(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

PCT

(43) International Publication Date
2 September 2010 (02.09.2010)

(10) International Publication Number
WO 2010/097584 A1

(51) International Patent Classification:
C12Q 1/48 (2006.01) *G01N 33/50* (2006.01)

(21) International Application Number:
PCT/GB2010/000335

(22) International Filing Date:
25 February 2010 (25.02.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0903373.9 27 February 2009 (27.02.2009) GB

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(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: DNA DAMAGE REPAIR ASSAY

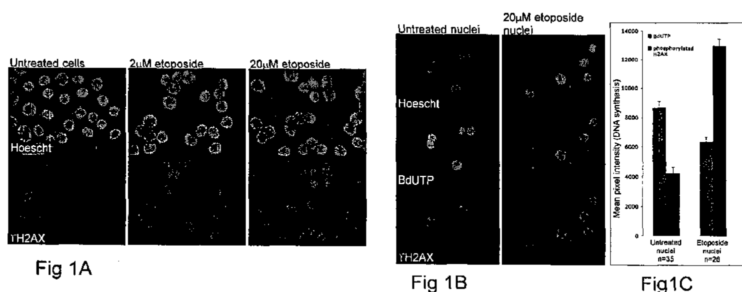

Fig 1A Fig 1B Fig1C

(57) Abstract: We describe an *in vitro* assay to monitor the repair of damaged DNA and its use in drug screening.

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DNA Damage Repair Assay

The invention relates to an assay to monitor the repair of damaged DNA and its use in drug screening.

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DNA damage can occur through a number of agents. For example, certain wavelengths of radiation, (e.g. gamma rays or X-rays), ultraviolet rays especially UV-C rays that are absorbed strongly by DNA, highly reactive oxygen radicals produced during respiration and other metabolic processes and chemical mutagens found in the environment which 10 may be man made or naturally occurring. DNA can be damaged in different ways. For example, the four bases that form DNA can be covalently modified at various positions. Deamination of an amino group is a common modification resulting in a mutation of cytosine to uracil. Other modifications include mismatches, for example the conversion of thymidine to uracil, single strand breaks in the phosphate backbone of the DNA 15 molecule and covalent crosslinks between bases which may be intra-strand or inter-strand. Several chemotherapeutic agents used in the treatment of cancer act as crosslinking agents. In addition it is important to determine whether new agents seeking regulatory approval have the potential to damage DNA. This would enable early detection of mutagenic activities in these agents and allow pharmaceutical companies to 20 screen out the problem agents an early stage.

Damaged DNA or inappropriate incorporation of bases into DNA can be corrected via several mechanisms. These include direct chemical reversal or excision repair. Excision repair results in removal of the damaged base and replacement with the correct base. 25 There are three mechanisms of excision repair utilised by cells to repair DNA damage.

Base excision repair involves the removal of the damaged base by a DNA glycosylase; removal of its deoxyribose phosphate to produce gapped DNA; replacement of the correct nucleotide by DNA polymerase β and ligation of the strand break by a DNA 30 ligase. Nucleotide excision repair involves recognition of the error by one or more protein factors; separating the DNA strands to produce a "bubble" by an enzyme called transcription factor IIH; scission at the 5' and 3' sides of the damaged area; replacement synthesis of the damaged area by DNA polymerases ε and ς and ligation of the strand break by a DNA ligase.

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Mismatch repair corrects mismatches in normal bases. The correction of mismatches

utilises enzymes involved in base excision repair and proteins that recognise the mismatch for example proteins encoded by MSH2 and scission around the mismatch by MLH 1 and other proteins. A mutation in either of these genes has been associated with an inherited form of colon cancer. The repair of the mismatch is completed by the DNA polymerases ε and ς.

In addition the repair of single and double strand breaks in DNA also involves a number of proteins. The repair of single strand breaks utilises many of the proteins involved in base excision repair. A double strand break is repaired either by direct ligation of the free ends of the break or by homologous recombination. Errors in direct ligation are associated with certain cancers, for example Burkitt's lymphoma and B-cell leukaemia.

This disclosure relates to an *in vitro* assay to monitor DNA repair. Cells depend on a functional DNA damage response (DDR) to prevent the propagation of potentially damaging mutations and support repair of potentially lethal DNA damage. Targeting this response is one method of improving the efficacy of chemo- and radiotherapy. We describe the development of a new method of studying the DDR using isolated nuclei and a soluble cell extract that is capable of uncoupling the cellular response to DNA damage from the damage itself. We also demonstrate its potential as a screening tool for both DNA damaging agents and inhibitors of the response. This has utility with respect to testing whether agents that can promote DNA repair and monitor agents that may potentially damage DNA. This allows the identification of undesirable DNA damaging properties of agents undergoing regulatory approval thereby allowing early genotoxic detection.

According to an aspect of the invention there is provided an *in vitro* method to screen for agents that modulate the DNA damage response in a eukaryotic cell comprising:

i) contacting a preparation comprising eukaryotic cells with at least one agent to be tested;
ii) rupturing the eukaryotic cells to form a cell free extract;
iii) separating the nuclear and cytosolic parts of said extract to form a nuclear extract and a substantially nuclear free cytosolic extract;
iv) forming a preparation comprising the cytosolic extract and deoxynucleotide triphosphates wherein said preparation is supplemented with a modified deoxynucleotide to allow the direct or indirect detection of DNA synthesis;

v) combining the preparation in (iv) with the nuclear extract formed in (iii) and incubating the preparation to initiate DNA synthesis and elongation; and optionally

vi) comparing the DNA synthesis activity of the preparation in (v) with a control wherein said control includes a nuclear and cytosolic extract prepared after contact with an agent known to induce a DNA damage response.

In a preferred method of the invention the cytosolic extract prepared in (iv) is prepared from a eukaryotic cell not contacted with an agent to be tested or a known DNA damage response inducing agent.

In an alternative preferred method of the invention the cytosolic extract prepared in (iv) is prepared from a eukaryotic cell contacted with said agent to be tested or a known DNA damage response inducing agent.

In a preferred method of the invention said eukaryotic cell is a mammalian cell.

In a preferred method of the invention said mammalian cell is a cancer cell.

As used herein, the term "cancer cell" refers to cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth. The term is meant to include all types of cancerous cells irrespective of histopathologic type or stage of invasiveness. The term "cancer cell" includes cells derived from, lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract, as well as cells derived from adenocarcinomas which include cells derived from colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumours, non-small cell carcinoma of the lung, cancer cells of the small intestine and cancer of the eosophagus. The term "carcinoma cell" is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term "carcinoma" also includes carcinosarcomas, e.g., which include malignant tumours composed of carcinomatous and sarcomatous cells. An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the

tumor cells form recognizable glandular structures. The term “sarcoma” is art recognized and refers to malignant tumors of mesenchymal derivation.

In a preferred method of the invention said cancer cell is a cervical cancer cell, for
5 example a Hela cell.

In a preferred method of the invention said eukaryotic cells are cell-cycle synchronized prior to contacting said agent.

10 In a preferred method of the invention said eukaryotic cells are synchronized in G1 or S phase; preferably S-phase.

In a preferred method of the invention said modified deoxynucleotide is adapted by the provision of a means by which said modified deoxynucleotide is detected as a measure
15 of DNA synthesis; preferably said modified deoxynucleotide is a biotinylated deoxynucleotide, for example dUTP, dATP, dCTP, dTTP, dGTP. Alternatives include digoxigenin modified dNTPs. Preferably said detection of DNA synthesis is by fluoresence detection.

20 In a preferred method of the invention said screening method includes the steps of: collating the activity data in (v) and (vi) above; converting the collated data into a data analysable form; and optionally providing an output for the analysed data. A number of methods are known which image and extract information concerning the spatial and temporal changes occurring in cells or nuclei expressing, for example fluorescent
25 molecules and other markers of gene expression or DNA synthesis, (see Taylor et al Am. Scientist 80: 322-335, 1992), which is incorporated by reference. Moreover, US5, 989,835 and US09/031,271, both of which are incorporated by reference, disclose optical systems for determining the distribution or activity of fluorescent reporter molecules in cells for screening large numbers of agents for biological activity. The
30 systems disclosed in the above patents also describe a computerised method for processing, storing and displaying the data generated.

The screening of large numbers of agents requires preparing arrays of cells for the handling of cells and the administration of agents. Assay devices, for example, include
35 standard multiwell microtitre plates with formats such as 6, 12, 48, 96 and 384 wells which are typically used for compatibility with automated loading and robotic handling

systems. Typically, high throughput screens use homogeneous mixtures of agents with an indicator compound which is either converted or modified resulting in the production of a signal. The signal is measured by suitable means (for example detection of fluorescence emission, optical density, or radioactivity) followed by integration of the signals from each well containing the cells, agent and indicator compound. The term "agent" includes any small molecule, antibody, polypeptide, peptide, aptamer, antisense or small inhibitory RNA. These can be an agonist or an antagonist.

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", means "including but not limited to", and is not intended to (and does not) exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

An embodiment of the invention will now be described by example only and with reference to the following figures:

Figure 1A illustrates immunofluorescence images showing detergent-resistant H2AX phosphorylation (red) in S phase HeLa cells treated with 2μM or 20μM etoposide as indicated (lower panels). Total nuclei are stained with H33258 (blue, upper panels);

Figure 1B illustrates products of cell-free reactions in which S phase nuclei isolated from cells treated with 20μM etoposide, or control nuclei, are incubated with undamaged extract from S phase HeLa cells. Incorporation of labelled nucleotide during in vitro DNA synthesis (green), H2AX phosphorylation (red) and total nuclei (blue) are shown;

Figure 1C illustrates quantification of the results represented in B, indicating relative levels of in vitro DNA synthesis (blue) and H2AX phosphorylation (red) in nuclei treated with or without 20μM etoposide before isolation. Histogram shows the average fluorescence intensity of nuclei with standard errors, and is representative data from one of four independent experiments;

Figure 2A illustrates products of cell-free reactions in which S phase nuclei are incubated with extract prepared from S phase cells treated with or without 20μM etoposide. Incorporation of labelled nucleotide during in vitro DNA synthesis (green), H2AX phosphorylation (red) and total nuclei (blue) are shown;

Figure 2B illustrates quantification of the results represented in A, indicating relative levels of in vitro DNA synthesis (blue) and H2AX phosphorylation (red) in nuclei incubated with extract from S phase cells treated with or without 20μM etoposide before isolation. Histogram shows the average fluorescence intensity of nuclei with standard errors, and is representative data from one of seven independent experiments;

Figure 2C illustrates *in vitro* replication initiation in cell-free reactions in which G1 phase nuclei are incubated with extract from S phase cells treated with or without 20μM etoposide before isolation. Table shows the proportion of nuclei undergoing in vitro DNA synthesis in extract from both untreated and etoposide-treated cells, with the percentage decrease seen in the etoposide-treated sample compared to its paired control, and the number of nuclei present in each replicate. This experiment was performed 6 times with 5 independent samples of isolated nuclei;

Figure 3A illustrates products of cell-free reactions in which S phase nuclei isolated from cells treated with 20μM etoposide, or control nuclei, are used to precondition undamaged extract from S phase cells during a 10 minute incubation and then removed from the extract by centrifugation. Results show the ability of these pre-conditioned soluble extracts to support elongation syntesis in undamaged nuclei. Incorporation of labelled nucleotide during in vitro DNA synthesis (green) and total nuclei (blue) are shown;

Figure 3B illustrates quantification of the results represented in B, indicating relative levels of in vitro DNA synthesis in nuclei incubated in extracts pre-exposed to nuclei treated with (dark blue) or without (light blue) 20μM etoposide before isolation.

Histogram shows the average fluorescence intensity of nuclei with standard errors, and is representative data from one of three independent experiments;

Figure 4 illustrates products of cell-free reactions in which S phase nuclei are incubated with extract prepared from S phase cells treated with 20μM etoposide in the presence or absence of 1mM aphidicolin. Incorporation of labelled nucleotide during in vitro DNA synthesis (green), H2AX phosphorylation (red) and total nuclei (blue) are shown; and

Figure 5A illustrates products of cell-free reactions in which S phase nuclei are incubated with extract from S phase cells treated with 20μM etoposide in the presence or absence of 200μM wortmannin. Incorporation of labelled nucleotide during in vitro DNA synthesis (green), H2AX phosphorylation (red) and total nuclei (blue) are shown; Figure 5B illustrates quantification of the results represented in B, indicating relative levels of in vitro DNA synthesis (blue) and H2AX phosphorylation (red) in nuclei incubated in extracts from etoposide-treated cells in the presence or absence of wortmannin. Histogram shows the average fluorescence intensity of nuclei with standard errors, and is representative data from one of four independent experiments.

Materials and Methods

Cell culture and synchrony

NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (PSG), on coated culture dishes (Nunclon). HeLa S3 cells were grown in DMEM supplemented with 10% FCS and 1% PSG, either in roller bottles saturated with CO_2 every 24 hours or on glass coverslips in 24 well plates.
HeLa S3 cells were synchronised in S phase by a 24 hour treatment with 2.5mM thymidine as described [1]. In order to harvest G1 phase cells, 3T3 cells were grown to confluence, and left quiescent for 72 hours before being subculturing at a 1:4 ratio in order to release them back into the cell cycle. Cells were harvested at 17 hours after release for preparation of 'replication competent' nuclei [2].

Preparation of nuclei and extracts for cell-free DDR

DNA damage was induced in HeLa S3 cells by exposure to a range of concentrations of etoposide (Sigma) as indicated, for a period of up to 3 hours. To create damaged S phase HeLa cell nuclei and extracts this treatment was carried out to coincide with the

final 3 hours of synchronisation. Cells were harvested under minimal lighting by centrifugation for 10 minutes at 1000rpm at 4°C, then washed once in ice cold PBS and once in ice cold hypotonic buffer (20 mM potassium-HEPES pH 7.8, 5 mM potassium acetate, 0.5 mM MgCl, 0.5 mM DTT). After centrifugation for 10 minutes at 1500rpm at 5 4°C cell pellets were resuspended in an equal volume of hypotonic buffer and disrupted using a Dounce Homogeniser (10 strokes with tight pestle for preparation of nuclei and 20 strokes for preparation of cytosolic extracts). Nuclei and extracts were separated in a microcentrifuge at 3000rpm for 10 minutes and the two fractions were immediately frozen as 5-50μl beads in liquid nitrogen. Materials for DDR studies were stored in 10 cryogenic vials in liquid nitrogen until required.

Reaction conditions

Replication initiation and elongation assays were carried out in cytosolic extract supplemented with energy regenerating system and nucleotides as described previously 15 [1]. Nuclei (~1x10^4/μl) and supplemented extracts were incubated together for 30 minutes at 37°C. 1nM biotinylated-dUTP (BdUTP, Roche) was added to reactions either at the start or after 5 minutes, as indicated, in order to label newly synthesised DNA. For "preconditioning" experiment, 20μl supplemented extract was incubated for 10 minutes at 37°C with 2μl nuclei. Reactions were then centrifuged at 3000rpm to pellet nuclei and 20 10μl extract was removed and applied to fresh nuclei. To inhibit PI3K-kinases, cell free extracts were supplemented with 200μM Wortmannin (Invitrogen). To inhibit DNA replication cell free reactions were supplemented with 1mM aphidicolin (Invitrogen). Reaction products were processed for visualisation of DNA synthesis as described previously [1], or for immunofluorescence.

25

Detection and imaging

Cells grown on coverslips were fixed in 4% PFA, after a brief pre-exposure to 0.1% Triton X-100 in PBS. γH2AX was detected with monoclonal antibody 05-636 (Upstate) diluted 1/2000 in antibody buffer (10 mg/ml BSA, 0.02% SDS, 0.1% Triton X-100 in 30 PBS) for 1 hour at 37°C. Alexa 568 secondary antibody (Molecular Probes) was used diluted 1/1000 in antibody buffer for 1 hour at 37°C. For combined detection of γH2AX and DNA synthesis, nuclei from *in vitro* DNA synthesis reactions were fixed in 4% PFA, after a brief pre-exposure to 0.1% Triton X-100 in PBS, spun through a sucrose cushion onto coverslips, and treated as described above except that streptavidin-FITC 35 (Amersham) was included in both primary and secondary antibody steps at a dilution of 1/500.

Quantification method

Nuclei from late G1 populations undergoing initiation of DNA replication *in vitro* were counted by eye at 600x magnification, using a Zeiss Axiovert microscope (Carl Zeiss, 5 Göttingen, Germany) with a 63/1.40 oil immersion objective. All nuclei with focal staining were scored as positives regardless of the extent of nucleotide incorporation. Typically 200-400 nuclei were scored per experiment.

To quantify the extent of elongation DNA synthesis in S phase nuclei and γH2AX immunofluorescence, nuclei within one experiment were photographed with an AxioCam 10 camera (Carl Zeiss Vision, Hallbergmoos, Germany) under constant exposure parameters. The intensity of BdUTP-dependent or γH2AX-dependent fluorescence within the largest rectangular area of individual nuclei was measured using using Openlab software (Improvision, Coventry, UK). The mean pixel intensity within the selected areas were collected and displayed in histograms along with standard errors 15 using Excel. Sample sizes for replication elongation assays ranged between 28 - 110 nuclei per experiment and were taken from a minimum of three images in each case. All experiments were performed at least 2 times (raw data is available in supplemental data file).

20 **Example 1**

DNA synthesis elongation and phosphorylation of histone H2AX can be used as markers of the DDR in vitro

25 Etoposide is a topoisomerase II inhibitor that stabilises the topoisomerase II cleavable complex (TOP2cc) [3], causing double strand breaks. We have used etoposide to illustrate the effect of induction of DNA damage on DNA synthesis (not shown) and on phosphorylation of H2AX (Figure 1A) in intact HeLa cells. More importantly, we show here that when combined with cell-free approaches, etoposide-treated HeLa cells can 30 form the basis of a highly exploitable assay for agents that induce the DDR. Moreover by reconstituting the process in a cell-free environment the signalling that takes place as a consequence of DDR activation can be isolated from and manipulated separate to the effect of the DNA damage itself.

35 To illustrate the effect of DNA damage on DNA synthesis *in vitro*, nuclei from etoposide-treated S phase cells were combined with S phase extract from undamaged cells. A

significant drop in replication elongation was observed in damaged nuclei compared to undamaged nuclei (Figure 1B, 1C), and this was particularly apparent when a 5 minute preincubation was included prior to the addition of biotinylated-UTP (BdUTP). Inclusion of a pre-incubation period would allow existing replication forks to encounter damage sites that block fork progression before monitoring begins. In the same nuclei phosphorylation of H2AX was increased in nuclei from cells treated with etoposide (Figure 1B, 1C). These data show that both DNA synthesis elongation and phosphorylation of histone H2AX can be used as markers of the DDR *in vitro*.

Example 2

Reconstitution of the DNA damage response in the absence of DNA damage

The experiment described above shows that the presence of double strand breaks in DNA is sufficient to inhibit replication elongation in vitro and to induce phosphorylation of H2AX that can be detected after incubation in undamaged extract. Using similar cell-free experiment but with *extract* derived from damaged cells we have also shown that damaged DNA is not required to sustain the DNA damage response once it has been activated. In fact when cytosolic extract from etoposide-treated S phase cells are incubated with nuclei from undamaged S phase cells the treated extracts were significantly less able to support replication elongation than untreated extracts (Figure 2A, 2B). This was accompanied by phosphorylation of H2AX in essentially the whole of the undamaged nuclear population (Figure 2A, 2B). These results show that soluble factors present in extract from damaged cells can efficiently impose the DDR even in the absence of damaged DNA. These experiments were also carried out using G1 phase nuclei to ask whether the activated extract can inhibit *initiation* of DNA replication in addition to elongation. In fact, etoposide-treated extract resulted in a consistent fall in levels of initiation across multiple batches of nuclei, when compared to undamaged extract (Figure 2C).

Example 3

Damaged nuclei can be used to condition S phase extracts to activate the DDR in untreated nuclei

Having demonstrated that etoposide-treated cell extracts can induce H2AX phosphorylation and supress elongation of DNA synthesis in the absence of physical

damage, we tested whether damaged nuclei could condition extracts to reproduce this effect. Etoposide-treated nuclei were incubated in untreated S phase extracts for 10 minutes, before removing nuclei and using the the conditioned extracts in replication elongation assays with untreated S phase nuclei. Conditioning extracts in this manner rendered them less able to sustain replication elongation compared to extracts exposed to untreated nuclei (Figure 3A, 3B), although the drop in BdUTP incorporation was only half that shown when directly treated extracts or nuclei were utilised.

Example 4

Reconstitution of the DDR in is independent of DNA replication elongation

Having demonstrated that etoposide-treated cell extracts can induce H2AX phosphorylation and supress elongation of DNA synthesis in the absence of physical damage, we tested whether this phosphorylation was dependent on the presence of DNA replication. Etoposide-treated extracts were supplemented with 1mM aphidicolin, an inhibitor of DNA replication, before conducting replication elongation assays with untreated S phase nuclei. After 4 minutes in aphidicolin-treated extract no BdUTP incorporation could be observed, yet H2AX phosphorylation occurred at similar levels to a non-aphidicolin-treated control (Figure 4).

Example 5

Reconstitution of the DDR in vitro can be used as a tool to quantify the effect of potential inhibitors of the DDR

To demonstrate potential applications of this system we used wortmannin, a well characterised inhibitor of ATM and ATR to block the effect on replication elongation and phosphorylation of H2AX. A concentration of 200μM, known to affect all PIKKs [4]. was used. When assays were carried out using etoposide-treated extracts, levels of replication-elongation were significantly increased in the presence of wortmannin and H2AX phosphorylation fell dramatically (Figure 5A, 5B). Thus, this system can be used to test the effect of candidate small molecule inhibitors on the signalling cascades that are activated by DNA damage, but in a system that is uncomplicated by the presence of the damage itself.

1. Krude, T., et al., *Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system.* Cell, 1997. **88**(1): p. 109-19.
2. Coverley, D., J. Marr, and J.F.-X. Ainscough, *Ciz1 promotes mammalian DNA replication.* Journal of Cell Science, 2005. **118**(1): p. 101-112.
3. Li, T.K. and L.F. Liu, *Tumor cell death induced by topoisomerase-targeting drugs.* Annu Rev Pharmacol Toxicol, 2001. **41**: p. 53-77.
4. Rodriguez-Bravo, V., et al., *Chk1- and claspin-dependent but ATR/ATM- and Rad17-independent DNA replication checkpoint response in HeLa cells.* Cancer Res, 2006. **66**(17): p. 8672-9.

Claims

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1 An *in vitro* method to screen for agents that modulate the DNA damage response in a eukaryotic cell comprising:

i) contacting a preparation comprising eukaryotic cells with at least one agent to be tested;

10 ii) rupturing the eukaryotic cells to form a cell free extract;

iii) separating the nuclear and cytosolic parts of said extract to form a nuclear extract and a substantially nuclear free cytosolic extract;

iv) forming a preparation comprising the cytosolic extract and deoxynucleotide triphosphates wherein said preparation is supplemented

15 with a modified deoxynucleotide to allow the direct or indirect detection of DNA synthesis;

v) combining the preparation in (iv) with the nuclear extract formed in (iii) and incubating the preparation to initiate DNA synthesis and elongation; and optionally

20 vi) comparing the DNA synthesis activity of the preparation in (v) with a control wherein said control includes a nuclear and cytosolic extract prepared after contact with an agent known to induce a DNA damage response.

25 2. A method according to claim 1 wherein the cytosolic extract prepared in (iv) is prepared from a eukaryotic cell not contacted with an agent to be tested or a known DNA damage response inducing agent.

3. A method according to claim 1 wherein the cytosolic extract prepared in (iv) is

30 prepared from a eukaryotic cell contacted with said agent to be tested or a known DNA damage response inducing agent.

4. A method according to any of claims 1-3 wherein said eukaryotic cell is a mammalian cell.

35

5. A method according to claim 4 wherein said mammalian cell is a cancer cell.

6. A method according to claim 5 wherein cancer cell is a cervical cancer cell.

7. A method according to claim 6 wherein said cervical cancer cell is a HeLa cell.

5 8. A method according to any of claims 1-7 wherein said eukaryotic cells are cell-cycle synchronized prior to contacting said agent.

9. A method according to claim 8 wherein said eukaryotic cells are synchronized in G1 phase.

10

10. A method according to claim 8 wherein said eukaryotic cells are synchronized in S phase.

11. A method according to any of claims 1-10 wherein said modified deoxynucleotide
15 is adapted by the provision of a means by which said modified deoxynucleotide is detected as a measure of DNA synthesis.

12. A method according to any of claims 1-11 wherein said screening method includes the steps of:
20 i) collating the activity data in (v) and (vi) above;
ii) converting the collated data into a data analysable form; and optionally
iii) providing an output for the analysed data.

13. A method according to any of claims 1-12 wherein said method is adapted for
25 high through put screening of agents.

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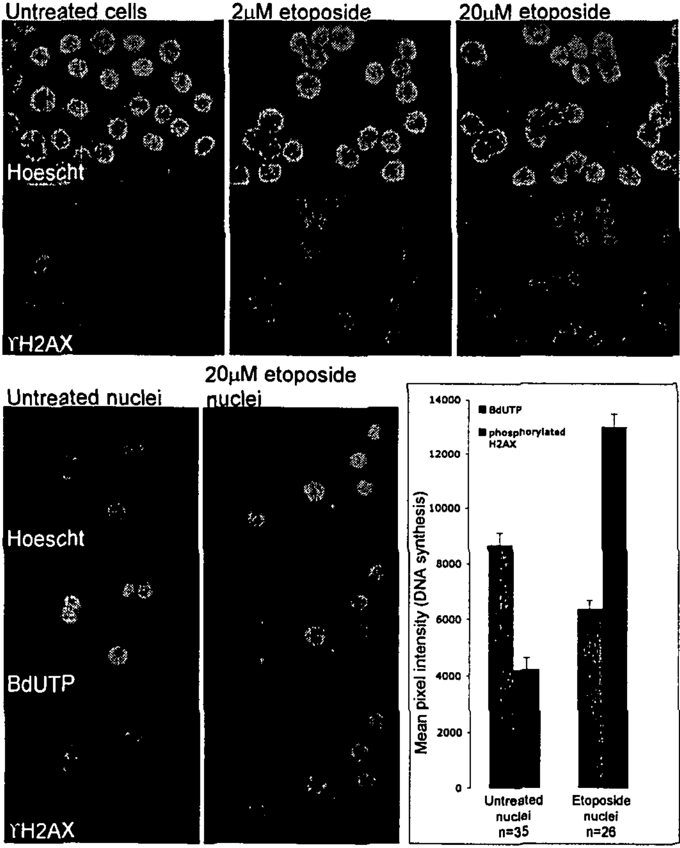


Fig 1A

Fig 1B

Fig1C

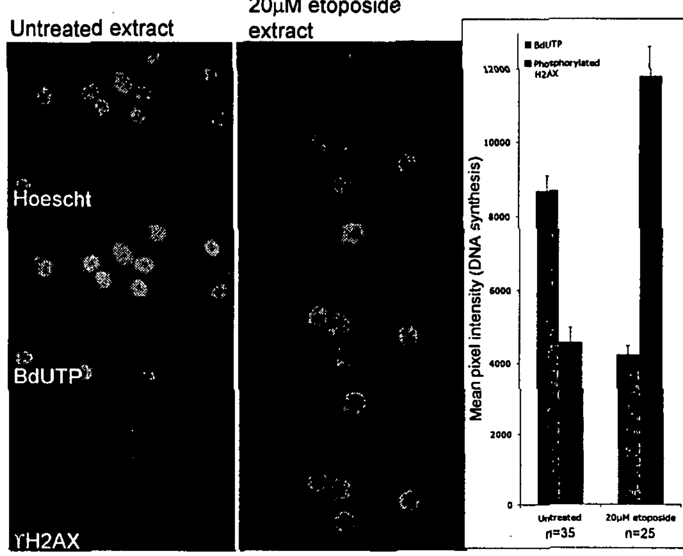


Fig 2A

Fig 2B

Replicate	Proportion of replicating nuclei in untreated sample	Proportion of replicating nuclei in treated sample	Percentage decrease in replication initiation	No. of nuclei
1	0.27	0.23	14.82%	225
2	0.269	0.233	13.39%	411
3	0.216	0.192	11.12%	227
4	0.077	0.065	15.59%	212
5	0.286	0.245	14.34%	218
6	0.304	0.214	29.61%	216
Mean	0.237	0.1965	16.48%	1509

Fig 2C

Fig 3A

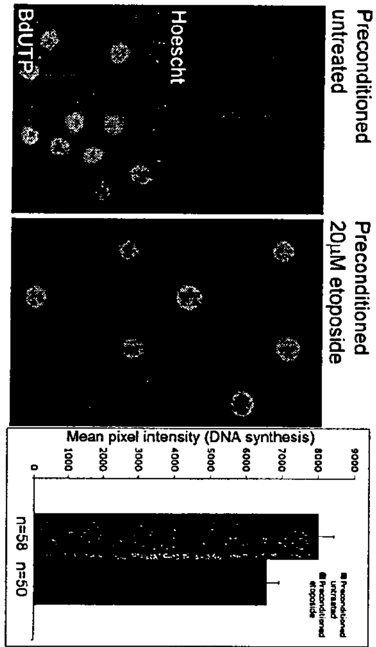


Fig 3B

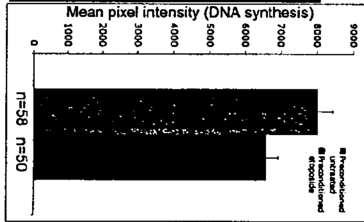


Fig 4

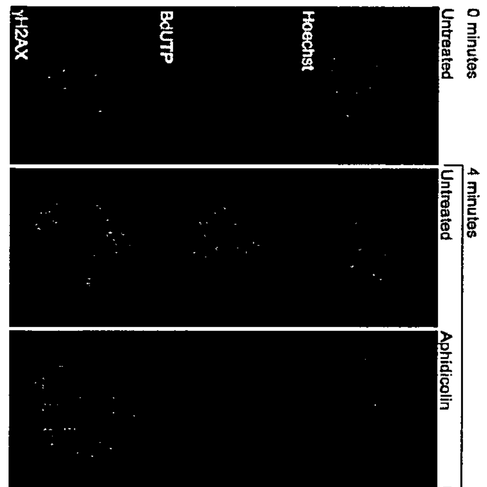


Fig5A

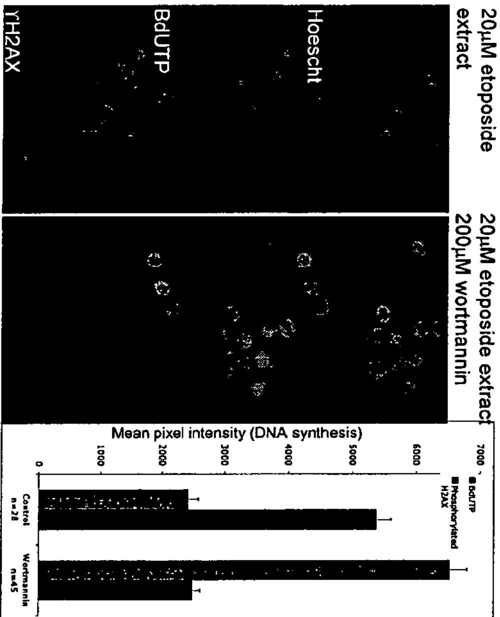
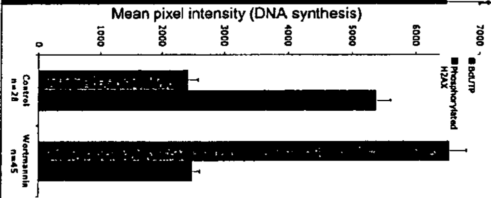


Fig 5B



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/000335

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/48 G01N33/50
ADD.

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)
C12Q G01N

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 practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SALLES B ET AL: "Repair of oxidative DNA damage in vitro: A tool for screening antioxidative compounds" FOOD AND CHEMICAL TOXICOLOGY, vol. 37, no. 9-10, September 1999 (1999-09), pages 1009-1014, XP002577869 ISSN: 0278-6915 the whole document figure 2 page 1011, column 2, paragraph 2-3 ----- -/--	1-13

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"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)

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"P" document published prior to the international filing date but later than the priority date claimed

"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

"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 April 2010	12/05/2010

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2010/000335

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SAWYER DENNIS E ET AL: "Altered nuclear activation parameters of rat sperm treated in vitro with chromatin-damaging agents" TOXICOLOGICAL SCIENCES, vol. 44, no. 1, July 1998 (1998-07), pages 52-62, XP002577870 ISSN: 1096-6080 the whole document abstract page 54	1-13
A	SEKI S ET AL: "BLEOMYCIN-INDUCED DNA SYNTHESIS IN A CELL-FREE SYSTEM USING A PERMEABLE MOUSE SARCOMA CELL EXTRACT" ACTA MEDICA OKAYAMA, vol. 41, no. 5, 1987, pages 195-200, XP008121504 ISSN: 0386-300X the whole document abstract page 196 figure 1	1-13
A	JENSEN T L ET AL: "DNA synthesis in isolated resting nuclei: Evidence for protease-dependent nonreplicative nucleotide incorporation" EXPERIMENTAL CELL RESEARCH, ACADEMIC PRESS, US LNKD- DOI:10.1016/0014-4827(90)90147-3, vol. 190, no. 1, 1 September 1990 (1990-09-01), pages 85-90, XP024854651 ISSN: 0014-4827 [retrieved on 1990-09-01] the whole document abstract page 85, column 2, paragraph 1 page 89, column 1, paragraph 2 page 90, column 2, paragraph 1	1-13
A	KRUDE TORSTEN ET AL: "Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system" CELL, vol. 88, no. 1, 1997, pages 109-119, XP002577871 ISSN: 0092-8674 the whole document abstract page 117	1-13