acid extract) is depolymerized to afford the constituent nucleosides (e.g. deoxyribonucleosides and/or ribonucleosides in the case of DNA and RNA, respectively). This is a step comprising depolymerizing the nucleic acid to afford the constituent nucleosides. As a person skilled in the art will be  
15 aware, nucleic acids such as DNA and RNA are polymeric in nature and are composed of monomeric units. The monomeric units are known as nucleotides. Nucleotides comprise three subunits: a "nitrogenous base", a 5-carbon sugar and at least one phosphate group. The "nitrogenous base" is covalently bound to the 5-carbon sugar, which  
20 is in turn covalently bound to the one or more phosphate groups. The "nitrogenous base" together with the 5-carbon sugar is known as a nucleoside. Consequently, it may be said that a nucleotide comprises a nucleoside covalently bound to a phosphate group. In the case of  
25 DNA, the 5-carbon sugar is deoxyribose, whereas in RNA, the 5-carbon sugar is ribose. The depolymerisation of the nucleic acid breaks the covalent bonds between the nucleosides and the phosphate groups, which yields the component nucleosides that were present in the nucleic acid.

There are methods known in the art for performing the  
30 depolymerisation, for example, DNA/RNA digestion, DNA/RNA hydrolysis. In some embodiments, an enzymatic process is used to perform the depolymerisation (i.e. an enzyme is used in the depolymerisation). Various enzymes are known to facilitate this depolymerisation and various techniques have been developed using  
35 enzymes for such purposes. For example, there are various phosphatases and phosphodiesterases that are commercially available

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(e.g. from Sigma Aldrich, MO, USA). In some embodiments, a phosphatase is used. In some embodiments, a phosphodiesterase is used. In some embodiments, both a phosphatase and a phosphodiesterase are used.

5 In some embodiments, the enzymatic process is performed at a pH of between about 6 and about 10, for example, between about 6 and about 9, between about 7 and about 10, between about 7 and about 9, between about 7 and about 8. In some embodiments, the enzymatic process is performed at near neutral pH (e.g. between about 7 and  
10 about 9). Advantageously, depolymerisation at or near neutral pH may be effective whilst exhibiting minimal degradation (e.g. solvolysis/hydrolysis) of the reduced cytidine analogue.

In some embodiments, the depolymerisation is performed in less than about 6 hours, for example, less than about 5 hours, less than about  
15 4 hours, less than about 3 hours, less than about 2 hours, less than about 1 hour, less than about 0.5 hours or less than about 0.3 hours. Reducing the depolymerisation time may reduce the amount of the reduced cytidine analogue lost due to degradation. Reducing the depolymerisation time too much (e.g. less than about 0.1 hours) does  
20 not allow sufficient time to effect complete depolymerisation. Therefore, it may be preferable to allow the depolymerisation to proceed for more than about 0.2 hours. In some particular embodiments, the depolymerisation is performed for about 0.5 to about 2 hours, more particularly about 1 hour.

25 In some embodiments, the depolymerisation is performed at less than about 60 °C, for example, less than about 50 °C or less than about 40 °C. As a person skilled in the art will appreciate, increased temperatures may lead to increased rates of degradation of the reduced cytidine analogue, but may also be more effective for  
30 depolymerisation. In some particular embodiments, depolymerisation may be effected at lower temperatures (e.g. less than about 40 °C) whilst exhibiting minimal degradation of the reduced cytidine analogue.

As a person skilled in the art will appreciate, enzyme type, pH,  
35 temperature and depolymerisation time may be varied to yield an

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optimum combination that achieves adequate depolymerisation with minimal degradation of the reduced cytidine analogue. A person skilled in the art will be able to judge suitable conditions for performing the depolymerisation.

- 5 The methods of the present invention provides a method or technique that is able to be used to determine, from a single sample, one or more of: intracellular concentrations of AZA; intracellular concentrations of DAC; RNA-incorporated AZA; DNA-incorporated DAC; DNA methylation; and RNA methylation. Advantageously, such
- 10 determinations may be made on samples obtained from a subject treated with AZA to thereby assess the cellular uptake of AZA in the subject. In addition, the distribution of AZA and AZA analogues (e.g. AZA metabolites) in the cells of a subject may be determined by the methods of the present invention.
- 15 The methods of the present invention may be used to determine the cellular uptake of AZA in a subject, thereby allowing a determination of how a subject is likely to respond to AZA. The methods of the present invention can therefore be used to identify patients or patient groups suffering from haematological neoplasms,
- 20 such as Myelodysplastic Syndrome (MDS) and Chronic Myelomonocytic Leukemia (CMML), that are likely to respond to treatment with AZA.

**EXAMPLES**

The present invention is further described below by reference to the following non-limiting Examples. Although described in the context of determining the amount of AZA, DAC, C and dC in a sample, a person skilled in the art will appreciate that the methods of the present invention may be used to determine the amount in a mixture of other cytidine analogues having an optionally substituted unsaturated 6-membered heterocyclyl ring comprising 3 ring carbon atoms and 3 ring nitrogen atoms.

**Example 1**

As described herein, a method (referred to herein as "AZA-MS") has been developed that is capable of simultaneously quantifying multiple parameters within the same fraction of AZA treated cells. With AZA-MS, it is possible to make direct measurements of (1.) AZA and DAC in RNA and DNA, while also (2.) quantifying RNA and DNA methylation, as well as (3.) measuring the abundance of unincorporated AZA and DAC in the cytoplasm within the same sample. Using AZA-MS, the inventors observed that while there is a linear, dose-dependent correlation between DAC incorporation into DNA and DNA demethylation, there is no correlation between AZA incorporation and RNA demethylation. The inventors applied AZA-MS to investigate the intracellular pharmacokinetics of AZA *in vivo* over the course of a cycle of AZA treatment in MDS and CMML patients undergoing treatment. The inventors observed quantitative differences in intracellular distribution of AZA and DAC in the cells of responders compared to non-responders. Additionally, AZA-MS has uncovered two distinct patterns of AZA dynamics within the non-responders, suggesting multiple mechanisms mediate AZA resistance.

**1 MATERIALS AND METHODS****1.1 Cell culture and treatment**

RKO cell line was cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 50 U/ml streptomycin, and 2 mM GlutaMAX (Thermo Fisher Scientific, CA, USA) and was maintained at 37°C in a humidified environment with 5% CO<sub>2</sub>. Cells were treated

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with different concentrations of 5-azacitidine (kind gift, Celgene, NJ, USA) for 72 hours, with media change containing fresh drug every 24 hours.

### 1.2 Primary bone marrow samples from MDS and CMML patients

5 Clinical samples used in this study were obtained from patients recruited from New South Wales, Australia on a compassionate access basis for AZA monotherapy. All samples were obtained with written informed consent in accordance with the Declaration of Helsinki and approval of the relevant institutional human research ethics  
10 committees. WHO classification, IPSS-R scoring and AZA response were based on published guidelines (WHO classification: Vardiman, J.W. et al. (2002) The World Health Organization (WHO) classification of the myeloid neoplasms *Blood*, **100**, 2292-2302; IPSS-R scoring: Greenberg, P.L. et al. (2012) Revised international prognostic scoring system  
15 for myelodysplastic syndromes *Blood*, **120**, 2454-2465; and AZA response: Cheson, B.D. et al. (2006) Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia *Blood*, **108**, 419-425).

AZA treatment consisted of standard 28-days cycles, each of which  
20 involves a consecutive 7-days treatment (75 mg/m<sup>2</sup>) followed by 21-days intermission. Immediately upon sample collection, mononuclear cells (MNCs) were isolated from the bone marrows by density centrifugation using Lymphoprep (Stem Cell Technologies, Canada). ~2\*10<sup>8</sup> MNCs per sample were then incubated with CD34+ magnetic beads  
25 (Miltenyi Biotec, Germany) and separated using an AutoMACS Pro machine (Miltenyi Biotec, Germany), per manufacturer's recommendations. Viably frozen vials of CD34<sup>-</sup> cells from the relevant time points were thawed, washed once in PBS and ~6\*10<sup>6</sup> cells per sample were used to prepare RNA and DNA for AZA-MS (as  
30 outlined below), while ~3\*10<sup>6</sup> cells were lysed to obtain the cytoplasmic extract for AZA-MS (as outlined below).

### 1.3 AZA-MS method

#### 1.3.1 DNA/RNA extraction

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Prior to harvest, cells were washed with PBS solution containing 100 µg/ml tetrahydrouridine (THU, Abcam, UK) and frozen at -80°C. DNA and RNA were purified from frozen cell pellets using the All-In-One DNA/RNA Miniprep Kit (Astral Scientific, NSW, Australia) following standard manufacturer recommendations, including RNase and DNaseI treatments to remove contaminating RNA or DNA as appropriate. Nucleic acids were quantified using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc., USA). Purified DNA and RNA samples were stored at -80°C until further processing.

#### 1.3.2 DNA/RNA preparation for LC-MS analysis

For duplicate measurement, 1 µg of extracted DNA, together with 5 µl of 50 µM 5-aza-2'-deoxycytidine-<sup>15</sup>N<sub>4</sub> (internal standard, Toronto Research Chemicals, Canada), was added to 10 µl of 20 mg/ml NaBH<sub>4</sub> solution. In case of RNA, 1 µg of extracted RNA, together with 5 µl of 50 µM 5-azacitidine-<sup>15</sup>N<sub>4</sub> (internal standard, Thermo Fisher Scientific, CA, USA), was added to 10 µl of 20 mg/ml NaBH<sub>4</sub> solution. The mixture was incubated at room temperature with agitation for 20 min, and neutralized with 1 µl 5M HCl. Reduced DNA/RNA were digested into individual (deoxy)ribonucleotides via a 1 hour incubation at 37°C following the addition of 40 µl of Digest Mix, an aqueous solution containing 50 U/mL Benzonase (Sigma Aldrich, MO, USA), 60 mU/mL Phosphodiesterase (Sigma Aldrich, MO, USA), 20 U/mL Alkaline Phosphatase (Sigma Aldrich, MO, USA), 20 mM Tris HCl pH 8, 100 mM NaCl, and 20 mM MgCl<sub>2</sub> as previously described (Quinlivan, E.P. and Gregory, J.F. (2008) *Analytical biochemistry*, **373**, 383-385). Samples were dried under vacuum (Savant Speedvac Plus SC210A, USA) and resuspended in 50 µl of CE buffer (10 mM TrisHCl pH 8.0 and 0.5 mM EDTA in Milli-Q water) for LC-MS analysis.

#### 1.3.3 Cytoplasmic extract preparation for LC-MS analysis

Frozen cell pellets were resuspended in 40 µl of PBS solution containing 100 µg/ml tetrahydrouridine. Following the addition of 5 µl of 50 µM 5-aza-2'-deoxycytidine-<sup>15</sup>N<sub>4</sub> and 5 µl of 50 µM 5-azacitidine-<sup>15</sup>N<sub>4</sub> internal standards, the cells were lysed by thorough mixing with 93 µl absolute MeOH. Subsequently, the sample was centrifuged at 12,000 rpm for 10 min at 4°C (Derissen, E.J. et al.

(2014) *Journal of pharmaceutical and biomedical analysis*, **90**, 7-14) and the supernatant was collected and dried under vacuum. The resulting precipitate was resuspended in 40 µl CE buffer and the mixture was reduced, neutralised, and digested with an enzymatic mix  
 5 (to dephosphorylate intracellular nucleotides) as described for DNA and RNA preparations (DNA and RNA extracts). Samples were then dried under vacuum, resuspended in 50 µl of CE buffer and utilised for LC-MS analysis.

#### 1.4 Mass spectrometry and data analysis

10 LC-MS analysis was performed utilizing an ultra-high performance liquid chromatography system (Dionex u3000 system, ThermoFisher Scientific, SJ, USA) interfaced to an Orbitrap mass spectrometer (Q Exactive Plus, ThermoFisher Scientific, SJ, USA) using a heated  
 15 electrospray interface operated in the positive ion mode. The chromatographic separation was performed on a 100 mm × 2.1 mm i.d., 3 µM, C30 column (Acclaim, ThermoFisher Scientific, SJ, USA) kept at 40°C. 20 µl of the sample was analysed using gradient elution with 0.1% formic acid in Milli-Q water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) at a flow of 0.4 ml/min over 8 min  
 20 (**Table 1**).

**Table 1.** UHPLC gradient conditions.

| Time (min) | A (%) <sup>a</sup> | B (%) <sup>b</sup> |
|------------|--------------------|--------------------|
| 0          | 100                | 0                  |
| 2.5        | 100                | 0                  |
| 3          | 0                  | 100                |
| 3.5        | 0                  | 100                |
| 4          | 100                | 0                  |
| 8          | 100                | 0                  |

<sup>a</sup> A: 0.1% formic acid in Milli-Q water

<sup>b</sup> B: 0.1% formic acid in acetonitrile

Mass spectra were acquired at a resolution of 140,000 over the range  
 25 of 220 to 260 Th. The electrospray voltage was set to 4000 V. The sheath gas pressure and the auxiliary gas pressure were 5 and 5 (ThermoFisher arbitrary units) respectively. The capillary

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temperature was 300°C and the s-lens was 80V. Data processing of chromatograms was performed using the Quanbrowser function of the Xcalibur Software package version 2.5 (ThermoFisher Scientific, NJ, USA). Quantification was performed on analyte-specific peaks obtained using accurate mass extracted ion chromatograms (XIC) (Table 2).

**Table 2.** Analytes of interest and their corresponding mass to charge (m/z) ratio.

| Analytes                                  | m/z       | Analytes                    | m/z       |
|-------------------------------------------|-----------|-----------------------------|-----------|
| dihydro-AZA                               | 247.1037  | deoxycytidine (dC)          | 228.09788 |
| dihydro-AZA- <sup>15</sup> N <sub>4</sub> | 251.09242 | 5-methyldeoxycytidine (mdC) | 242.11353 |
| dihydro-DAC                               | 231.10878 | cytidine (C)                | 244.0928  |
| dihydro-DAC- <sup>15</sup> N <sub>4</sub> | 235.09747 | 5-methylcytidine (mC)       | 258.10845 |

Calibration standard mixes were prepared by dilution in CE buffer of the following chemicals: 5-azacitidine & decitabine (Selleckchem, TX, USA), cytidine triphosphate & deoxycytidine triphosphate (Promega, WI, USA), and 5-methylcytidine & 5-methyl-2'-deoxycytidine (MP Biomedicals, CA, USA). To 5 µl of each standard mix were added 5 µl of 50 µM 5-aza-2'-deoxycytidine-<sup>15</sup>N<sub>4</sub> and 5 µl of 50 µM 5-azacitidine-<sup>15</sup>N<sub>4</sub> internal standards. The mixture was reduced, neutralised, and digested as described in the RNA/DNA sample preparation section above. Samples were dried under vacuum and resuspended in 50 µl of CE buffer, bringing the final concentration ranges to: AZA & DAC (0.01 µM - 1 µM), C & dC (1 µM - 50 µM), and mC & mdC (0.02 µM - 1 µM). Standard curves were created by plotting the analyte: internal standard ratio against analyte concentration. The concentration of AZA, DAC, C, dC, mC and mdC in the samples was calculated from the corresponding standard curves by interpolation.

Global RNA methylation level was assessed by calculating the ratio of mC over total C (mC+C), and global DNA methylation level was determined from the ratio of mdC over total dC (mdC + dC) (Quinlivan, E.P. and Gregory, J.F. (2008) *Nucleic acids research*,

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36, e119-e119). Intra-assay variability was calculated by running eight replicate 500 nM AZA standard samples consecutively within the same run. Inter-assay variability was determined by assaying eight replicate 1000 nM AZA standard samples on 8 different days.

5 Correlation was measured by calculating the Pearson product-moment coefficient. All statistical analyses were performed in GraphPad Prism.

## 2 RESULTS

### 10 2.1 Establishing a high-resolution mass spectrometry method to directly quantify intracellular DAC

It was believed desirable to develop a quantitative LC-MS based method to accurately measure the amount of DAC within cells without interference from deoxycytidine (dC), a mass difference of just one Dalton (Figure 1A). As a first step, the inventors evaluated the applicability of a previously established LC-MS method for quantifying dC and 5-methyl-2'-deoxycytidine levels in DNA (Liu, J. et al. (2013), *Journal of Proteomics & Bioinformatics*) based on the premise that DAC would possess similar chemical properties to dC (Figure 1A-B). The original method was developed using a triple quadrupole mass spectrometer that possesses very high sensitivity, though its resolving power is lower than other types of commercially available mass spectrometers. The inventors tested the ability of the triple quadrupole to accurately discriminate between dC and DAC. Two samples, one containing only dC and another containing only DAC were analysed. If there is clear discrimination between the two molecules, there should only be a signal for dC in the dC-containing sample and only for DAC in the DAC-containing sample. Both dC and DAC eluted with a retention time of 1.34 min (Figure 1C). In the DAC-only sample, a clean signal was detected corresponding to the appropriate mass to charge ratio (m/z) for DAC, and nothing corresponding to dC (Figure 1C), as expected. However, in the dC-only sample, an interference signal was observed in the m/z window corresponding to DAC (Figure 1D), wherein there should be no signal. The inventors considered that the interference was arising from the <sup>15</sup>N- and <sup>13</sup>C- containing natural isotopes of dC (Figure 1E).

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Given the very close m/z values for DAC (229.093), <sup>15</sup>N-dC (229.095) and <sup>13</sup>C-dC (229.101), the inventors considered that to accurately quantify DNA-incorporated DAC at a resolution of 0.001 m/z would require the use of a mass spectrometer with a resolving power of at least 229,000 (full width at half maximum, FWHM), much greater than possible on the triple quadrupole machine. The inventors reasoned that a new generation Orbitrap mass spectrometer (Q Exactive Plus, ThermoFisher) with a mass resolution of 280,000 (FWHM) would have more than sufficient capability to separate out DAC from the different isotopes of dC. Using spiked test samples as before, the inventors were able to achieve direct mass resolution of DAC from <sup>15</sup>N-dC and <sup>13</sup>C-dC on the Orbitrap (Figure 1F).

## 2.2 Quantifying DNA-incorporated DAC intracellularly with high sensitivity

Having established that accurate detection and quantification of DAC using the Orbitrap was possible, the inventors set about establishing a sensitive method to isolate nucleic-acid incorporated nucleosides including AZA and DAC. Intracellularly, nucleosides can exist as mono-, di- and tri-phosphorylated nucleotides. In order to reduce the analytical complexity associated with measuring the various isoforms of all of the different nucleotides, the inventors adopted a DNA-fragmentation method utilizing a mix of enzymes which yields dephosphorylated nucleosides from DNA (Quinlivan, E.P. and Gregory, J.F. (2008) *Analytical biochemistry*, **373**, 383-385). However, a major complication is the marked chemical instability and hydrolysis of AZA and DAC in aqueous environments, with a reported half-life of as low as seven hours at 37°C. Since the reported DNA fragmentation method required a six-hour incubation step at 37°C, the inventors tested whether the prolonged incubation at this temperature would be detrimental to DAC (and by corollary, AZA). Indeed, the inventors observed a more than two-fold decrease in detectable signal by MS consistently across a range of DAC concentrations tested, following a six-hour incubation at 37°C (Figure 2A).

The inventors sought to mitigate this problem by a two-pronged approach. Firstly, the inventors decided to establish the minimum

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amount of time required to completely fragment DNA. Testing a range of fragmentation times (1, 2 or 6 hours) on different input DNA concentrations (1, 2 and 5 µg), the inventors determined that 1 hour was sufficient to fragment up to 5 µg of DNA (Figure 2B). Secondly, the inventors aimed to decrease the rate of spontaneous hydrolysis of AZA or DAC in aqueous solutions. The inventors believed that a major mode of decomposition of AZA and DAC is through hydrolytic ring opening of the labile 5,6-bond of the triazine ring, followed by deformylation. To decrease the reactivity (i.e. increase the stability) of the triazine ring, the inventors reduced DAC to the more stable dihydro-DAC using the reducing agent sodium borohydride, to thereby improve the detection sensitivity. Across a range of DAC concentrations tested (0.5 - 1000 nM), the inventors observed a greater than two-fold improvement in signal for DAC (Figure 2C). Additionally, the inventors set about evaluating a range of different parameters to identify attributes that might further improve the sensitivity of detection. The inventors also ascertained that removing ammonium formate from the sample solution (the solution comprising the analyte) resulted in a twenty seven-fold improvement in signal intensity for DAC across a range of DAC concentrations tested (Figure 2D). Evaluating a range of chromatographic columns, including a C18 HSS, C18 BEH and a C30 column, the inventors identified the C30 column as the most optimal (data not shown). The inventors also assessed the m/z scan range and MS source conditions via continual infusion of standard solution (as outlined in the Materials and Methods section above). The combination of these features provided a method having sufficient resolution and sensitivity to directly quantify DAC intracellularly (Figure 2E).

### 2.3 Establishing the multi-parameter AZA-MS method

As a first step towards applying the method to primary samples, the inventors set about establishing a minimum quantity of input DNA in which it would still be possible to detect DAC incorporation, as it was anticipated that the cell numbers and resultant DNA yields from primary samples would be crucial limiting factors. In addition, there was the added complexity that the exact *in vivo* doses

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encountered by hematopoietic cells residing in the bone marrow, and therefore the amounts of AZA incorporated into their DNA, upon AZA therapy was not known. The inventors devised a two-factorial experiment employing the colorectal cancer cell line, RKO, in which the molecular mechanisms following decitabine treatment have been well characterised (Hesson, L.B. et al. (2013) *PLoS Genet*, **9**, e1003636). The inventors treated RKO cells with a wide range of AZA concentrations (100 nM - 1250 nM) that were expected to flank the range of reported *in vivo* dosages. The cells were quickly washed with buffer containing the cytidine deaminase inhibitor tetrahydrouridine (Ebrahim, Q. et al. (2012) *Oncotarget*, **3**, 1137-1145; Lavelle, D. et al. (2012) *Blood*, **119**, 1240-1247) in order to dampen any deamination of AZA during the subsequent processing steps. DNA was subsequently isolated from these cells and different input concentrations of DNA (100 ng - 1250 ng) were treated and fragmented as per the method described above. Across the entire range of AZA treatment dosages, it was possible to reliably detect DNA-incorporated DAC from a minimum of 500 ng of input DNA (Figure 3A).

Having established that the method could successfully detect DNA incorporated DAC, the inventors next sought to expand the capability of the method to also simultaneously detect AZA incorporation into RNA, as well as to quantify unincorporated, cytoplasmic AZA and DAC. Mutations or altered expression of the enzymes involved in AZA metabolism have been attributed as causes of AZA and DAC resistance in cell lines and patients. Additionally, drug resistance could also potentially arise because of reduced cellular abundance as a result of decreased influx or increased efflux. Mutations of the drug efflux protein MDR1 have been shown to promote AZA resistance in a cell line, though somatic mutations of this gene have not been observed to correlate with AZA resistance in patients. From RKO cells treated across a range of relevant AZA concentrations (100 nM - 1250 nM) as before, the inventors isolated intact total RNA. The fragmentation mix used for complete fragmentation of DNA was also suitable for degrading RNA into its constituent ribonucleosides. The inventors determined that the fragmentation conditions established

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for complete DNA fragmentation (1 hour, 37°C) were also sufficient for complete fragmentation of RNA (data not shown). The inventors also reduced RNA-incorporated AZA using sodium borohydride to decrease degradation of AZA in aqueous solutions and followed the fragmentation steps already established for DNA and DAC. The inventors then assessed signals for RNA-incorporated AZA across a range of different input amounts of RNA (100 ng - 1250 ng, Figure 3B). As with DNA, it was established that a minimum input amount of 500 ng of RNA yielded reliably quantifiable AZA signals across the entire range of AZA treatment doses tested (Figure 3B). Finally, to quantify unincorporated free AZA and DAC in the cytoplasm, a further alteration was made to the method by incorporating a methanol-based extraction step to isolate cytoplasmic nucleotides (Derissen, E.J. et al. (2014) *Journal of pharmaceutical and biomedical analysis*, **90**, 7-14). In the altered method, a fraction of AZA treated cells was set aside for extracting unincorporated nucleotides from the cytoplasm, while DNA and RNA were simultaneously extracted from the remaining cells (Figure 3C).

#### 2.4 Applying AZA-MS to survey intracellular dynamics of AZA treatment in vitro

To survey the intracellular pharmacokinetics of AZA therapy comprehensively using AZA-MS, the inventors treated RKO cells with 1.25  $\mu$ M AZA, a dose that was established as sufficient to demethylate DNA (Figure 3D) and re-induce expression of the MLH1 tumour suppressor gene (Figure 3E). AZA- or DMSO-treated cells were harvested and DNA, RNA and cytoplasmic extracts were prepared and analysed by the AZA-MS method described above. By spiking samples with defined quantities of isotopically labelled  $^{15}\text{N}_4$ -AZA and  $^{15}\text{N}_4$ -DAC as internal reference standards, it was possible to obtain precise quantifications of intracellular AZA and DAC in different subcellular fractions with robust reproducibility by AZA-MS, evidenced by low variability in inter-assay (%RSD = 5.03%, n = 8, Table 3) and intra-assay (%RSD = 0.80%, n = 8, Table 3) runs. Free, unincorporated AZA was readily detected in the cytoplasm of treated cells at  $11.46 \pm 0.11$  pmol AZA/ million cells whereas there was no signal in DMSO control cells (Figure 3F). RNA-incorporated AZA was

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also detected in treated cells ( $1.32 \pm 0.03$  pmol of AZA per 1  $\mu$ g of RNA) with no signal in the DMSO control (Figure 3F). Free DAC was detected in the cytoplasmic extracts ( $9.59 \pm 0.16$  pmol DAC per 1 million cells) as well as incorporated into DNA ( $5.65 \pm 0.12$  pmol of DAC per 1  $\mu$ g of DNA), while no signal was observed in control cells (Figure 3G). Neither AZA incorporation into DNA, nor DAC incorporation into RNA was observed (data not shown), consistent with the highly evolved specificity of the different polymerases for their cognate nucleotides and also the activity of repair mechanisms that remove misincorporated nucleotides.

Table 3

| Sample <sup>a</sup> | Inter-assay AZA measurements |                                               |                    | Intra-assay AZA measurements |                                               |                    |
|---------------------|------------------------------|-----------------------------------------------|--------------------|------------------------------|-----------------------------------------------|--------------------|
|                     | Analyte <sup>b</sup>         | [ <sup>15</sup> N <sub>4</sub> ] <sup>c</sup> | Ratio <sup>d</sup> | Analyte <sup>b</sup>         | [ <sup>15</sup> N <sub>4</sub> ] <sup>c</sup> | Ratio <sup>d</sup> |
| 1                   | 711603                       | 4375184                                       | 0.163              | 24417361                     | 293257441                                     | 0.083              |
| 2                   | 1596919                      | 9807888                                       | 0.163              | 15419854                     | 190758878                                     | 0.081              |
| 3                   | 1631353                      | 10209937                                      | 0.160              | 1416859                      | 16397612                                      | 0.086              |
| 4                   | 1086512                      | 6758246                                       | 0.161              | 1529706                      | 19905537                                      | 0.077              |
| 5                   | 1011977                      | 6337687                                       | 0.160              | 849735                       | 10663726                                      | 0.080              |
| 6                   | 891294                       | 5496896                                       | 0.162              | 926884                       | 10415279                                      | 0.089              |
| 7                   | 816006                       | 5095821                                       | 0.160              | 934163                       | 10787802                                      | 0.087              |
| 8                   | 758596                       | 4691786                                       | 0.162              | 842053                       | 9719439                                       | 0.087              |
| Mean                | 1063033                      | 6596681                                       | 0.161              | 5792077                      | 70238214                                      | 0.084              |
| %RSD                | 34.06                        | 34.13                                         | 0.80               | 156.22                       | 155.98                                        | 5.03               |

<sup>a</sup>Eight replicates of an AZA standard solution were independently analysed by LC-MS: 1000 nM AZA solution was used for inter-assay and 500 nM AZA for intra-assay measurements.

<sup>b</sup>Peak area of the analyte, AZA.

<sup>c</sup>Peak area of the [<sup>15</sup>N<sub>4</sub>]-labeled AZA internal standard.

<sup>d</sup>Ratio of analyte to [<sup>15</sup>N<sub>4</sub>]-labeled internal standard.

The inventors used the AZA-MA method to explore the relationship between DAC or AZA incorporation to DNA or RNA methylation respectively. RKO cells were treated with a range of AZA concentrations (0 nM – 1250 nM) for three days, DNA and RNA were extracted and prepared for LC – MS as before. The amount of DNA-incorporated DAC increased linearly with increasing AZA doses ( $R^2 = 0.9973$ , Figure 4A). Conversely, global DNA methylation level, assessed as the percentage of methylated dC over total dC (i.e. dC + mdC) decreased linearly and inversely with increasing AZA treatment concentration ( $R^2 = 0.9483$ , Figure 4B). DNA methylation levels were

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~40% of levels observed in untreated cells. This data fits neatly with the well-established DNA demethylating role of AZA.

Unexpectedly however, a similar trend was not observed in RNA. AZA incorporation increased proportionally with increasing AZA treatment dosage ( $R^2 = 0.9896$ , Figure 4D). Global RNA methylation, assessed as the percentage of methylated C over total C (i.e. C + mC) remained unchanged. Even after three days of 1250 nM AZA treatment, RNA methylation levels were ~125% of that observed in untreated cells. Therefore, these results indicate that there is no correlation between AZA incorporation into RNA and RNA demethylation, even though AZA incorporation can trap RNA methyltransferases.

#### *2.5 AZA-MS reveals differences in the intracellular pharmacokinetics of AZA during in vivo therapy*

The inventors used the AZA-MS method to study AZA intracellular dynamics *in vivo*, using primary samples obtained from MDS or CMML patients undergoing AZA therapy. Archived bone marrow CD34<sup>+</sup> cells were available from eight patients (MDS, n=4 ; CMML, n =4) who had received at least 6 cycles of standard AZA therapy. From each patient, three longitudinal samples had been collected over the course of treatment: immediately before the start of treatment, i.e. pre-treatment; after 7 consecutive days of the first cycle of AZA therapy, C1d8; and at the end of the first cycle of treatment (C1d28), at a period after twenty days off the drug (Figure 5A). Four patients (R1-R4) were assessed to have been AZA complete responders by IWG criteria, while the other four (N1-N4) were non-responders (Figure 5B).

In all eight patients, DNA-incorporated DAC could be observed at C1d8, though it was significantly more in AZA responders compared to non-responders (responders, mean = 0.23 pmol of DAC per  $\mu$ g of DNA; non-responders, mean = 0.12 pmol of DAC per  $\mu$ g of DNA; p value = 0.03, Figure 5C). DAC incorporation was also correlated with DNA demethylation, with increased demethylation observed in AZA responders (mean = 75.3% of pre-treatment levels, Figure 5D) compared to non-responders (mean = 80.5% of pre-treatment levels, Figure 5D). While DAC levels in DNA dropped by C1d28, residual amounts were still detectable in the cells of all patients

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(responders, mean = 0.079 pmol of DAC per  $\mu$ g of DNA; non-responders, mean = 0.0169 pmol of DAC per  $\mu$ g of DNA, Figure 5C). DNA methylation, conversely, bounced back to almost pre-treatment levels in all patients at Cld28 (Figure 5D).

5 The patterns of interplay between DAC incorporation and DNA demethylation were even more clearly observed when analysing individual patients. The levels of demethylation at Cld8 were greatest amongst individuals who had the highest levels of DNA-incorporated DAC, both in responders ( $R4 > R3 > R2 > R1$ , Figure 5E)  
10 and non-responders ( $N4 > N3 > N1$  and  $N2$ , Figure 5F). Furthermore, two patterns were observed in the non-responders: in non-responders  $N1$  and  $N2$ , there were very low levels of AZA incorporated into DNA at Cld8 (0.01 and 0 pmol of DAC per  $\mu$ g of DNA respectively, Figure 5F). As a consequence, there was no demethylation in these patients  
15 (Figure 5F). In the remaining two non-responders  $N3$  and  $N4$ , however, AZA incorporation was much higher (0.16 and 0.3 pmol of DAC per  $\mu$ g of DNA respectively, Figure 5F) and so was DNA demethylation, dropping to ~60% of pre-treatment levels (Figure 5F).

#### 2.6 Low DAC incorporation into DNA is not a result of impaired 20 intracellular AZA metabolism

The low DNA incorporation of DAC observed in the AZA non-responders could arise because of low intracellular accumulation of AZA, either as a result of ineffective drug import or elevated drug efflux, or as a result of low conversion of AZA into DAC intracellularly. To  
25 investigate these possibilities, the inventors compared the intracellular concentrations of AZA in the bone marrow CD34<sup>+</sup> cells of responders and non-responders. The levels of intracellular AZA at Cld8 were almost seventy-folds higher in non-responders compared to responders (responders, mean = 0.0146 nmol of AZA per  $\mu$ mol cytidine;  
30 non-responders, mean = 1.0 nmol of AZA per  $\mu$ mol cytidine, Figure 6A). In fact, non-responders  $N1$  and  $N2$ , who had almost no DNA incorporated DAC, had the highest quantities of intracellular AZA at Cld8 (Figure 6A). Intracellular concentrations of DAC were also much higher at Cld8 in AZA non-responders, whereas it was undetectable in  
35 the responders (responders, mean = 0 nmol of DAC per  $\mu$ mol deoxycytidine; non-responders, mean = 0.023 nmol of DAC per  $\mu$ mol

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deoxycytidine, Figure 6B). Interestingly, in the non-responders, the patients with the highest levels of free DAC ( $N4 > N2 > N1$ , Figure 6B) had the lowest levels of free AZA ( $N1 > N2 > N4$ , Figure 6A), suggesting that ribonucleotide reductase responsible for converting AZA to DAC is subject to feedback inhibition by these substrates. Whilst feedback inhibition of RNR by endogenous nucleotides has been extensively studied before, these results provide evidence that AZA and DAC are also capable of regulating RNR activity *in vivo*. The increased amount of unincorporated AZA in non-responders was also reflected by greater amount of AZA incorporation into RNA (responders, mean = 0.36 pmol of AZA per  $\mu\text{g}$  RNA; non-responders, mean = 0.70 pmol of AZA per  $\mu\text{g}$  RNA, Figure 6C). The non-responders with the highest amounts of free AZA, N1 and N2, also had the highest amounts of RNA incorporated AZA (1.57 and 0.71 pmol of AZA per  $\mu\text{g}$  RNA respectively, Figure 6C). This is likely a result of the shift in azacitidine : cytidine nucleotide ratios in the cytoplasm of non-responders, enabling a greater likelihood for incorporation into transcripts in AZA non-responders.

### 3. DISCUSSION

In order to gain a detailed picture of the intracellular dynamics of AZA, the inventors developed the quantitative LC-MS based analytical method AZA-MS (discussed above) to quantify AZA and DAC in different subcellular components. Utilising the AZA-MS method in conjunction with the ultra-high mass accuracy and resolution of the hybrid quadrupole Orbitrap mass spectrometer, the inventors achieved mass separation of DAC from all of the naturally occurring endogenous isotopic forms of deoxycytidine. The interference of DAC signal from natural isotopes of dC has confounded other analytical attempts to accurately quantify DAC (Mompalmer, R.L. et al. (1985) *Pharmacol Ther*, **30**, 277-286). Previous studies have attempted to resolve this by either attempting to identify molecule-specific fragments in tandem MS, without success (Derissen, E.J. et al. (2014) *Journal of pharmaceutical and biomedical analysis*, **90**, 7-14), or to mathematically correct the signal based on an assumption of a constant isotopic distribution (Jansen, R.S. et al. (2012) *Cancer chemotherapy and pharmacology*, **69**, 1457-1466). The AZA-MS method

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described herein, when implemented in conjunction with the new generations of mass spectrometers with very high resolving power, provides a method to directly separate the different AZA, DAC and related molecules based on their relative mass differences.

5 The added confidence obtained from these direct measurements have enabled the inventors to trace the intracellular fate of AZA (and DAC) and quantitatively measure its abundance in DNA, RNA and the cytoplasm, while also simultaneously measuring its biological impact through the measurement of DNA and RNA methylation. Previous  
10 attempts have only focused piece-meal on individual components, which has limited a fuller understanding of the effects of AZA on the cell. The closest efforts to measuring multiple parameters have either; 1.) been performed *ex vivo*, by treating primary cells with tritiated AZA, followed by measuring DAC incorporation and total  
15 intracellular abundance through scintillation counts (Öz, S. et al. (2014) *Nucleic acids research*, gku775), or 2.) directly, using mass spectrometry, but only to measure DAC incorporation into DNA and corresponding DNA methylation following decitabine treatment *in vitro* of cell lines or *in vivo* in mice, without showing any  
20 corresponding capability to measure quantities in humans (Anders, N.M. et al. (2016) *J Chromatogr B Analyt Technol Biomed Life Sci*, **1022**, 38-45). In the former case, it is unclear what artefactual effects *ex vivo* treatment of cells would cause. In the latter case, the method also did not quantify unincorporated DAC in the  
25 cytoplasm.

The comprehensive simultaneous measurements of multiple parameters by AZA-MS yields significant insights into the intracellular dynamics of AZA treatment. The AZA-MS method can also be readily adapted to measure other nucleic acid modifications of clinical and  
30 biological importance, including cytosine hydroxymethylation in DNA and RNA, or RNA adenine methylation, as well as to study the intracellular pharmacology of other nucleotide analogs used as therapeutic agents.

By applying the AZA-MS method to the RKO cell line where the  
35 molecular effects of DNA demethylation through DAC treatment had been previously studied, it is believed that while there is a clear

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dose-dependent correlation between DAC incorporation into DNA and DNA demethylation, there is no correlation between the amount of AZA incorporated into RNA and RNA demethylation. Indeed, RNA demethylation was not decreased even at the highest doses of AZA treatment, a level sufficient to decrease DNA methylation by 50%. While the amount of AZA incorporated into RNA (1.2 pmol of AZA per ug of RNA at 1250 nM of AZA) was considerably less than that of DAC per an equivalent unit of DNA (6 pmol of DAC per ug of DNA), these findings could be explained by the fact that there are significantly more molecules of RNA per cell compared to DNA. In addition, the average half-life of RNA transcripts are considerably shorter than that of DNA molecules. This greater turnover would mean that RNA-incorporated AZA would be degraded into its various decomposition products more rapidly than DNA-incorporated DAC.

Finally, by applying the AZA-MS method to primary bone marrow samples from patients, it was possible to measure the cellular pharmacokinetics of AZA therapy *in vivo*. The amount of DNA incorporated DAC detectable after 7 consecutive days of AZA therapy ranged from 0.11 pmol/ug - 0.43 pmol/ug (median = 0.19 pmol/ug) in the bone marrows of AZA responders to ranges of 0 pmol/ug - 0.30 pmol/ug (median = 0.08 pmol/ug) in AZA non-responders. These values are an order higher than those previously reported based on scintillation counts from bone marrow mononuclear cells from nine AML patients treated *ex vivo* for twenty-four hours with a fixed dose (100 nM) of decitabine (Öz, S. et al. (2014) *Nucleic acids research*, gku775). These higher signals in these measurements, made directly on primary samples that have not been extensively manipulated *ex vivo*, merely highlight the enhanced sensitivity afforded by the AZA-MS method described herein. Extrapolating from the DAC incorporation signals observed in RKO cells treated at various AZA concentrations, the *in vivo* DAC signals could equate to bone marrow cells being exposed to *in vivo* AZA doses of 27 nM - 100 nM (median = 44 nM) in responders, decreasing to 0 nM - 34 nM (median = 20 nM) by the end of cycle 1. In AZA non-responders, the doses were 4 nM - 70 nM (median = 20 nM) at Cld8, dropping down to 0 nM - 16 nM (median = 0 nM) by the end of the cycle. However, these measurements are indirect inferences and do not take into account differences in the

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cell cycle behaviour of different populations of hematopoietic cells in the bone marrow, or the morphological and vascular complexity of the bone marrow. Direct determination of AZA in bone marrow serum collected simultaneously at the appropriate time points from patients could provide validation of these inferences.

In the small set of longitudinal samples analysed, it was observed that the biggest difference between AZA responders and non-responders was the uniformly higher levels of DAC incorporation into DNA in AZA responders. The magnitude of DAC incorporation also correlated with DNA demethylation. However, there were two patterns observed in AZA non-responders: some AZA non-responders (n=2) showed minimal DAC incorporation into DNA and thus minimal DNA demethylation. In these patients, it was possible to detect AZA and DAC intracellularly, as well as AZA incorporation into RNA, suggesting that neither cellular uptake nor intracellular metabolism could be potential reasons to explain the low rates of DNA incorporation of DAC in these patients. It is therefore likely that in these patients, an increased proportion of the bone marrow cells might be quiescent and not undergoing DNA replication, therefore not incorporating DAC into their DNA. Increased cell cycle quiescence as a result of the overexpression of the cytokines CXCL4 and CXCL7 has been observed in CMML patients who fail to respond to AZA therapy.

However, in other AZA non-responders, DAC incorporation into DNA and resultant DNA demethylation at levels similar to AZA responders was observed. This suggests that there might be multiple mechanisms driving primary AZA resistance. Recently, it has been detected that DAC treatment of cancer cell lines induces transcription of endogenous retroviral elements, leading to an interferon response in cells. It is possible that in the AZA non-responders who incorporate DAC into DNA but fail to respond, AZA therapy might not lead to the induction of an interferon response necessary for proper AZA response. Alternatively, these patients could have increased tolerance to, or defective, immune-cell mediated clearance of dysplastic cells.

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Having applied AZA-MS to clinically annotated samples, the inventors considered that AZA refractoriness is not simply due to failure of AZA uptake in cells and incorporation in DNA, but is more complex.

#### **Example 2**

- 5 The reducing agents sodium borohydride ( $\text{NaBH}_4$ ) and sodium triacetoxyborohydride ( $\text{NaBH}(\text{OAc})_3$ ) were compared using the substrates AZA and DAC. The results are summarised graphically in Figure 7, wherein the values are displayed relative to  $\text{NaBH}_4$ .

#### *Procedure*

- 10 To 1000nM of 5-aza-2'-deoxycytidine or 5-azacytidine (Toronto Research Chemicals, Canada), 10  $\mu\text{l}$  of 20 mg/ml  $\text{NaBH}_4$  or 20mg/ml  $\text{NaBH}(\text{OAc})_3$  was added. The mixture was incubated at room temperature with agitation for 20 min, and neutralized to pH7.0 with 5M HCl (for  $\text{NaBH}_4$ ) or 1M NaOH (for  $\text{NaBH}(\text{OAc})_3$ ). Samples were dried under vacuum
- 15 (Savant Speedvac Plus SC210A, USA) and resuspended in 50  $\mu\text{l}$  of CE buffer (10 mM TrisHCl pH 8.0 and 0.5 mM EDTA in Milli-Q water) for LC-MS analysis. The reduced analytes were then analysed by AZA-MS using the LC-MS procedures detailed in section 1.3 and 1.4 above.

- 20 It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art, in Australia or any other country.

- In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to
- 25 express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in
- 30 various embodiments of the invention.

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**CLAIMS:**

1. A method for determining the amount of a cytidine analogue in a mixture, the cytidine analogue having an optionally substituted unsaturated 6-membered heterocyclyl ring comprising 3 ring carbon  
5 atoms and 3 ring nitrogen atoms, the method comprising treating the mixture to reduce the 6-membered heterocyclyl ring of the cytidine analogue and then determining the amount of the reduced cytidine analogue.
- 10 2. The method according to claim 1, wherein the amount of the reduced cytidine analogue is determined by a quantification technique selected from mass spectroscopy, liquid chromatography, LC-MS and NMR.
- 15 3. The method according to claim 1, wherein the amount of the reduced cytidine analogue is determined by mass spectroscopy.
4. The method according to any one of claims 1 to 3, wherein the mixture is a cellular extract.
- 20 5. The method according to claim 4, wherein the cellular extract is an aqueous cellular extract.
6. The method according to claim 4 or 5, wherein the cellular  
25 extract is a cytoplasmic extract.
7. The method according to claim 4, wherein the cellular extract is a nucleic acid extract.

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8. The method according to claim 7, wherein the nucleic acid extract is a DNA extract.

9. The method according to claim 7, wherein the nucleic acid  
5 extract is an RNA extract.

10. The method according to claim 7, wherein the method further comprises:

a) after treating the nucleic acid extract to reduce the 6-  
10 membered heterocyclyl ring of the cytidine analogue, depolymerising the nucleic acid extract to afford the constituent deoxyribonucleosides and ribonucleosides; and

b) determining the amount of the reduced cytidine analogue in the nucleic acid extract to thereby determine the amount of the  
15 cytidine analogue that had been in the nucleic acid extract.

11. The method according to claim 8, wherein the method further comprises:

a) after treating the DNA extract to reduce the 6-membered  
20 heterocyclyl ring of the cytidine analogue, depolymerising the DNA to afford the constituent deoxyribonucleosides; and

b) determining the amount of the reduced cytidine analogue in the extract to thereby determine the amount of the cytidine analogue that had been in the DNA.

25

12. The method according to claim 9, wherein the method further comprises:

a) after treating the RNA extract to reduce the 6-membered  
heterocyclyl ring of the cytidine analogue, depolymerising the RNA  
30 to afford the constituent ribonucleosides; and

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b) determining the amount of the reduced cytidine analogue in the extract to thereby determine the amount of the cytidine analogue that had been in the RNA.

5 13. The method according to any one of claims 10 to 12, wherein the depolymerisation is performed by an enzymatic process.

14. The method according to claim 13, wherein the enzymatic process comprises the use of a phosphatase, phosphodiesterase or  
10 both a phosphatase and a phosphodiesterase.

15. The method according to claim 14, wherein the enzymatic process is performed at a pH of between about 7 and about 9.

15 16. The method according to any one of claims 10 to 15, wherein the depolymerisation is performed in less than about 1 hour.

17. The method according to any one of claims 10 to 16, wherein the depolymerisation is performed at less than about 40 °C.

20

18. The method according to any one of claims 1 to 17, wherein the mixture is reduced by contacting the mixture with a reducing agent.

19. The method according to claim 18, wherein the reducing agent  
25 is NaBH<sub>4</sub>.

20. The method according to any one of claims 1 to 19, wherein the amount of the reduced cytidine analogue is determined using a mass

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spectrometer having a resolving power of at least 229,000 full width at half maximum (FWHM).

21. The method according to any one of claims 1 to 20, wherein the  
5 step of determining the amount of the reduced cytidine analogue comprises subjecting the reduced cytidine analogue to liquid chromatography, mass spectroscopy or LC-MS in a solution comprising less than about 0.01 mol L<sup>-1</sup> ammonium formate buffer.

10 22. The method according to any one of claims 1 to 21, wherein the method further comprises determining the amount of an analyte other than the reduced cytidine analogue.

23. The method according to claim 22, wherein the amount of the  
15 reduced cytidine analogue and the amount of the analyte other than the reduced cytidine analogue are determined by liquid chromatography, mass spectroscopy or LC-MS.

24. The method according to claim 23, wherein the amount of the  
20 reduced cytidine analogue and the amount of the analyte other than the reduced cytidine analogue are determined simultaneously by liquid chromatography, mass spectroscopy or LC-MS.

25. A method for determining the amount of 5-aza-2'-deoxycytidine  
25 (DAC) incorporated into the DNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

- a) obtaining cells from the subject;
- b) extracting nucleic acid from the cells to afford a nucleic acid extract;

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c) treating the nucleic acid extract to reduce the 6-membered heterocyclyl ring of the 5-aza-2'-deoxycytidine (DAC) incorporated into the DNA;

d) treating the nucleic acid extract to depolymerise the DNA;  
5 and

e) determining the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) in the product of step d) to thereby determine the amount of the 5-aza-2'-deoxycytidine (DAC) that had been incorporated into the DNA of the subject.

10

26. The method according to claim 25, wherein in step e), the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) is determined by mass spectroscopy.

15 27. A method for determining the amount of 5-azacitidine (AZA) incorporated into the RNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

a) obtaining cells from the subject;

b) extracting nucleic acid from the cells to afford a nucleic  
20 acid extract;

c) treating the nucleic acid extract to reduce the 6-membered heterocyclyl ring of the 5-azacitidine (AZA) incorporated into the RNA;

d) treating the nucleic acid extract to depolymerise the RNA;  
25 and

e) determining the amount of the reduced 5-azacitidine (reduced AZA) in the product of step d) to thereby determine the amount of the 5-azacitidine (AZA) that had been incorporated into the RNA of the subject.

30

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28. The method according to claim 27, wherein in step e), the amount of the reduced 5-azacitidine (reduced AZA) is determined by mass spectroscopy.

5 29. A method for determining the amount of 5-aza-2'-deoxycytidine (DAC) that had not been incorporated into the DNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

a) obtaining cells from the subject;

b) extracting the cytoplasm from the cells to afford a  
10 cytoplasmic extract;

c) treating the cytoplasmic extract to reduce the 6-membered heterocyclyl ring of the 5-aza-2'-deoxycytidine (DAC) in the extract; and

d) determining the amount of the reduced 5-aza-2'-  
15 deoxycytidine (reduced DAC) in the extract to thereby determine the amount of the 5-aza-2'-deoxycytidine (DAC) in the cytoplasm of the cells.

30. The method according to claim 29, wherein in step d), the  
20 amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) is determined by mass spectroscopy.

31. A method for determining the amount of 5-azacitidine (AZA) that had not been incorporated into the RNA of a subject that has  
25 been administered 5-azacitidine (AZA), the method comprising:

a) obtaining cells from the subject;

b) extracting the cytoplasm from the cells to afford a  
cytoplasmic extract;

c) treating the cytoplasmic extract to reduce the 6-membered  
30 heterocyclyl ring of the 5-azacitidine (AZA) in the extract; and

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d) determining the amount of the reduced 5-azacitidine (reduced AZA) in the product of step c) to thereby determine the amount of the 5-azacitidine (AZA) in the cytoplasm of the cells.

5 33. The method according to claim 31, wherein in step d), the amount of the reduced 5-azacitidine (reduced AZA) is determined by mass spectroscopy.

34. The method according to any one of claims 25 to 33, wherein  
10 the method further comprises determining the amount of 1, 2, 3 or 4 analytes selected from deoxycytidine (dC), 5-methyldeoxycytidine (mdC), cytidine (C) and 5-methylcytidine (mC).

35. The method according to claim 34, wherein the amount of the 1,  
15 2, 3 or 4 analytes is determined by mass spectroscopy.

36. A method for determining the amount of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) incorporated into the DNA and RNA, respectively, of a subject that has been administered 5-azacitidine  
20 (AZA), the method comprising:

a) obtaining cells from the subject;

b) extracting nucleic acid from the cells to afford a nucleic acid extract;

c) treating the nucleic acid extract to reduce the 6-membered  
25 heterocyclyl ring of the 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) incorporated into the DNA and RNA;

d) treating the extract to depolymerise the DNA and RNA; and

e) determining the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA)  
30 in the product of step d) to thereby determine the amount of the 5-

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aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) that had been incorporated into the DNA and RNA of the subject.

37. The method according to claim 36, wherein in step e), the amounts of the reduced 5-aza-2'-deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA) are determined by mass spectroscopy.

38. A method for determining the amount of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) that had not been incorporated into the DNA or RNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

- a) obtaining cells from the subject;
- b) extracting the cytoplasm from the cells to afford a cytoplasmic extract;
- c) treating the cytoplasmic extract to reduce the 6-membered heterocyclyl ring of the 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) in the extract; and
- d) determining the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA) in the product of step c) to thereby determine the amount of the 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) in the cytoplasm of the cells.

39. The method according to claim 38, wherein in step d), the amounts of the reduced 5-aza-2'-deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA) are determined by mass spectroscopy.

40. A method for determining the amount of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) that i) has and ii) has not been

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incorporated into the DNA or RNA of a subject that has been administered 5-azacitidine (AZA), the method comprising:

a) obtaining cells from the subject;

5 b) taking a portion of the cells obtained in step a) and extracting nucleic acid from the cells to afford a nucleic acid extract, treating the nucleic acid extract to reduce the 6-membered heterocyclyl rings of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) incorporated into the DNA and RNA, and treating the extract to depolymerise the DNA and RNA to afford a first sample; and

10 c) taking a portion of the cells obtained in step a) and extracting the cytoplasm from the cells to afford a cytoplasmic extract, and treating the cytoplasmic extract to reduce the 6-membered heterocyclyl rings of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) to afford a second sample;

15 d) determining the amount of the reduced 5-aza-2'-deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA) in the first sample and the second sample to thereby determine the amount of 5-aza-2'-deoxycytidine (DAC) and 5-azacitidine (AZA) that had and had not been incorporated into the DNA and RNA of the  
20 subject.

41. The method according to claim 40, wherein in step d), the amounts of the reduced 5-aza-2'-deoxycytidine (reduced DAC) and reduced 5-azacitidine (reduced AZA) are determined by mass  
25 spectroscopy.

42. A method for identifying a subject suffering from haematological neoplasms who will respond to treatment with AZA, the method comprising:

30 a) administering AZA to the subject;

b) obtaining cells from the subject after administration of AZA;

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c) determining the amount of DAC incorporated into the DNA of the subject by the method of claim 25 or 26; and

d) identifying the subject as a subject likely to respond to AZA treatment when DAC is incorporated into the DNA of the subject  
5 and is accompanied by the induction of double stranded RNA intracellularly and increased levels of pro-inflammatory cytokines.

43. A method for identifying and treating subjects suffering from haematological neoplasms which will respond to AZA, the method  
10 comprising:

a) administering AZA to the subject;

b) obtaining cells from the subject after administration of AZA;

c) determining the amount of DAC incorporated into the DNA of  
15 the subject by the method of claim 25 or 26;

d) identifying the subject as a subject likely to respond to AZA treatment when DAC is incorporated into the DNA of the subject and is accompanied by the induction of double stranded RNA intracellularly and increased levels of pro-inflammatory cytokines;  
20 and

e) administering an effective amount of AZA to the identified subject.

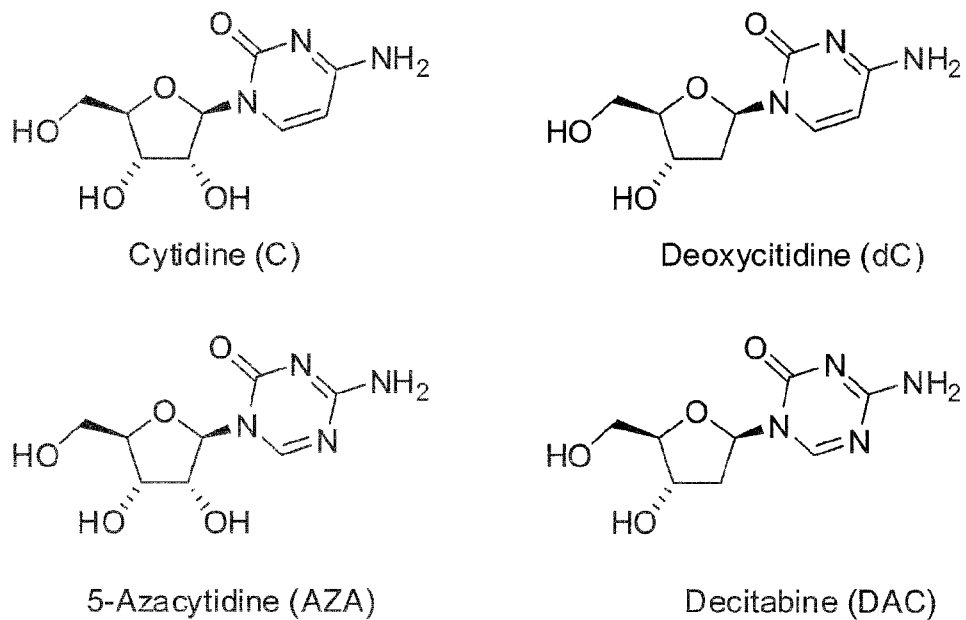


Figure 1(A)

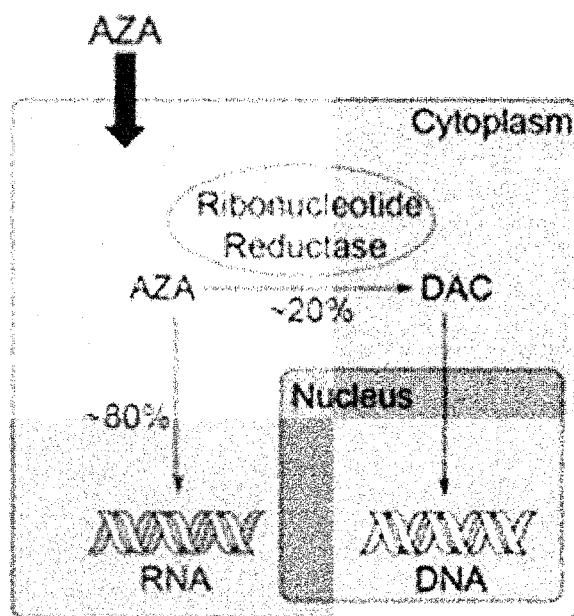


Figure 1(B)

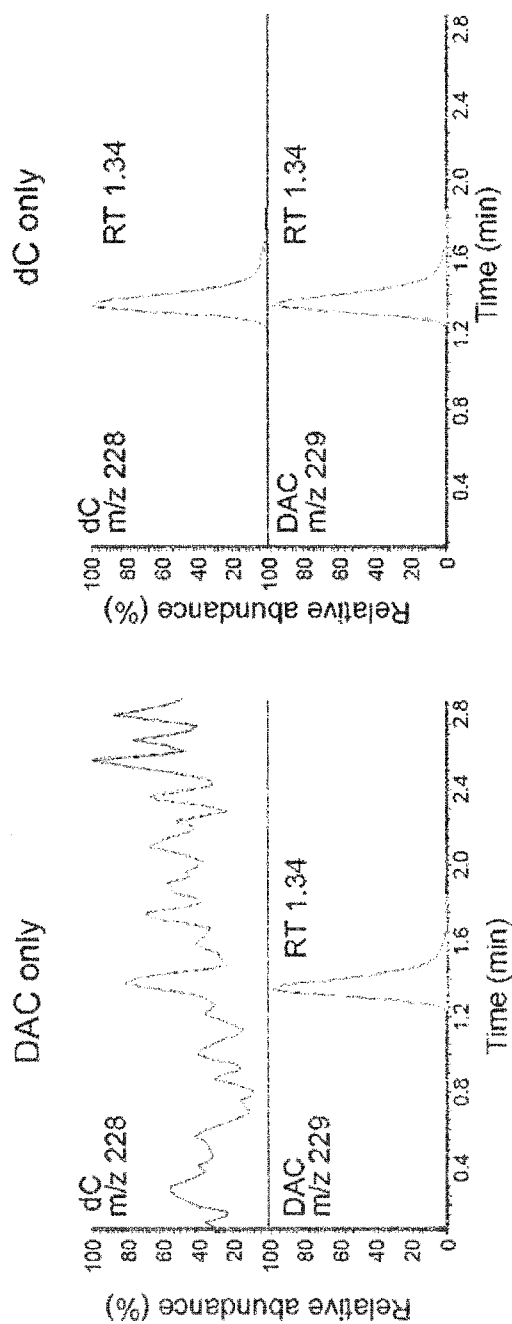


Figure 1(C)

TQMS mass spectrum - DAC and dC

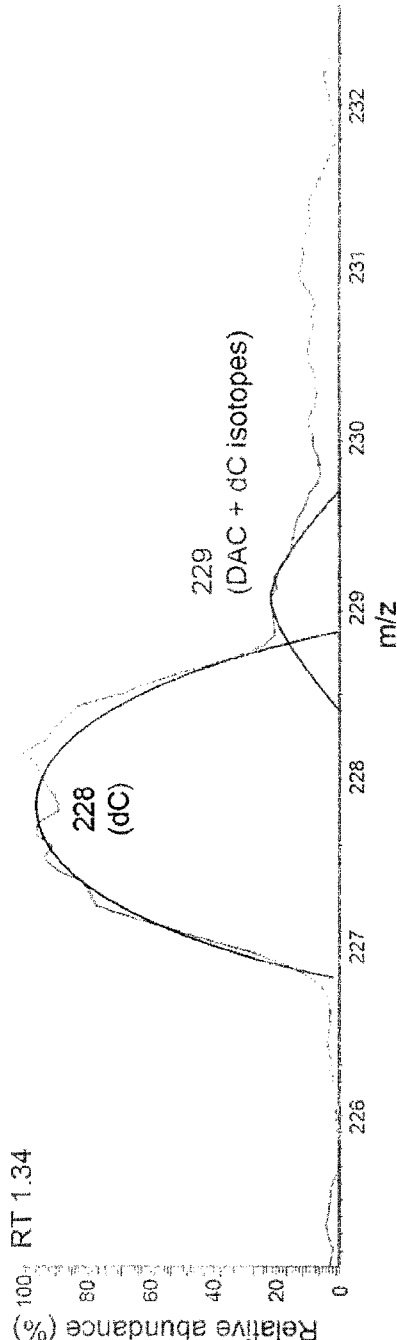


Figure 1(D)

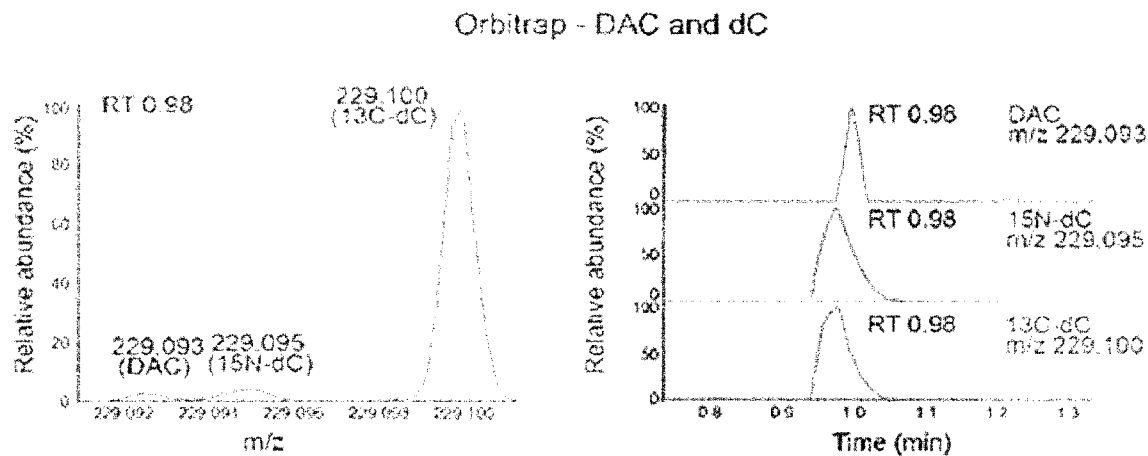


Figure 1(E)

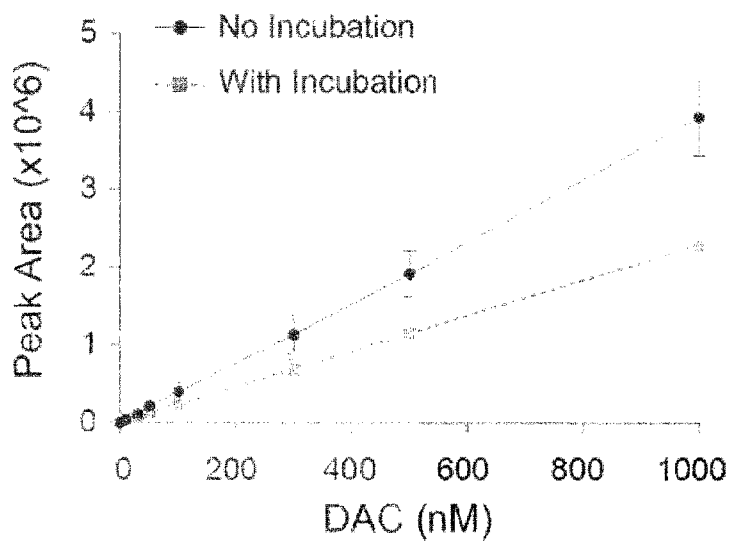


Figure 2(A)

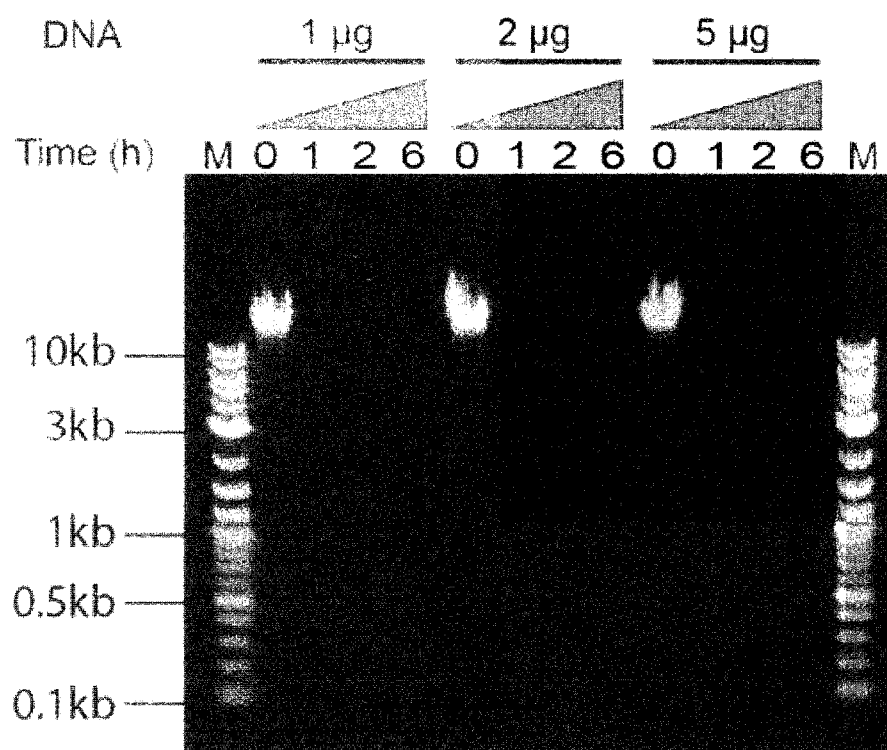


Figure 2(B)

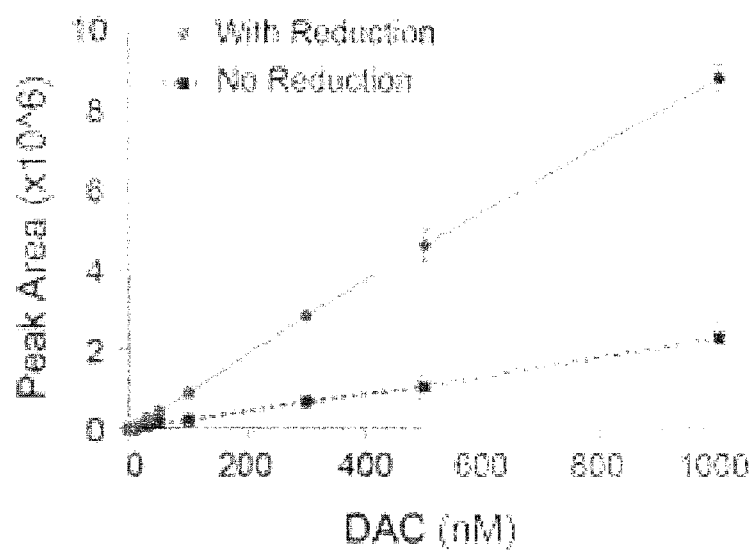


Figure 2(C)

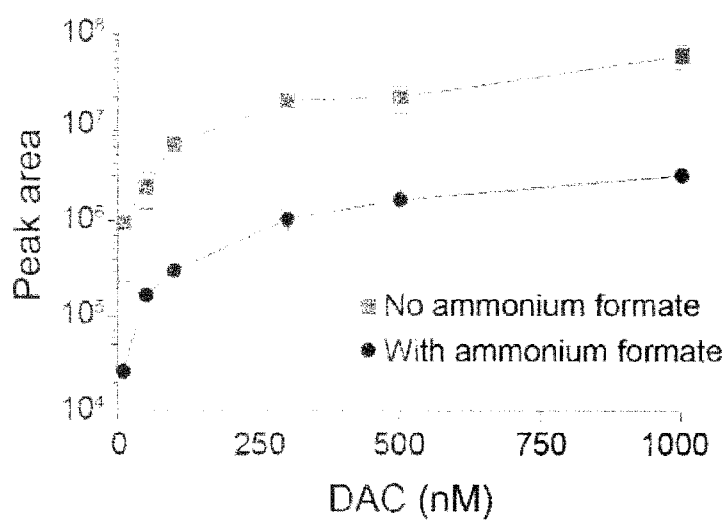


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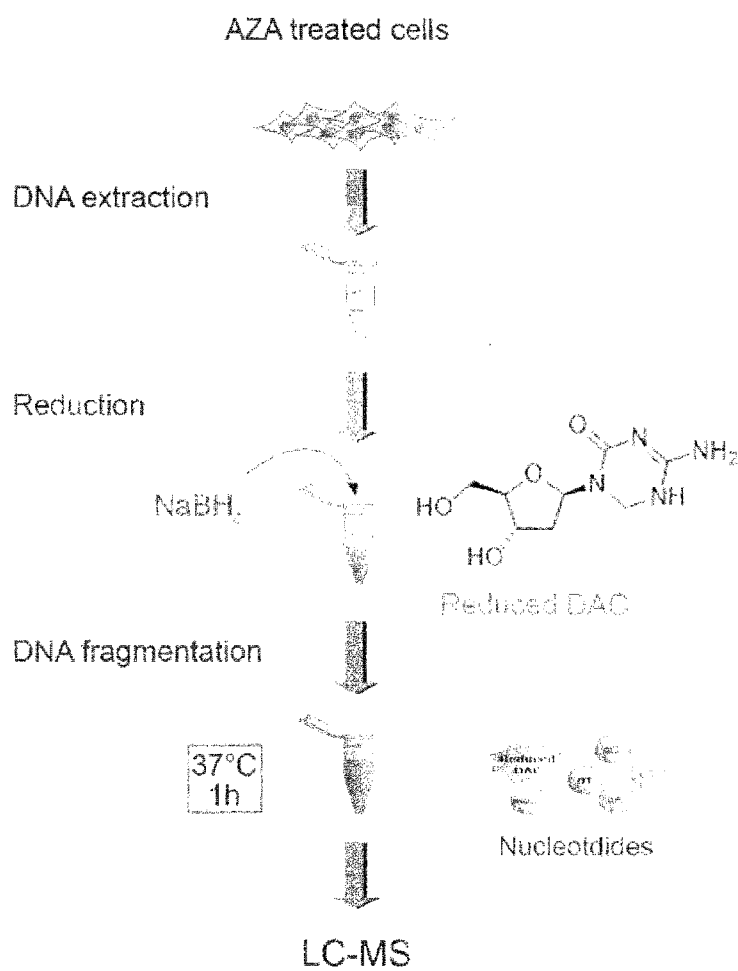


Figure 2(E)

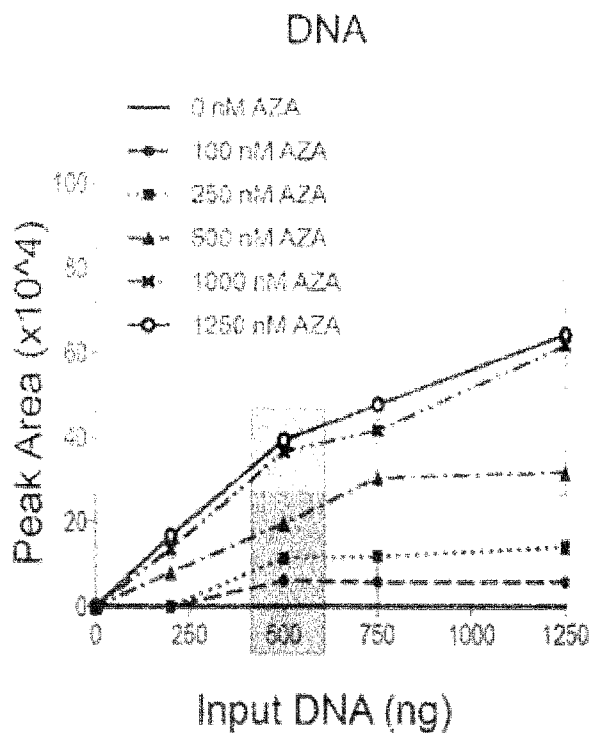


Figure 3(A)

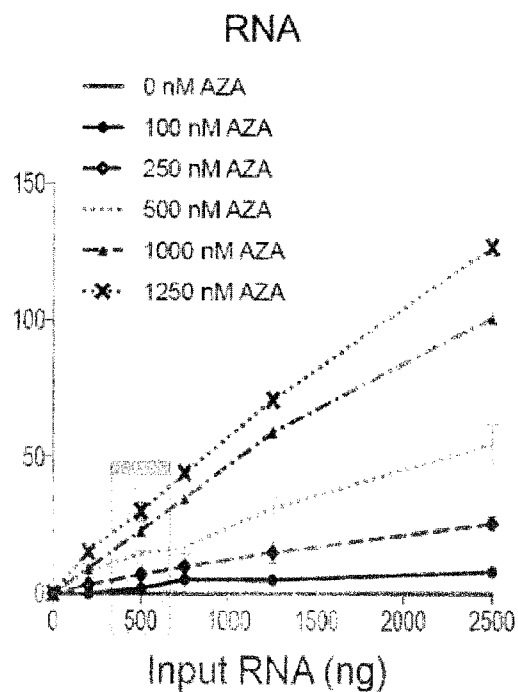


Figure 3(B)

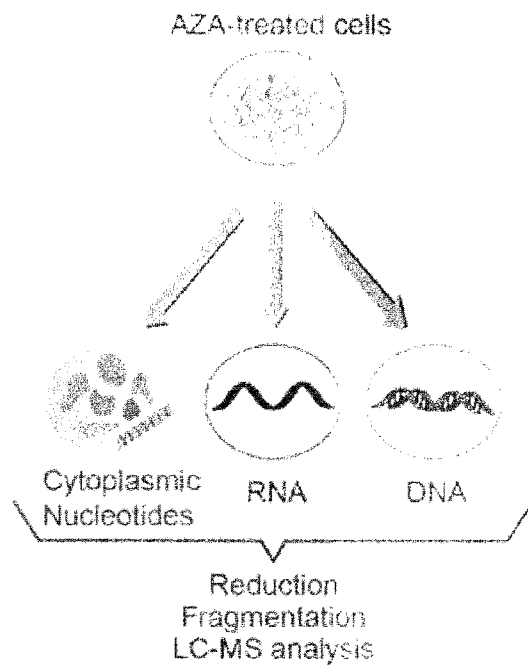


Figure 3(C)

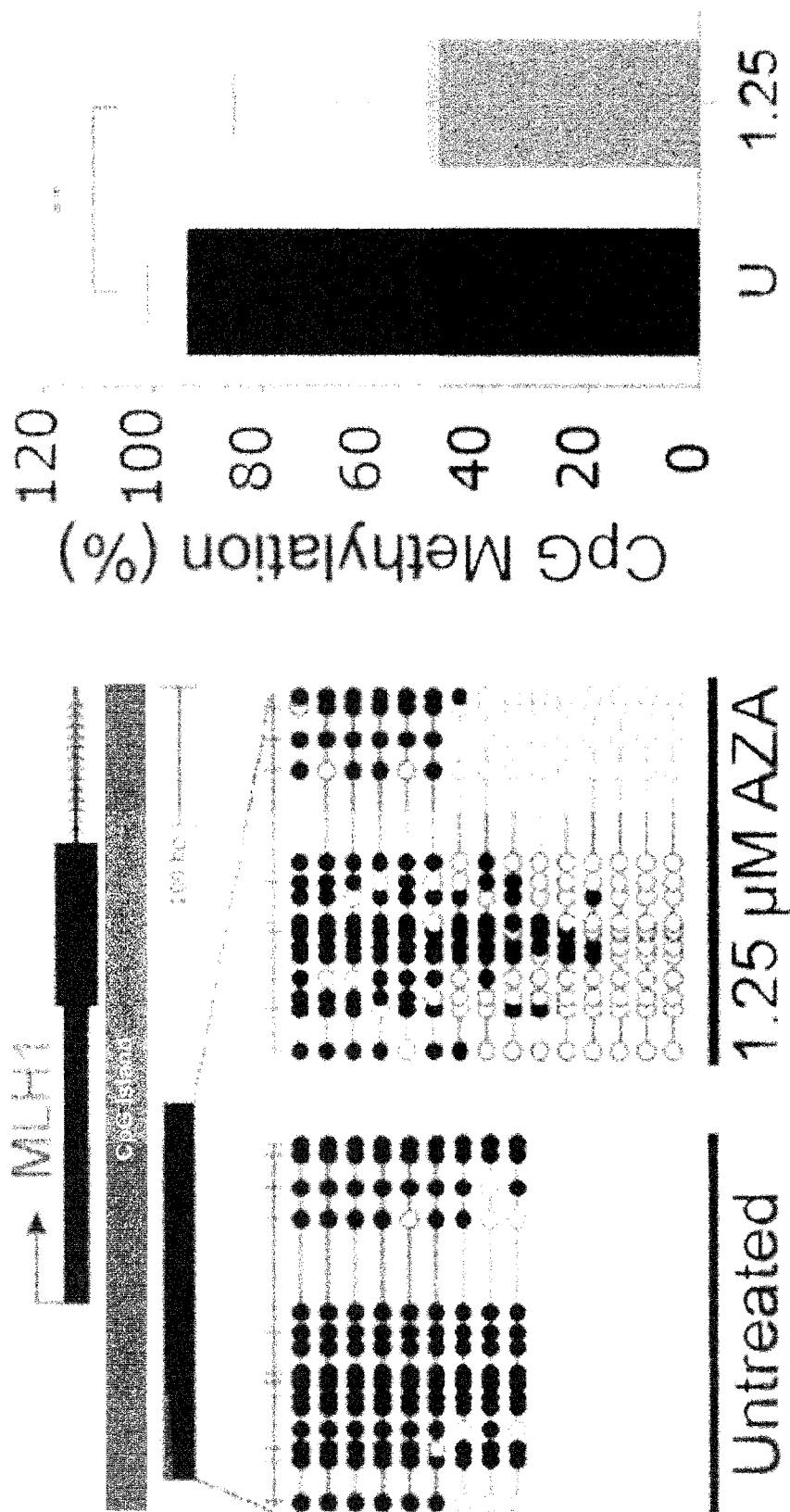


Figure 3(D)

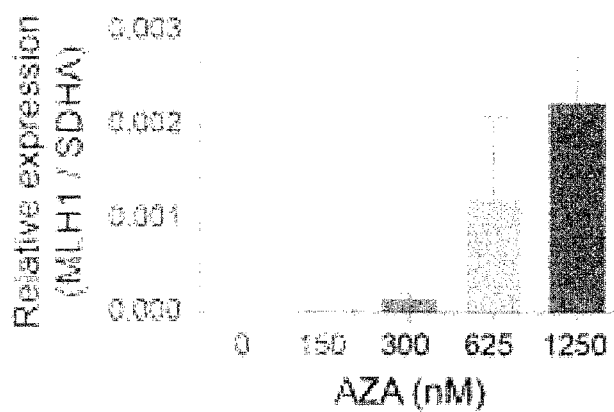


Figure 3(E)

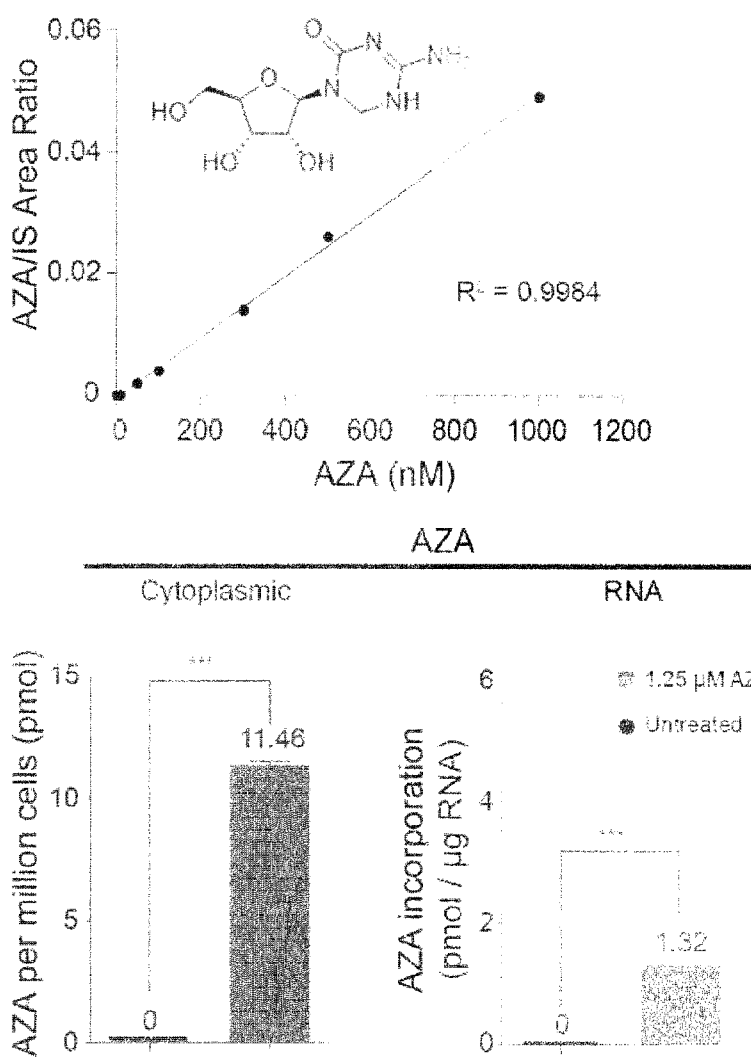


Figure 3(F)

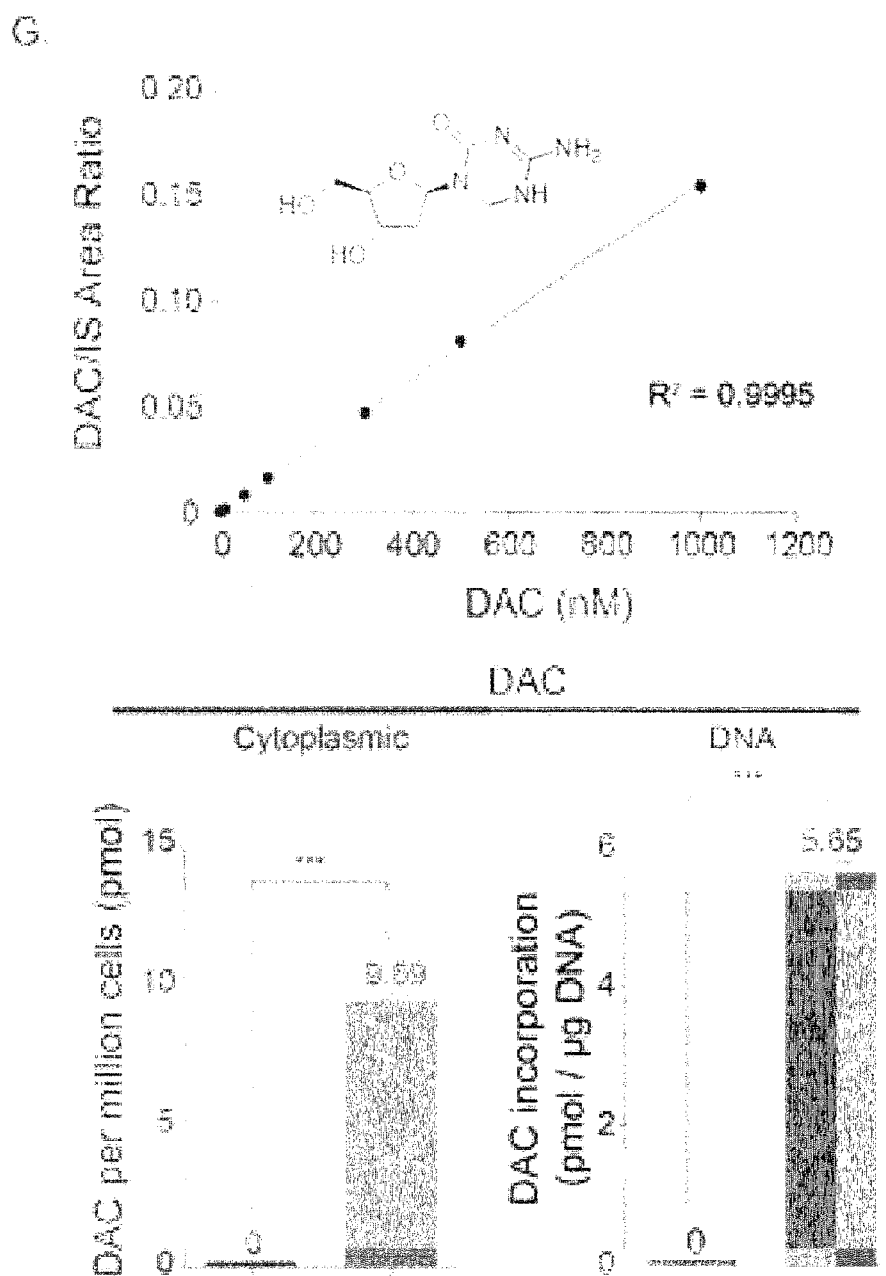


Figure 3(G)

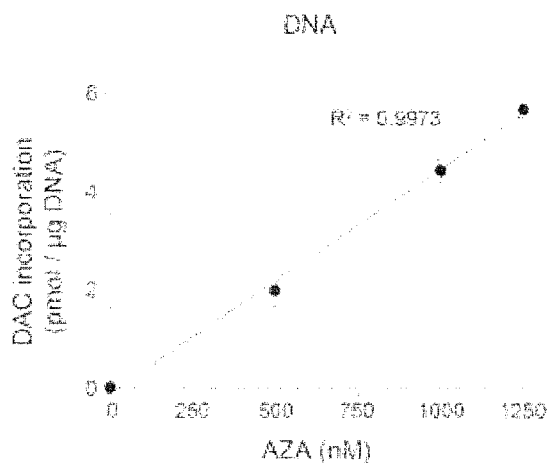


Figure 4(A)

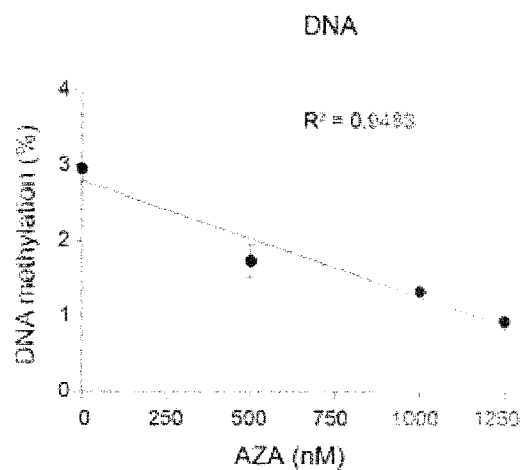


Figure 4(B)

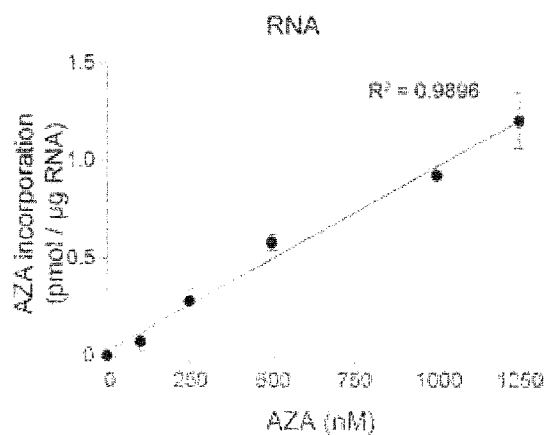


Figure 4(C)

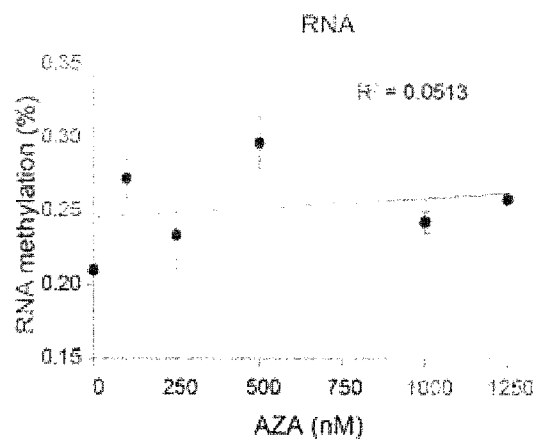


Figure 4(D)

AZA 75g mg/m2

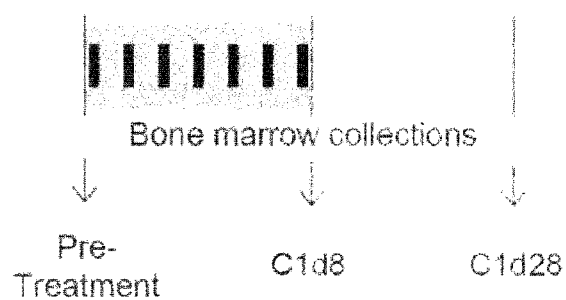


Figure 5(A)

- 11/16 -

| Patient | Disease (WHO Classification) | Gender | Age | IPSS-R                         | AZA response  |
|---------|------------------------------|--------|-----|--------------------------------|---------------|
| R1      | CMML                         | M      | 59  | Score=4/Intermediate           | Responder     |
| R2      | CMML                         | F      | 70  | Score=3.5/Intermediate         | Responder     |
| R3      | MDS/RAEB-2                   | F      | 69  | Score=5.5/High                 | Responder     |
| R4      | MDS/RAEB-2                   | M      | 73  | Neutrophils>8x 10 <sup>9</sup> | Responder     |
| N1      | MDS/RAEB-2                   | F      | 68  | Score= 7 /Very High            | Non-Responder |
| N2      | CMML                         | M      | 68  | Neutrophils>8x 10 <sup>9</sup> | Non-Responder |
| N3      | MDS/RAEB-2                   | M      | 69  | Score=5/High                   | Non-Responder |
| N4      | CMML                         | F      | 65  | Neutrophils>8x 10 <sup>9</sup> | Non-Responder |

Figure 5(B)

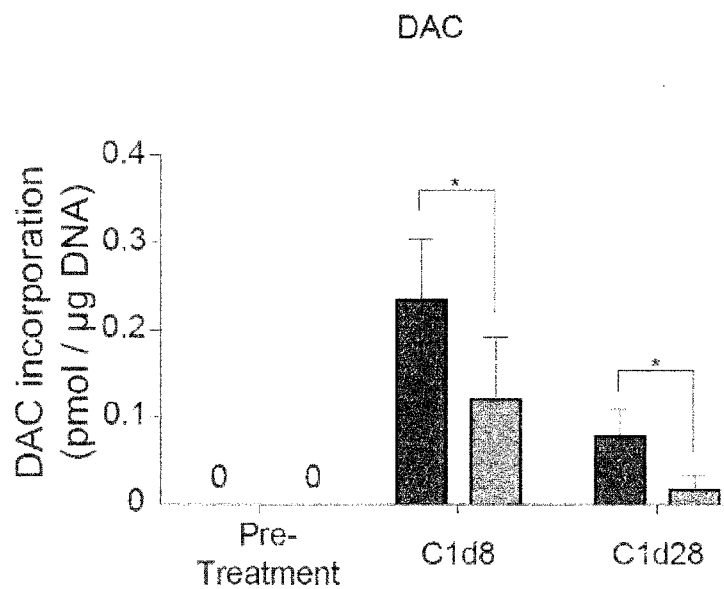


Figure 5(C)

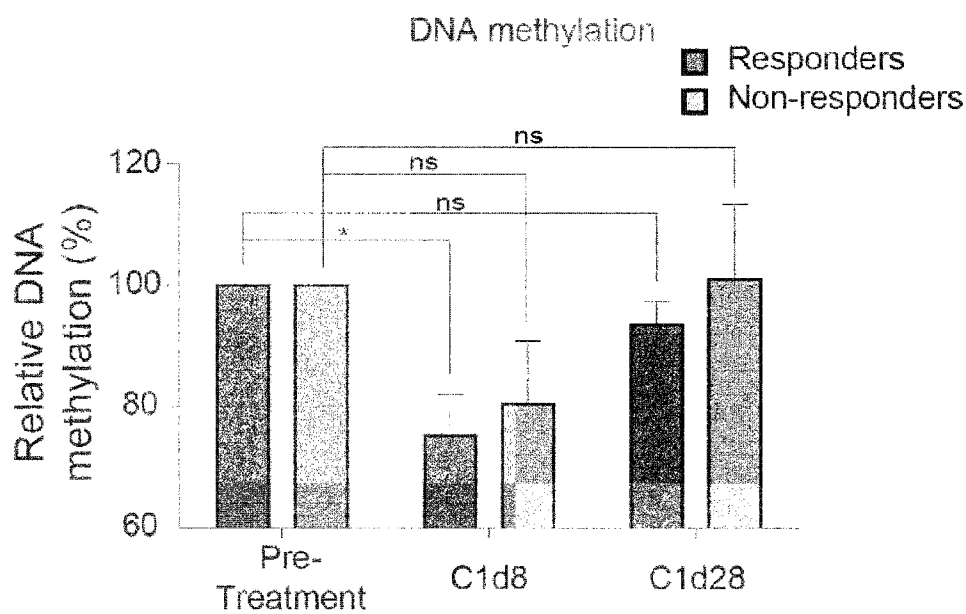


Figure 5(D)

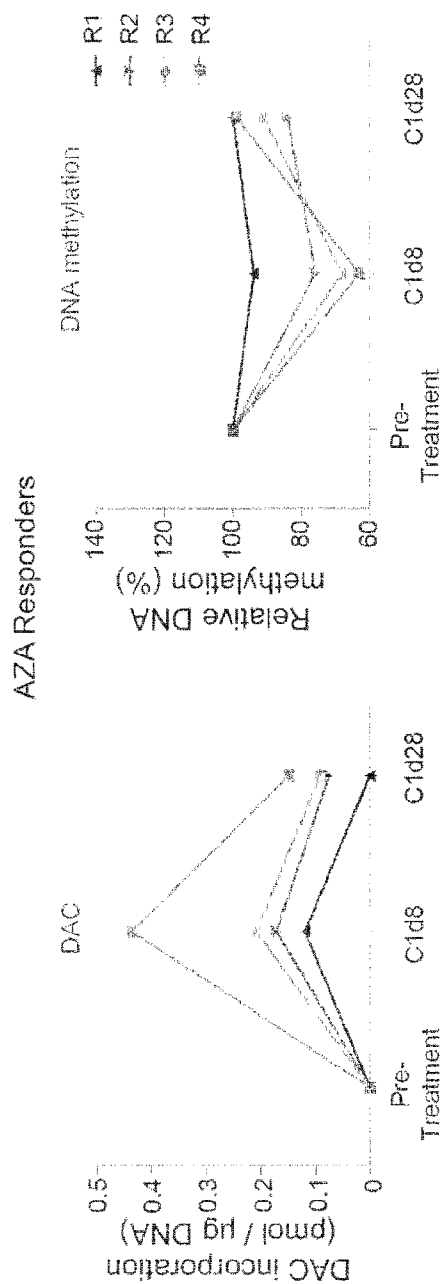


Figure 5(E)

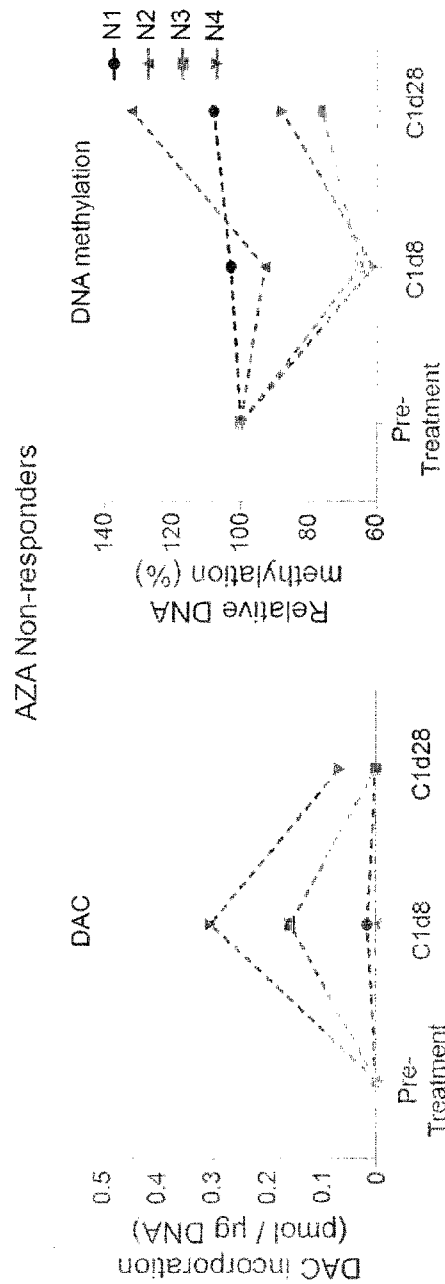


Figure 5(F)

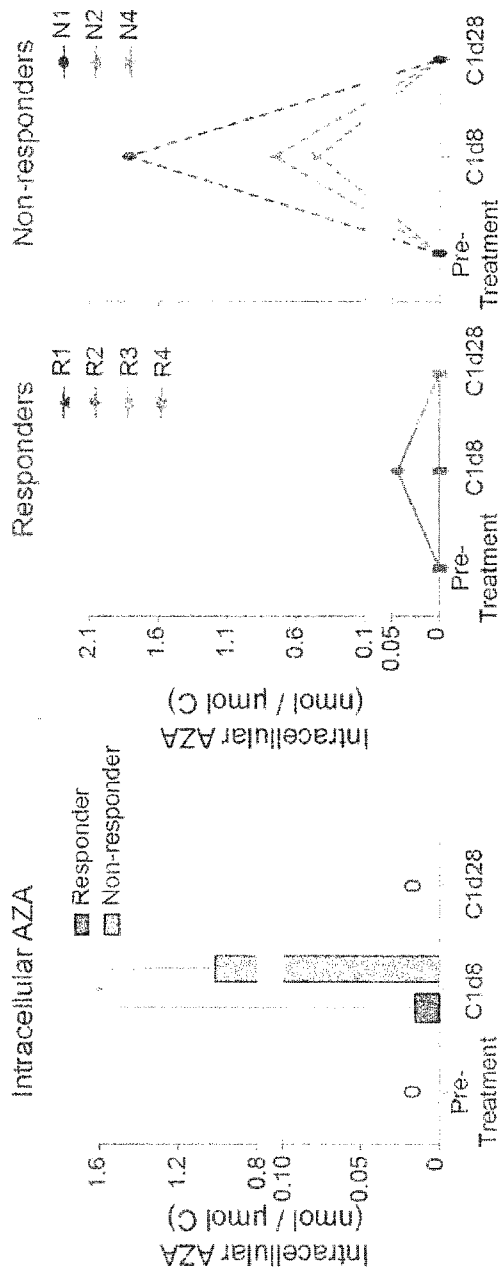


Figure 6(A)

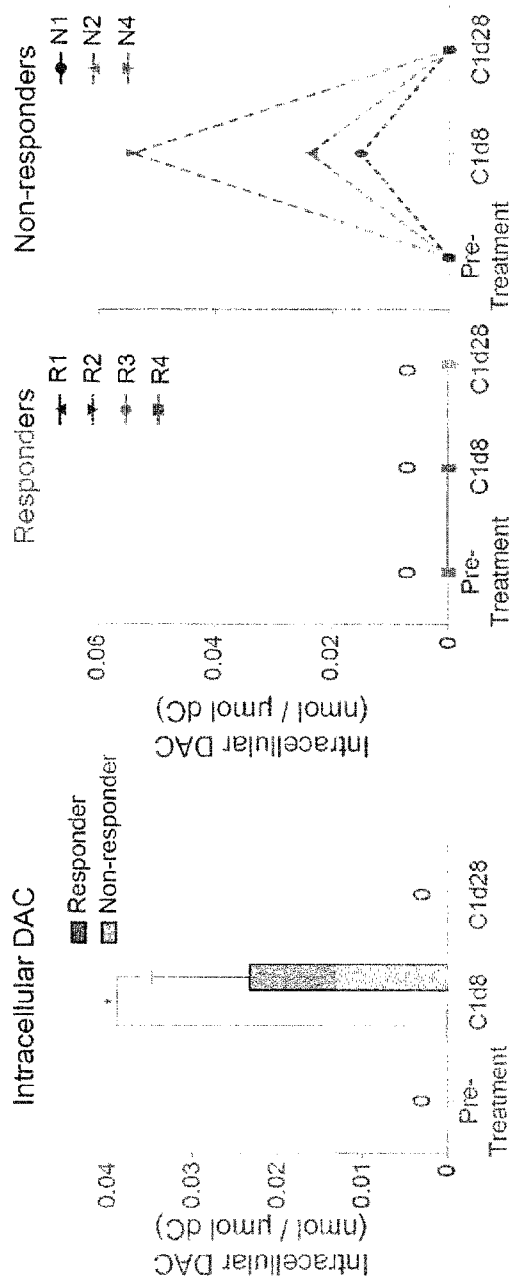


Figure 6(B)

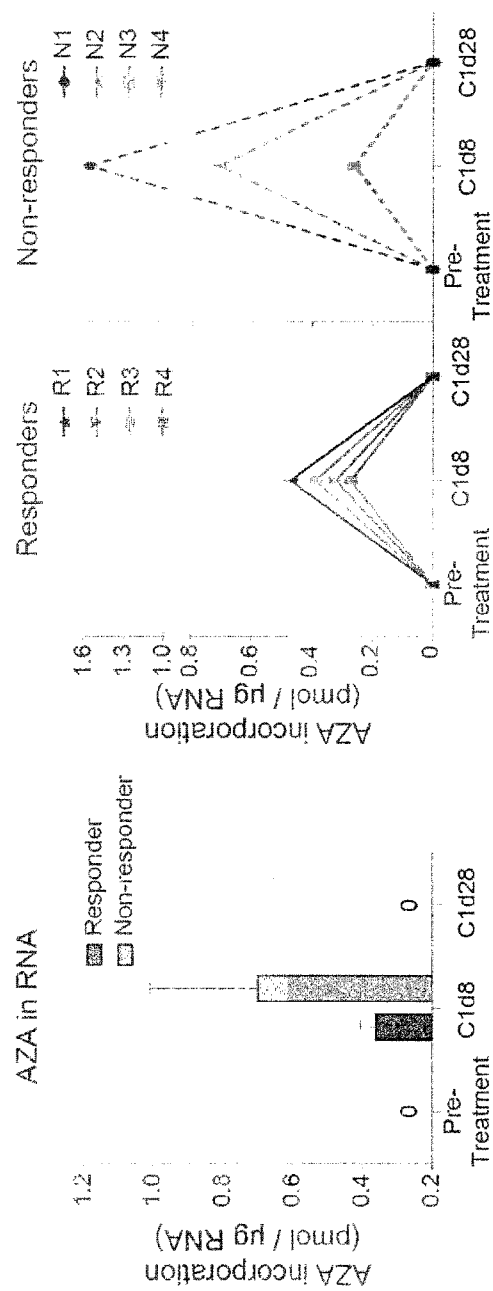


Figure 6(C)

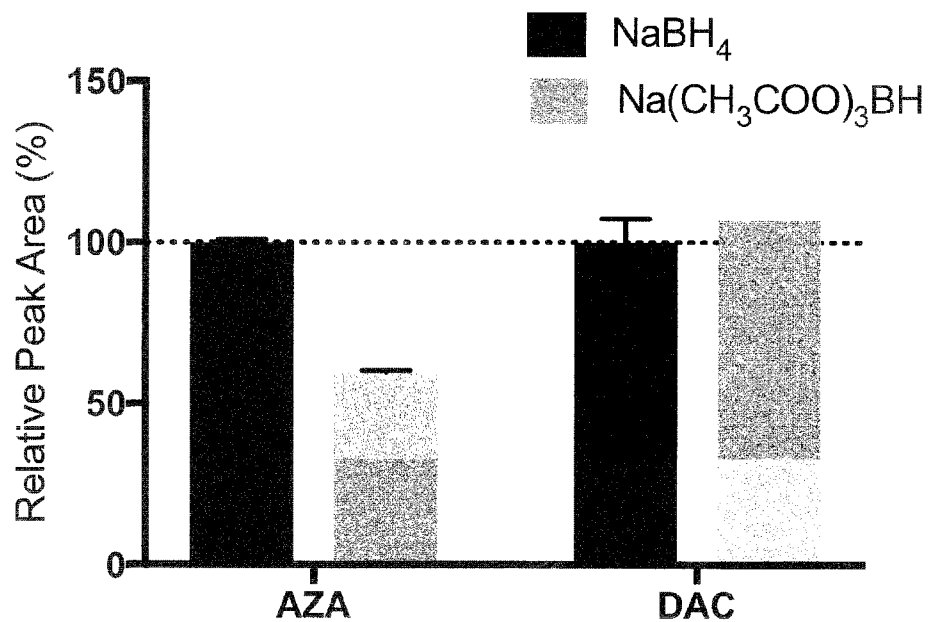
**Quantity of reduced AZA or DAC**

Figure 7

## INTERNATIONAL SEARCH REPORT

International application No.  
**PCT/AU2018/050597**

## A. CLASSIFICATION OF SUBJECT MATTER

**G01N 33/50 (2006.01) G01N 33/48 (2006.01)**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

**PATENW, MEDLINE, BIOSIS, HCAPLUS, EMBASE:** azacytidine, 5-AZA, 5-aza-2'-deoxycytidine, cytidine analogue, reducing agent, borohydride, G01N (IPC/CPC), detect, quantitate, quantify, incorporation, integration, intracellular dynamics, responder, prediction and related terms.

**HCAPLUS:** Structure search for cytidine analogue of Formula (I)

**eSpaceNet:** NewSouth Innovations AND G01N; AZA or cytidine or "5-azacitidine" AND NewSouth Innovations; Unnikrishnan, Ashwin; Pimanda, John; Vo, Anh; Pickford, Russell;

**PubMed:** Unnikrishnan, Ashwin; Pimanda, John; Vo, Anh; Pickford, Russell;

Applicant/Inventor names searched in internal databases provided by IP Australia.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------|-----------------------|
|           | Documents are listed in the continuation of Box C                                  |                       |



Further documents are listed in the continuation of Box C



See patent family annex

|          |                                                                                                                                                                     |     |                                                                                                                                                                                                                                              |
|----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| *<br>"A" | Special categories of cited documents:<br>document defining the general state of the art which is not considered to be of particular relevance                      | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention                                              |
| "E"      | earlier application or patent but published on or after the international filing date                                                                               | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone                                                                     |
| "L"      | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "O"      | document referring to an oral disclosure, use, exhibition or other means                                                                                            | "&" | document member of the same patent family                                                                                                                                                                                                    |
| "P"      | document published prior to the international filing date but later than the priority date claimed                                                                  |     |                                                                                                                                                                                                                                              |

Date of the actual completion of the international search  
20 July 2018

Date of mailing of the international search report  
20 July 2018

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| INTERNATIONAL SEARCH REPORT                           |                                                                                                                                                                                                                                                                                                                                                                                                                                           | International application No. |
|-------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|
| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                                                           | PCT/AU2018/050597             |
| Category*                                             | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                        | Relevant to claim No.         |
| X                                                     | AIMIUWU <i>et al.</i> : "RNA-dependent inhibition of ribonucleotide reductase is a major pathway for 5-azacytidine activity in acute myeloid leukemia" <i>Blood</i> , 2012, vol. 119, no. 22, pp. 5229-5238<br>See whole document, in particular abstract, page 5230 right column paragraph 2, page 5230 left column paragraph 4, page 5233 right column paragraph 3 and page 5234 left column paragraph 1.                               | 1-41                          |
| Y                                                     | See whole document, in particular abstract, page 5230 right column paragraph 2, page 5230 left column paragraph 4, page 5233 right column paragraph 3 and page 5234 left column paragraph 1.                                                                                                                                                                                                                                              | 42 AND 43                     |
| X                                                     | BEISLER <i>et al.</i> : "Synthesis and Antitumor Activity of Dihydro-5-azacytidine, a hydrolytically stable analogue of 5-Azacytidine" <i>Journal of Medicinal Chemistry</i> , 1977, vol. 20, no. 6, pp. 806-812<br>See whole document, in particular abstract, page 806 left column paragraph 1, page 807 left column paragraph 3 and page 807 right column paragraph 1.                                                                 | 1-3 AND 18-24                 |
| X                                                     | US 4058602 A (BEISLER <i>et al.</i> ) 15 November 1977<br>See whole document, in particular abstract, column 2 line 67 – column 3 line 28 and column 3 lines 43-68.                                                                                                                                                                                                                                                                       | 1-3 AND 18-24                 |
| Y                                                     | Öz <i>et al.</i> : "Quantitative determination of decitabine incorporation into DNA and its effect on mutation rates in human cancer cells" <i>Nucleic Acids Research</i> , 2014, vol. 42, no. 19, e152<br>See whole document, in particular abstract, page 7 left column – right column bridging paragraph and page 7 right column paragraph 4.                                                                                          | 42 AND 43                     |
| Y                                                     | WANG <i>et al.</i> : "In Vivo Quantification of Active Decitabine-Triphosphate Metabolite: A Novel Pharmacanalytical Endpoint for Optimization of Hypomethylating Therapy in Acute Myeloid Leukemia" <i>The AAPS Journal</i> , 2013, vol. 15, no. 1, pp. 242-249<br>See whole document, in particular abstract, page 246 left column paragraph 2, page 246 right column paragraph 2 and page 248 right column paragraph 1.                | 42 AND 43                     |
| P,X                                                   | UNNIKRISHNAN <i>et al.</i> : "AZA-MS: a novel multiparameter mass spectrometry method to determine the intracellular dynamics of azacitidine therapy <i>in vivo</i> " <i>Leukemia</i> , April 2018, vol. 32, no. 4, pp. 900-910<br>See whole document, in particular abstract, page 901 left column paragraph 5 - right column paragraph 3, page 903 right column, page 906 left column paragraph 1 and page 909 left column paragraph 2. | 1-43                          |
| Form PCT/ISA/210 (fifth sheet) (January 2015)         |                                                                                                                                                                                                                                                                                                                                                                                                                                           |                               |

|                                                                                                                                                                                                                                                                       |                         |                                                           |                         |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|-----------------------------------------------------------|-------------------------|
| <b>INTERNATIONAL SEARCH REPORT</b><br>Information on patent family members                                                                                                                                                                                            |                         | International application No.<br><b>PCT/AU2018/050597</b> |                         |
| This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information. |                         |                                                           |                         |
| <b>Patent Document/s Cited in Search Report</b>                                                                                                                                                                                                                       |                         | <b>Patent Family Member/s</b>                             |                         |
| <b>Publication Number</b>                                                                                                                                                                                                                                             | <b>Publication Date</b> | <b>Publication Number</b>                                 | <b>Publication Date</b> |
| US 4058602 A                                                                                                                                                                                                                                                          | 15 November 1977        | US 4058602 A                                              | 15 Nov 1977             |
| <b>End of Annex</b>                                                                                                                                                                                                                                                   |                         |                                                           |                         |
| <div> <p>Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.</p> <p>Form PCT/ISA/210 (Family Annex)(January 2015)</p> </div>                                                                    |                         |                                                           |                         |