Incorporation of metabolic activation potentiates cyclophosphamide-induced DNA damage response in isogenic DT40 mutant cells

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Abstract

Elucidating the DNA repair pathways that are activated in the presence of genotoxic agents is critical to understand their modes of action. Although the DT40 cell-based DNA damage response (DDR) assay provides rapid and sensitive results, the assay cannot be used on genotoxic compounds that require metabolic activation to be reactive. Here, we applied the metabolic activation system to a DDR and micronucleus (MN) assays in DT40 cells. Cyclophosphamide (CP), a well-known cross-linking agent requiring metabolic activation, was preincubated with liver S9 fractions. When DT40 cells and mutant cells were exposed to the preactivated CP, CP caused increased cytotoxicity in FANC-, RAD9-, REV3- and RAD18-mutant cells compared to isogenic wild-type cells. We then performed a MN assay on DT40 cells treated with preactivated CP. An increase in the MN was observed in REV3- and FANC-mutant cells at lower concentrations of activated CP than in the parental DT40 cells. These results demonstrated that the incorporation of metabolic preactivation system using S9 fractions significantly potentiates DDR caused by CP in DT40 cells and their mutants. In addition, our data suggest that the metabolic preactivation system for DDR and MN assays has a potential to increase the relevance of this assay to screening various compounds for potential genotoxicity.

Introduction

Every year, a large number of new chemicals are added to the already vast Toxic Substances Control Act Inventory. A number of conventional genotoxicity assays, such as the in vitro gene mutations test in bacteria, the in vitro test for chromosomal damage and/or gene mutations in cultured mammalian cells and the in vivo test for cytogenetic effects in rodent bone marrow cells, have played an important role in identifying hazardous chemicals. However, the US National Research Council (NRC) committee suggested that a more modern approach be considered for toxicity testing in the 21st century (1). The overall toxicity testing framework developed by the committee focuses on four major components: chemical characterisation, toxicity pathways and targeted testing, dose–response and extrapolation modelling and population-based and human exposure data. With regards to the conventional genotoxicity assays, little attention has been paid to the dose–response pattern and toxicity pathways activated by genotoxic agents. Recently, a mechanistic understanding and quantitative analysis of genotoxic agents were highlighted in order to determine acceptable exposure levels in humans (2–6). The use of a comprehensive set of in vitro tests to identify the pathways affected in the presence of genotoxic agents would provide much stronger, mechanistically based, predictive tools for human health risk assessment.

For this purpose, the US Tox21 program adopted a DNA damage response (DDR) assay utilising isogenic chicken DT40 cell lines
that broadly probed biological targets, pathways and mechanisms in relation to genotoxicity and/or cytotoxicity endpoints for a large number of chemicals (7,8). The reverse genetic approach provides a powerful method for studying gene function and regulation. DT40 cells originated from a chicken B-lymphocyte line derived from an avian leucosis virus-induced bursal lymphoma isolated in 1985 (9). We established a multiwell-plate-based method that makes use of the DT40 isogenic cell line and its dozens of available mutants knocked out in DNA repair and cell cycle pathways (10–12). This assay, which is based on increased cytotoxicity in DNA repair-deficient DT40 mutants versus the parental DT40 cells, is a rapid and simple method to evaluate the genotoxicity of xenobiotics and is suitable for high throughput screening (8).

In order to screen a broader range of chemicals, the current DT40 cell-based DDR assay needs to incorporate metabolic activation because some xenobiotics show genotoxic potential only after metabolic activation. In this study, we applied a metabolic activation system using S9 to the DT40 cell-based DDR assay. We first utilised a cell-washing method for the metabolic activation system. The washing method is an established procedure for metabolic activation in the genotoxicity study; however, this process may introduce physical stress to the cells from centrifugation and loss of cells by media change. In particular, DT40 cells are very sensitive to various environmental stressors, such as pipetting pressure and temperature (11); therefore, it is better to avoid unnecessary stress derived from washing, centrifugation and handling errors. Furthermore, the washing method is not practical to screen for many chemicals particularly in the high-throughput format. We decided to incorporate the S9 metabolic activation system using a convenient method that requires only the addition of the reagents in the DT40 cell-based DDR analysis. Consequently, DT40 cells need to be cultivated in the presence of S9 fractions. However, cytochrome P450 metabolises lipids that make up S9 microsomes and result in the formation of toxic microsomal lipid peroxides (13,14). It is also known that cytochrome P450, in the absence of substrates, cycle electrons and could produce reactive oxygen species (15). Using preincubation method, we investigated the ability of cyclophosphamide (CP), a genotoxin requiring metabolic activation (16), to induce differential cytotoxicity across the different DNA repair-deficient DT40 cell lines.

### Materials and methods

**DT40 cell culture and maintenance**

Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Atlanta Biologicals (Norcross, GA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. RPMI 1640 culture medium (+glutamine, -pheno1 red) and chicken serum (CS) were acquired from Life Technologies (Grand Island, NY, USA). Dulbecco’s phosphate-buffered saline (PBS) was obtained from Life Technologies (Grand Island, NY, USA). CP was dissolved in PBS, and XTT cocktail was prepared with 500 mg of the XTT salt, 10.2 mg PMS and 3.3 ml DMSO. The S9 mixture was prepared prior to use following the manufacturer’s instructions and stored on ice until use.

**Chemicals**

Cyclophosphamide (CP), 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT), 1-methoxy-5-methylphenazinium methyl sulfate (PMS) and dimethylsulfoxide (DMSO) were obtained from Sigma. The Arocior 1254-induced male rat liver S9, G-6-P, NADP Regensys™ B and MgCl2/KCl solution were purchased from MOLTOX (Boone, NC, USA).

### Table 1. A list of examined DT40 mutant cell lines

<table>
<thead>
<tr>
<th>DNA repair/tolerance pathway</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base excision repair (BER)</td>
<td>POL8, FEN1, PARP1</td>
</tr>
<tr>
<td>Nucleotide excision repair (NER)</td>
<td>XPA</td>
</tr>
<tr>
<td>Non-homologous end-joining (NHEJ)</td>
<td>LIGIV</td>
</tr>
<tr>
<td>Homologous recombination (HR)</td>
<td>RAD54</td>
</tr>
<tr>
<td>Fanconi anaemia pathway (FANC)</td>
<td>FANCD2, FANC1, FANCG, FANCC, FANCL, BRCA2</td>
</tr>
<tr>
<td>DNA damage checkpoint (DDC)</td>
<td>RAD9, ATM</td>
</tr>
<tr>
<td>Mis-match repair (MMR)</td>
<td>MSH3</td>
</tr>
<tr>
<td>Trans-lesion synthesis (TLS)</td>
<td>REV3, POLδ, POLκ, POLθ, RAD18</td>
</tr>
</tbody>
</table>

**DT40 cell-based DDR assay with metabolic activation**

For the DDR assay, ~1200 cells were seeded in each well of a 96-well plate in 75 μl of medium. These plates were kept in an incubator at 39.3°C until the start of treatment. Cells were exposed to S9, CP or a CP-S9 mixture, with a slight modification of our previously reported version of the assay (11). To determine the cytotoxic concentration of S9, cells were incubated with 0.052–0.25% S9 as a final concentration. The S9 was mixed with cofactor (0.4 mol/l MgCl2, 1.65 mol/l KCl, 1 mol/l glucose 6-phosphate and 0.5 mol/l NADPH in 0.1 mol/l phosphate buffer) to make a 10% mixture, and this mixture was diluted with PBS. To further optimise S9 incorporation into the DDR assay, two different methods were examined. The first method was a cell-washing method, in which cells were treated with CP in the presence of 1% S9 for 2 h. After treatment, plates were centrifuged (200g, 5 min), cells were washed twice with fresh medium to remove the chemical and S9, and plates were incubated for 2–3 days to obtain a sufficient number of cells for the XTT assay. The second method was a preincubation method, in which the mixture of CP and 1% S9 was preincubated at 37°C for 30 min, and 8.35 μl of the reaction mixture was transferred to the 96-well plate in which 75 μl of the cell suspension was seeded to set the final concentration of S9 at 0.1%. A preliminary test to find the optimal dose was conducted in DT40 wild-type cells to select the appropriate concentration range that includes at least one concentration showing severe cytotoxicity and no cytotoxicity at lower concentrations. To observe the cytotoxic effect of CP without metabolic activation, cells were treated with CP without S9 on the same plate. Duplicate wells for each concentration of CP and four untreated wells as controls and four as blanks were set up (Figure 1A). In both methods, PBS was used as a negative control. Once treated or washed, cells were incubated for at least 48 h after which they were observed microscopically to determine the growth of the cells. Once the control cells were nearly contiguous, cells were exposed to the XTT cocktail. The plates were returned to the incubator until the dye developed sufficient colour for detecting absorbance on the plate reader, which normally takes 4–5 h. Absorbance was measured at 450 nm with a reference of 620 nm using a Tecan Sunrise (Tecan Systems, San Jose, CA, USA) plate reader with XFluor™ software (Tecan, version 6.4). All data are reported as the means ± standard deviation of experiments done at least in triplicate.
Micronucleus frequency
For the micronucleus (MN) assay, flow cytometry-based MN measurements were performed using an In Vitro MicroFlow™ Kit (Litron Laboratories, Rochester, NY, USA) according to the manufacturer’s instructions (17). Briefly, 150 µl of DT40 cells adjusted at 2×10^5 cells/ml was seeded into 96-well plates at 39°C and 5% CO₂ in a humid atmosphere. In a separate plate, the mixture of CP and 1% S9 was preincubated at 37°C for 30 min, which is the same procedure used in the DDR assay, and 16.7 µl of CP-S9 mixture was transferred to the 96-well plate to set the final concentration of S9 at 0.1%. Cells were cultured at 39°C for 16 h, which corresponds to approximately two cell cycles. After treatment, the plate was centrifuged (200g, 5 min) to collect cells, and the treatment medium was discarded. The plate was then placed on ice and incubated for 20 min, and 50 µl of nucleic acid dye A working solution was added to each well. A light source was placed ~15 cm above the samples for 30 min. Subsequently, the samples were washed once with cold 1× buffer solution. Next, 100 µl of complete lysis solution 1 (including nucleic acid dye B) was added to each well, and the samples were incubated for 1 h in the dark at 37°C. Subsequently, 100 µl of complete lysis solution 2 (including nucleic acid dye B) was added to each well, and the samples were incubated for 30 min in the dark at room temperature.

Samples for the MN assay were analyzed by flow cytometry (LSRII, BD Biosciences), using an excitation wavelength of 488 nm. The frequency of MN was determined by acquiring at least 10 000 gated

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**Figure 1.** A 96-well plate format for treatment and cell survival in DT40 cells treated with CP and S9 using the washing method. (A) 96-well plate format for treatment. Cells were exposed to CP in duplicate wells with or without S9. Eight concentrations of CP and PBS as a control [C] was added to corresponding cell lines. The outside 2 wells were used as blank controls [B], and the average absorbance was subtracted from each well in the XTT analysis. (B) In the cell washing method, DT40 cells were treated with CP in the presence or absence of 1% S9 for 2 h, and cells were washed with medium and further incubated at 39°C for 2–3 days. After the incubation, cell survival was examined in the XTT assay. Two independent experiments were conducted and the mean relative survival at each concentration was plotted.
nuclei per sample. The data are summarised as a mean of two or more independent experiments. The extent of survival in the treated cultures, described as relative population doubling (RPD), was evaluated as the reduction in population doubling (PD) (18), as compared to the vehicle control. The RPD was calculated using the following formula.

\[
\text{RPD} = \frac{(\text{Number of PD in treated cultures}) - (\text{Number of PD in control cultures}) \times 100}{\log(\text{post-treatment cell number/initial cell number}) - \log 2}
\]

Data and statistical analysis
We calculated the lethal concentration 50 (LC\textsubscript{50}) values for each cell line using Graphpad Prism 5 (La Jolla, CA, USA). Based on the wild-type LC\textsubscript{50}, the two-sided 95% confidence interval was calculated to judge whether the LC\textsubscript{50} in mutant cells are biologically significant. In order to represent the point of departure (PoD) values for concentration–response in the MN assay, benchmark dose (BMD) values (US EPA/100/R-12/001 2012) were obtained using the EPA BMD software v 2.5. These values represent a one standard deviation departure from control values, and the BMDL refers to the corresponding lower limit of a one-sided 95% confidence interval on the BMD. A \(\log_{10}\) (concentration +0.5) was used to avoid taking logarithms of zero control values. In each instance, the best fitting result from Hill, exponential and polynomial response non-constant variance models was used. Additionally, Fisher’s exact test was performed to evaluate the statistical significance in MN frequency in the MN assay.

Results
Cell toxicity caused by CP in the presence of S9 (washing method)
The S9 microsomal fraction of rat liver homogenate is widely used in the conventional in vitro genotoxicity assay to evaluate the toxicity of reactive metabolites. A final concentration of 1% S9 is the concentration typically used that maintains sufficient activity of S9 to generate genotoxic metabolites, as per the guidelines (19,20). We first utilised a cell washing method in parental DT40 cells. DT40 cells were exposed to CP in the presence of 1% S9 for 2 h. On the same plate, cells were exposed to CP in the absence of S9. CP with S9 caused cell toxicity in DT40 cells in a concentration-dependent manner (Figure 1B).

Assessment of S9 cytotoxicity in DT40 cells
Considering the toxicity of S9, the concentration of S9 was first adjusted in the cytotoxicity assay in order to minimise damage to DT40 cells. To determine the cytotoxic concentration of S9 in DT40 cells during the incubation period, cells were incubated for 2–3 days in the presence of serially diluted S9 (Experiment I in Figure 2A). This exposure period allows cells to grow sufficiently for the XTT assay. As expected, the relative survival decreased as S9 concentration increased (Figure 2B). At a final concentration of 0.2% S9, almost no cells survived, while at 0.1% S9 no cytotoxicity was observed. Considering this steep cytotoxicity pattern and variability between experiments, we set the S9 concentration at 0.1% in the subsequent experiments.

Cell toxicity caused by preactivated CP using S9 (preincubation method)
To minimise S9 fraction-derived cytotoxicity in DT40 cells but still maintain sufficient metabolic activity, DT40 cells were exposed to preactivated CP in the presence of S9 (Experiment II in Figure 2A). To determine the metabolic capacity of S9 after a variety of preincubation times with CP, CP was incubated in the presence of 1% S9 for 10, 20, 30, 40 and 50 min, and the CP-S9 mixture was then exposed to DT40 cells by adding 1/9 volume of the CP-S9 preincubation mixture. The plots in Figure 2C show that the cytotoxicity of CP increases in a time-dependent manner. The survival curve after 50 min of incubation was comparable to that of the washing methods. The LC\textsubscript{50} dropped sharply within 30 min of incubation, and it continued to drop moderately until 50 min of incubation. These results also indicate that the metabolite of CP is stable for ~50 min. Because a small difference in LC\textsubscript{50} was noted at incubation periods of 30 min or longer, CP was preincubated with S9 for 30 min in the DDR assay using a variety of DT40 mutants.

DDR assay using a panel of DT40 mutants treated with CP
To address whether the DT40 cell-based DDR assay incorporating metabolic activation can elucidate the repair mechanism of CP, 20 isogenic DT40 mutant cell lines were exposed to the CP-S9 mixture after 30 min preincubation at 37°C. The LC\textsubscript{50} in each mutant was compared to the concurrent LC\textsubscript{50} in DT40 cells. The LC\textsubscript{50} in parental DT40 cells was 5.19 ± 2.11 μM in eight independent assays. The calculated 95% confidence interval was 3.43–6.95 μM, which correspond to 66–134% in relative LC\textsubscript{50}. For the mutant cell lines, the means of three or more independent experiments are plotted as the relative LC\textsubscript{50}. The mutant cell lines cover a variety of DNA repair/tolerance pathways (e.g. BER, base excision repair; NER, nucleotide excision repair; FANC, Fanconi anaemia pathway; HR, homologous recombination; NHEJ, non-homologous end-joining; DDC, DNA damage checkpoint; MMR, mismatch repair; TLS, translesion DNA synthesis) (Supplementary Table 1, available at Mutagenesis Online). Among these cell lines, DT40 cells deficient in FANC genes showed marked hypersensitivity to activated CP, mainly due to interstrand DNA cross-links (Figure 3 and Supplementary Figure 1, available at Mutagenesis Online). They were ~10-fold or more sensitive to activated CP than DT40 wild-type cells (relative LC\textsubscript{50} was less than 10% of DT40 cells) while no cytotoxicity was observed at the LC\textsubscript{50} in the absence of metabolic activation both in DT40 wild-type and mutant cells (Supplementary Figure 1, available at Mutagenesis Online). In addition, severe toxicity (relative LC\textsubscript{50} was less than 40% of DT40 cells) was observed in the RAD9-, RAD18- and REV3-deficient DT40 cell lines, suggesting DDC, and TLS pathways are involved in the DDR to activated CP.

MN induction in DT40 and mutant cells treated with CP in the presence of S9
Because CP is a well-known genotoxic agent that induces MN formation in mammalian cells (21,22), the DT40 wild-type cells and some of the mutant cell lines hypersensitive to CP were exposed to CP after preincubation in the presence of S9, and the induced MN levels were determined. The RPD was also examined based on the cell numbers after the treatment. While activated CP induced MN in DT40 wild-type and all mutant cell lines tested in this study (Figure 4), a significant increase in the MN frequency was observed at lower concentrations in REV3- and FANC-G- and FANC-D2-deficient cell lines than wild-type cells. The calculated PoD was 0.099, 0.110 and 0.027 μM in REV3, FANC-G and FANC-D2 cells, respectively, as compared to 0.176 μM in DT40 wild-type cells. At the highest concentration, FANC-D2-deficient cells showed hypersensitivity to the CP-S9 mixture while REV3- and FANC-G-deficient cells showed ~50% cytotoxicity.
Figure 2. Experimental procedure and cell survival results in DT40 cells treated with either S9 alone or CP plus S9 using the preincubation method. (A) In Experiment I, wild-type cells were exposed to S9 alone at concentrations ranging from 0.052 to 0.25% as a final concentration to determine the non-cytotoxic concentration of S9 for the DDR assay. In Experiment II, 1% S9 and different concentrations of CP were first preincubated for 10–50 min. The mixture solution was transferred to each well to expose to the wild-type cells. The final concentration of S9 was 0.1% for all wells. (B) To examine S9-mediated toxicity, DT40 cells were treated with S9 alone at different concentrations. DT40 cells were incubated with serially diluted S9. After 2–3 days, XTT was added and the absorbance measured by a plate reader to assess cell survival. Results show an average of five independent experiments. (C) Cell survival was measured in DT40 cells treated with CP and S9 using the preincubation method. Prior to cell treatment, CP was preincubated with 1% S9 for 10, 20, 30, 40, and 50 min. These preincubated mixtures were then exposed to DT40 cells by addition of 1/9 volume of the preincubation mixture to each well, and survival was examined using the XTT assay. The CP toxicity in the washing method was similar to toxicity caused by the 30- to 50-min preincubation method. The data shows the mean of two independent experiments.
Discussion

In this study, we demonstrated that the incorporation of metabolic preactivation system using S9 fractions significantly potentiates DDR caused by CP in DT40 cells and their mutants using Aroclor 1254-induced rat liver S9 fractions. Previously, we reported a multi-well plate-based DDR assay using DT40 mutant cell lines that can be used as a high-throughput genotoxicity assay to characterise mechanisms of action of various compounds (10–12). While this assay is both rapid and sensitive, it is not applicable for genotoxic compounds requiring metabolic activation. Incorporation of metabolic activation is mandatory in regular genotoxicity testing according to OECD guidelines (19,20). Here, we expanded the capability of the assay by incorporating metabolic activation. In this study, CP preincubated with S9 and the resulting mixture, including metabolites, was mixed with the cell suspension at a ratio of 1:9 so that the final concentrations of S9 were non-toxic for the entire incubation period. This process prevents suspension cells from undergoing stress by centrifugation and loss of cells by medium change, which is routinely conducted for the conventional genotoxicity assays. The LC$_{50}$ levels obtained from the washing and preincubation methods were almost comparable (Figure 2C), indicating our methods can generate sufficient amounts of metabolites. Although many genotoxic chemicals other than CP need to be tested, we believe that this convenient preincubation method could substitute the cell washing method used in the conventional genotoxicity assays in mammalian cells. Recently, the in vitro MN assay has also been conducted in a multi-well plate format using a washing method (23). Thus, our S9 preincubation method for the MN assay would be more suitable, require less time and resources, and reduce handling errors.

When cells were exposed to the CP-S9 mixture after 10- to 50-min preincubation periods, DT40 cells showed a time-dependent decrease in LC$_{50}$ after 50 min. While the LC$_{50}$ dropped sharply within 30 min, it continued to drop moderately between 30 and 50 min.
This suggests that S9 activity becomes gradually depleted, the metabolites could be partly degraded, or most of the CP was metabolically activated within 30 min. Taking these points into consideration, cells were exposed to the CP-S9 mixture after a 30-min preincubation period in our study.

The exceptional advantage of the DDR assay is the ability to elucidate DNA repair or tolerance pathways, which helps to understand the mechanism of action of any chemical of interest. Due to technical complexity, genotoxic agents requiring metabolic activation, including CP, have rarely or never been characterised in DDR using the DT40 cell system. We investigated the DDR of CP using 20 mutant cell lines that cover major DNA repair or tolerance pathways. Our results showed that a series of FANC-deficient cells were hypersensitive to CP metabolised with the S9 mix (activated CP), indicating that the FANC pathway is critical for repairing DNA damage caused by activated CP. CP has been used as a chemotherapeutic agent in the treatment of leukaemia and solid tumours for nearly 70 years (24). The ability of CP to induce interstrand crosslinks (ICLS) on DNA is key for its function and one that requires metabolic activation. Fanconi anemia, a genetic disorder that results in sensitivity to ICLs (25–27), is caused by the mutation of one of at least 16 different genes. Although their functions have not been completely elucidated, there is substantial evidence to suggest that these genes participate in a common pathway of ICL repair (28). Our finding is very consistent with these reports. It is also noteworthy that FANC-deficient DT40 cells were ~10-fold or more sensitive to CP in the presence of S9 than CP in the absence of metabolic activation (Supplemental Figure 1, available at Mutagenesis Online), which indicates that the preincubation method employed in this study worked well to generate genotoxic metabolites. These results also imply that the metabolic preactivation system could be useful for researchers to investigate not only genotoxicity but also the mechanism of action of a wide range of agents.

In addition, DT40 mutants lacking REV3 and RAD18 genes, which are associated with the translesion synthesis (TLS) pathway, showed severe toxicity in the presence of activated CP. Previous work demonstrated that mutants of the TLS polymerases REV1 and Pol-ζ (a complex of REV3 and REV7) are exquisitely sensitive to cross-linking agents (29). It was also reported that suppression of REV3 sensitises drug-resistant lung tumours to chemotherapeutics, and suppression of REV1 inhibits both cisplatin- and CP-induced mutagenesis (30,31). The higher toxicity observed in REV3 and RAD18-deficient mutants compared to that seen in the parental DT40 cells is consistent with these reports. It is worthwhile to note that activated CP could produce mono adducts as well as intranuclear crosslinks. The high cellular toxicity in REV3, and RAD18-deficient mutants in the presence of activated CP could be partly due to the inability to bypass mono adducts (32).

The DDR assay focuses on cytotoxicity, but CP is known to induce MN as a marker of genotoxicity. MN induction was investigated in DT40 wild-type and CP-sensitive mutant cell lines. The results were analyzed by bench mark dose analysis software v2.5 provided by the US EPA, which enables us to calculate PoD using several types of fitting models. The PoD concentrations in hypersensitive mutant cell lines were lower than for the parental DT40 wild-type cell lines, indicating that activated CP-derived DNA damage is repaired or tolerated by FANC and/or TLS pathways.

Overall, our DT40 cell-based DDR assay with metabolic activation could provide a valuable tool to elucidate the DNA damage repair/tolerance pathways of a wide range of genotoxic agents. Although more chemicals need to be examined for further validation, this assay could be incorporated as part of the HTS-based bioassay such as Tox21 (33), which will enable us to evaluate chemicals in the presence and absence of metabolic activation. Our assay has the potential of broadening the range of chemicals and will greatly help in evaluating uncharacterised xenobiotics.

**Supplementary data**

Supplementary Table 1 and Figure 1 is available at Mutagenesis Online.

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**References**


