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Non-homologous end joining is the responsible pathway for the repair of fludarabine-induced DNA double strand breaks in mammalian cells

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ABSTRACT

Fludarabine (FLU), an analogue of adenosine, interferes with DNA synthesis and inhibits the chain elongation leading to replication arrest and DNA double strand break (DSB) formation. Mammalian cells use two main pathways of DSB repair to maintain genomic stability: homologous recombination (HR) and non-homologous end joining (NHEJ).

The aim of the present work was to evaluate the repair pathways employed in the restoration of DSB formed following replication arrest induced by FLU in mammalian cells.

Replication inhibition was induced in human lymphocytes and fibroblasts by FLU. DSB occurred in a dose-dependent manner on early/middle S-phase cells, as detected by γH2AX foci formation. To test whether conservative HR participates in FLU-induced DSB repair, we measured the kinetics of Rad51 nuclear foci formation in human fibroblasts. There was no significant induction of Rad51 foci after FLU treatment. To further confirm these results, we analyzed the frequency of sister chromatid exchanges (SCE) in both human cells. We did not find increased frequencies of SCE after FLU treatment.

To assess the participation of NHEJ pathway in the repair of FLU-induced damage, we used two chemical inhibitors of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), vanillin and wortmannin. Human fibroblasts pretreated with DNA-PKcs inhibitors showed increased levels of chromosome breakages and became more sensitive to cell death. An active role of NHEJ pathway was also suggested from the analysis of Chinese hamster cell lines. XR-C1 (DNA-PKcs-deficient) and XR-V15B (Ku80-deficient) cells showed hypersensitivity to FLU as evidenced by the increased frequency of chromosome aberrations, decreased mitotic index and impaired survival rates. In contrast, CL-V4B (Rad51C-deficient) and V-C8 (Brca2-deficient) cell lines displayed a FLU-resistant phenotype. Together, our results suggest a major role for NHEJ repair in the preservation of genome integrity against FLU-induced DSB in mammalian cells.

1. Introduction

DNA double strand breaks (DSB) are important forms of DNA damage. They occur endogenously as natural and highly regulated processes, such as V(D)J recombination and immunoglobulin G class switch or can be induced exogenously by chemical or physical agents. If left unrepaired, DSB can lead to cell death [1]. If incorrectly repaired, they can result in tumorigenesis through translocations, inversions or deletions [2,3].

Several agents (e.g. arabinosylcytosine and arabinosyladenine monophosphate) have been shown to arrest the replication fork during DNA synthesis [4]. These compounds are metabolized to endogenous nucleosides and nucleotides. Active metabolites interfere with the *de novo* synthesis of nucleosides and nucleotides or inhibit the DNA chain elongation after being incorporated into the DNA strand as terminators [5]. The action of the analogue 9-beta-D-arabinofuranosyl-2-fluoroadenine (FLU, fludarabine) on DNA synthesis has been reported both in vitro and in vivo, and resulted in termination of DNA strand elongation [4]. The underlying mechanisms by which FLU triphosphate affects DNA synthesis include: (a) a competition with the normal substrate dATP to be incorporated into DNA which results in repression of further polymerization; (b) the inhibition of ribonucleotide reductase that results in lowering the normal cellular pool of deoxynucleotides; (c) the inhibition of human DNA polymerases α, β, γ and ε; and (d) the inhibition of DNA ligase I [6].

Replication blocks are known to induce DSB [7]. In response to DSB, cells activate a complex network of cellular processes. These include the regulation of a subset of genes, among which are those

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associated with DNA damage signal transduction, cell cycle regulation, DNA repair, and eventually, cell death [8].

Mammalian cells use at least two distinct pathways to rejoin DSB: homologous recombination (HR) and non-homologous end joining (NHEJ) [9].

HR is a process in which a homologous chromosome or a sister chromatid is used as a template to repair and is essentially error-free [10]. Previous data reported that recombination between sister chromatids participates in mitotic HR at least 100-fold more frequently than the homologous chromosome [11–13] and is one of the principal mechanisms responsible for sister chromatid exchange (SCE) in vertebrate cells [14]. In mammalian cells HR requires, among others, the homologous of the *Saccharomyces cerevisiae* Rad52 epistasis group of proteins, including Rad51, Rad52 and Rad54, and other factors, the breast cancer susceptibility proteins Brca1 and 2. Rad51 is an essential component of HR because it catalyzes the DNA strand exchange reaction [15]. Early reports showed that Rad51 re-localizes in nuclear foci after a genotoxic stress [16].

NHEJ requires the DNA-dependent protein kinase (DNA-PK), which comprises the catalytic subunit DNA-PKcs and the DNA end-binding heterodimer Ku70/Ku80. This complex is involved in DNA end recognition and end joining. The proteins XRCC4 and DNA ligase IV are involved in the final step catalyzing the ends ligation. NHEJ directly ligates severed DNA ends with no apparent requirement for extensive sequence homology. The rejoining process may result in the deletion of short stretches of nucleotides and is therefore potentially mutagenic [17].

Current models for explaining differential activation of both pathways are controversial. A competition between Rad52 and Ku for DNA end binding has been suggested to explain the channeling to a particular pathway [18,19]. However, the work from Ristick et al. suggests that both proteins can bind to different DNA substrates in vitro [20]. On the other hand, there is some evidence for a differential contribution of both NHEJ and HR to DSB repair depending on the moment of the cell cycle when DSB are introduced [21].

The recognition and repair of the replication fork-associated DSB induced by FLU remains unclear. The aim of the present work was to evaluate the main system involved in the repair of DSB resulting from the action of FLU on mammalian cells through the study of different cellular responses. In this way, our data suggest a principal role for NHEJ with no evidence for Rad51-dependent HR activity in the repair of DSB induced by FLU.

2. Materials and methods

2.1. Chemicals

FLU (CAS no. 75607-67-9; Schering Argentina), mitomycin C (MMC; CAS no. 50-07-7; Sigma), hydroxyurea (HU; CAS no. 127-07-1; Microsules Argentina S.A.), bromodeoxyuridine (BrdU; CAS no. 59-14-3; Sigma) and vanillin (VN; CAS no. 121-33-5; Sigma) were dissolved in bidistilled water. Wortmannin (WTM; CAS no. 19545-26-7; Sigma) was dissolved in DMSO.

2.2. Cell cultures and drug treatments

Heparinized peripheral blood samples were obtained from four healthy donors (three women and one man, aged from 32 to 46 years), free of any known exposure to genotoxic agents. Whole blood (0.8 ml) was added to 10 ml of F-10 medium containing 15% fetal bovine serum (FBS) and 2% phytohemagglutinin M (PHA). Low passages of the human foreskin fibroblast cell line PTP were kindly provided by Dr. M.I. Tous (Servicio de Cultivo de Tejidos, Depto Virología, ANLIS "CG Malbrán", Buenos Aires, Argentina). The cell line was grown in Minimum Essential Medium supplemented with 10% FBS and 2 mM L-glutamine. Mutant hamster cell lines deficient in NHEJ (XR-C1 and XR-V15B) and HR (CL-V4B and V-C8) with their correspondent parental cell lines (CHO9, V79B and V79) were used. CHO9, XR-C1 and XR-V15B were grown in F-12 medium supplemented with 10% FBS and 2 mM L-glutamine. V79, V79B, CL-V4B and V-C8 were cultured in Mc Coy's 5A medium (without hypoxanthine and thymidine) supplemented with 10% FBS. The hamster cell lines were kindly provided by Prof. Dr. M.Z. Zdzienicka and Dr. W. Wiegant (Leiden University, Leiden, The Netherlands). All the cultures were incubated at 37 °C under a 5% CO_2 humidified atmosphere. Different doses of FLU, MMC or HU were applied as indicated in the text.

2.3. Cell synchronization, BrdU labelling and γH2AX detection

Human fibroblast cell line was arrested in G1-phase by contact inhibition and maintained under serum starvation conditions for 48 h. Cells were replated at low density on coverslips and released for 24 h in complete medium to enrich the cell population in the S-phase. The percentage of cells in the S-phase obtained with this methodology was checked by flow cytometry with propidium iodide and was higher than 70%. Then, the cultures were exposed for 2 h to FLU 1 μg/ml, HU 1 mM or mock treated. Cells were pulsed with 150 μM BrdU for 10 min before fixation/permeabilization with 2% paraformaldehyde containing 0.5% Triton X-100 in PBS for 20 min. Nuclei were denatured with 0.05 M NaOH for 30 min. Indirect immunofluorescence was performed using primary antibodies against BrdU (1:100; Santa Cruz Biotechnology) and γH2AX (1:300; Cell Signaling), followed by exposure to secondary antibodies (Vector Laboratories). One hundred BrdU-positive nuclei were scored and classified by their BrdU labelling pattern as focal (early S-phase), intermediate and distributed (middle S-phase), and heterochromatic (late S-phase) [22]. The percentage of γH2AX-positive cells in each BrdU labelling pattern was scored. Two independent experiments were carried out for each treatment.

2.4. Cell proliferation and mitotic index

Both human lymphocytes and fibroblasts were cultured for two complete rounds of replication in presence of 10 μg/ml BrdU. Twenty-four hours after the culture onset, lymphocytes were treated with FLU and incubated in darkness for 48 h. Colcemid (0.2 μg/ml) was added 90 min before harvesting. Exponentially growing fibroblasts were treated with FLU and incubated in the dark for 48 h. Colcemid (0.2 μg/ml) was added 4 h before harvesting and then cells were trypsinized. Negative and positive (MMC) control cultures were grown under identical conditions. The cells were then collected by centrifugation, exposed to 0.075 M KCl hypotonic solution and fixed in methanol:acetic acid (3:1). Air-dried chromosome preparations were made and a modification of the fluorescence-plus-Giemsa method [23] was applied to obtain harlequin chromosomes. For cell cycle analysis, each metaphase was classified as being in the first (M_1), second (M_2), or third and further (M_{3+}) division and 200 metaphases per culture were examined. The replication index (RI) was calculated as follows: $RI = 1 \times (\%\text{ of cells in } M_1) + 2 \times (\%\text{ of cells in } M_2) + 3 \times (\%\text{ of cells in } M_{3+})/100$. The MI was calculated as the number of metaphases among 2000 nuclei and expressed as a percentage. Three to five independent experiments were carried out for each end point.

2.5. Analysis of SCE

Human lymphocytes and fibroblasts were incubated in presence of 10 μg/ml BrdU and FLU for two complete rounds of replication (48 h). The SCE average was taken from the analysis of 30 metaphases during the second cycle of division in three to five independent experiments.

2.6. γH2AX fluorescent immunostaining

Exponentially growing human fibroblasts were grown on coverslips for at least 24 h before a 16 h treatment with different doses of FLU. The cells were fixed with methanol:acetone (1:1) for 2 min at room temperature. After washing with PBS, the cells were exposed to blocking solution (3% BSA and 0.25% Tween 20 in PBS) for 1 h and then incubated with rabbit anti-γH2AX (1:300; Cell Signaling) or rabbit anti-Rad51 (1:200; Santa Cruz Biotechnology) antibodies for 2 h. The secondary fluorescein isothiocyanate-conjugated anti-rabbit antibody (1:250; Vector Laboratories) was incubated for 1 h and the slides were mounted in 4′,6′-diamidino-2-phenylindole containing antifade solution (DAPI; Vector Laboratories). Two hundred nuclei were scored per slide in three independent experiments.

2.7. Assessment of chromosome alterations

Exponentially growing human fibroblasts were pretreated with VN 300 μM or WTM 8 μM for 1 h, and then cells were exposed to FLU 1 μg/ml during 48 h in presence of BrdU 10 μg/ml. Both VN and WTM were present in the culture medium during the whole time of treatment. Colcemid (0.2 μg/ml) was added 4 h before harvesting and then cells were trypsinized, exposed to hypotonic solution and fixed. The slides were processed according to Perry and Wolff [23]. Thirty metaphases in second division (bifilarly stained) were analyzed for SCE and 50 metaphases in first division (unifilarly stained) were evaluated for chromosome breakages (CB) in two or three independent experiments. Chromatid and chromosome breaks were scored as one break and chromatid exchange configurations, dicentric and ring chromosomes were scored as two breaks. Gaps were excluded in the result of CB frequencies.

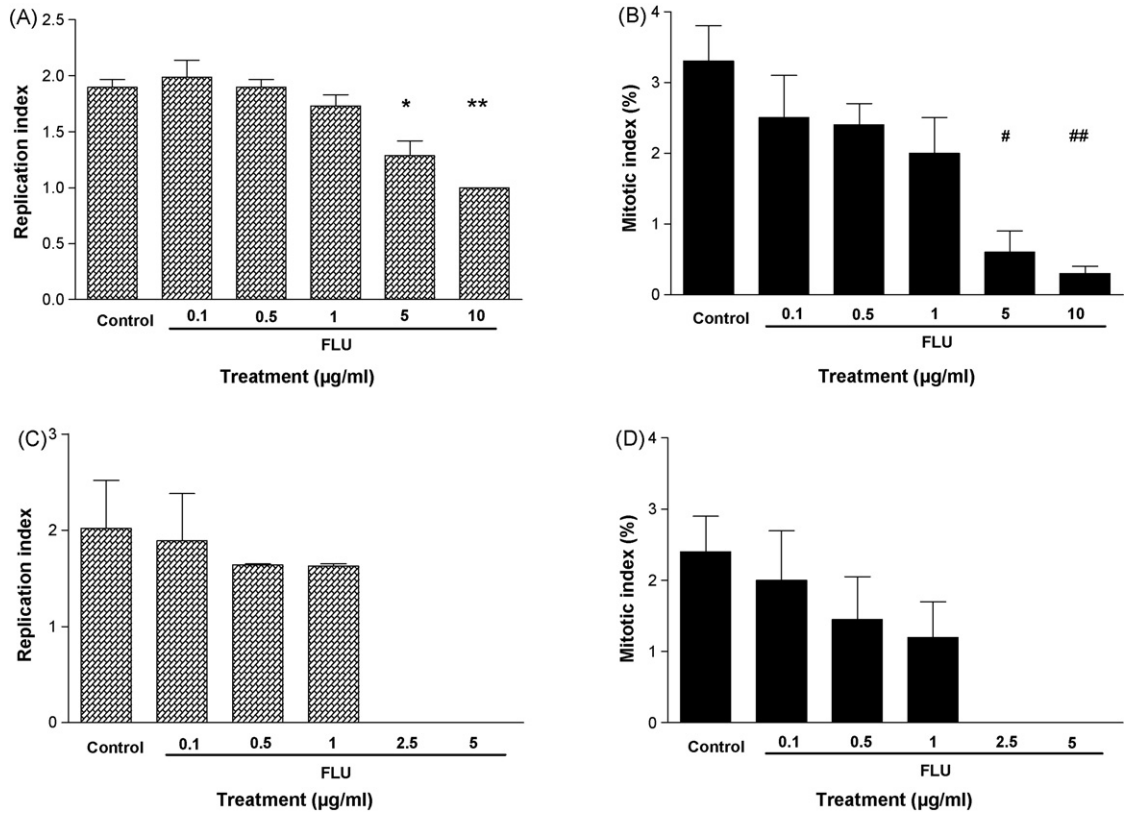

Fig. 1. Proliferation in normal human cells treated with different concentrations of FLU for 48 h. Replication indexes in human lymphocytes (A) and fibroblasts (C) and mitotic indexes of lymphocytes (B) and fibroblasts (D) are presented. $^{*}p = 0.014$ and $^{**}p = 0.0001$ (vs. control). $^{\#}p = 0.01$ and $^{\#\#}p = 0.004$ (vs. control). Bars represent the S.E.M.

2.8. Cell death analysis

Exponentially growing fibroblasts were mock or pretreated with either VN 300 μM or WTM 8 μM for 1 h and treated with FLU. After 36 h, 100 μl of 1% trypan blue solution was added directly into each well containing 100 μl of complete medium. The percentage of cell death was determined in 300 cells from at least eight different fields. Analysis was performed using an inverted microscope. From two to five independent experiments were done.

2.9. DNA damage in NHEJ- and HR-deficient cell lines

Chinese hamster cell lines were plated in 60 mm-diameter dishes and left to attach for 4 h. FLU was added to the medium during 34–36 h. Colcemid (0.2 μg/ml) was added 2–2.5 h before harvesting and then cells were trypsinized, exposed to hypotonic solution, fixed and stained with Giemsa 10% for 4 min. CB were evaluated in 50 metaphases. The MI was calculated as the number of metaphases among 1000 nuclei. Three independent experiments were carried out for each end point.

2.10. Cell survival in NHEJ- and HR-deficient cell lines

Chinese hamster cells were plated at low density (300–1000 cells/dish), allowed to attach for 4 h and treated with graded concentrations (1–30 μg/ml) of FLU during 20 h. After drug exposure, cells were washed twice with PBS and cultured in fresh medium for 7–10 days. The culture dishes were rinsed with PBS, methanol fixed and stained with crystal violet. Only colonies containing more than 50 cells were scored. Survival fraction was calculated as the ratio of colonies in drug-treated cultures compared with control cultures and expressed in percentages. Three independent experiments were carried out for each end point.

2.11. Statistical analysis

Differences between the mean RI and MI, Rad51 and γH2AX foci formation, CB percentages, cell death and colony formation were analyzed using the Student's *t*-test. The means of the frequencies of SCE were statistically analyzed by the Kruskal–Wallis test [24] (comparisons between groups were done by the Dunn procedure). The dose–response relationships were determined by means of the regression coefficients (Primer of Biostatistics, version 3.0 by S.A. Glantz, McGraw-Hill, Inc., 1992).

3. Results

3.1. Kinetics of cell proliferation

To evaluate the effect of FLU on cell proliferation we studied the kinetics of replication and mitotic indexes in the normal human cells assayed (Fig. 1). The RI estimates the average number of generations passed during a specified lapse of time. During 48 h of treatment, FLU induces a significant delay in cell proliferation in both stimulated human lymphocytes and human fibroblasts. There was also a progressive reduction in the percentage of cells in M_2 and in M_3 with an increase of M_1 cells. The RI of lymphocytes (Fig. 1A) treated with FLU 5 μg/ml decreased significantly ($p = 0.014$) to 1.29 ± 0.13 from 1.90 ± 0.07 in control cultures. At 10 μg/ml, all lymphocytes were arrested at the first metaphase (RI = 1.00). Following treatment with FLU the RI decreased in a concentration-dependent manner ($r = 0.976$, $p = 0.001$). In the fibroblast cell line (Fig. 1C), the RI of cells treated with FLU 1 μg/ml decreased to 1.63 ± 0.02 from 2.02 ± 0.5 in controls. A severe reduction in the number of cells was found at the highest concentrations of FLU assayed (2.5 and 5 μg/ml), and metaphases were no longer present. The lowest MI was induced in lymphocyte cultures exposed to the highest concentration of the drug (Fig. 1B). Fibroblasts decreased the MI at FLU 1 μg/ml to 1.2 ± 0.5 from 2.4 ± 0.5 in controls (Fig. 1D).

3.2. FLU induces DNA DSB detectable in the early S-phase

To assess whether FLU induces DNA DSB, we analyzed γH2AX nuclear foci in human fibroblasts. After 16 h of incubation, FLU-induced γH2AX nuclear foci in a dose-dependent manner (Fig. 2A and B). FLU increased the frequency of γH2AX-positive cells from $19.65\% \pm 3.7$ with 0.5 μg/ml to $40.05\% \pm 2.9$ ($p = 0.005$) with

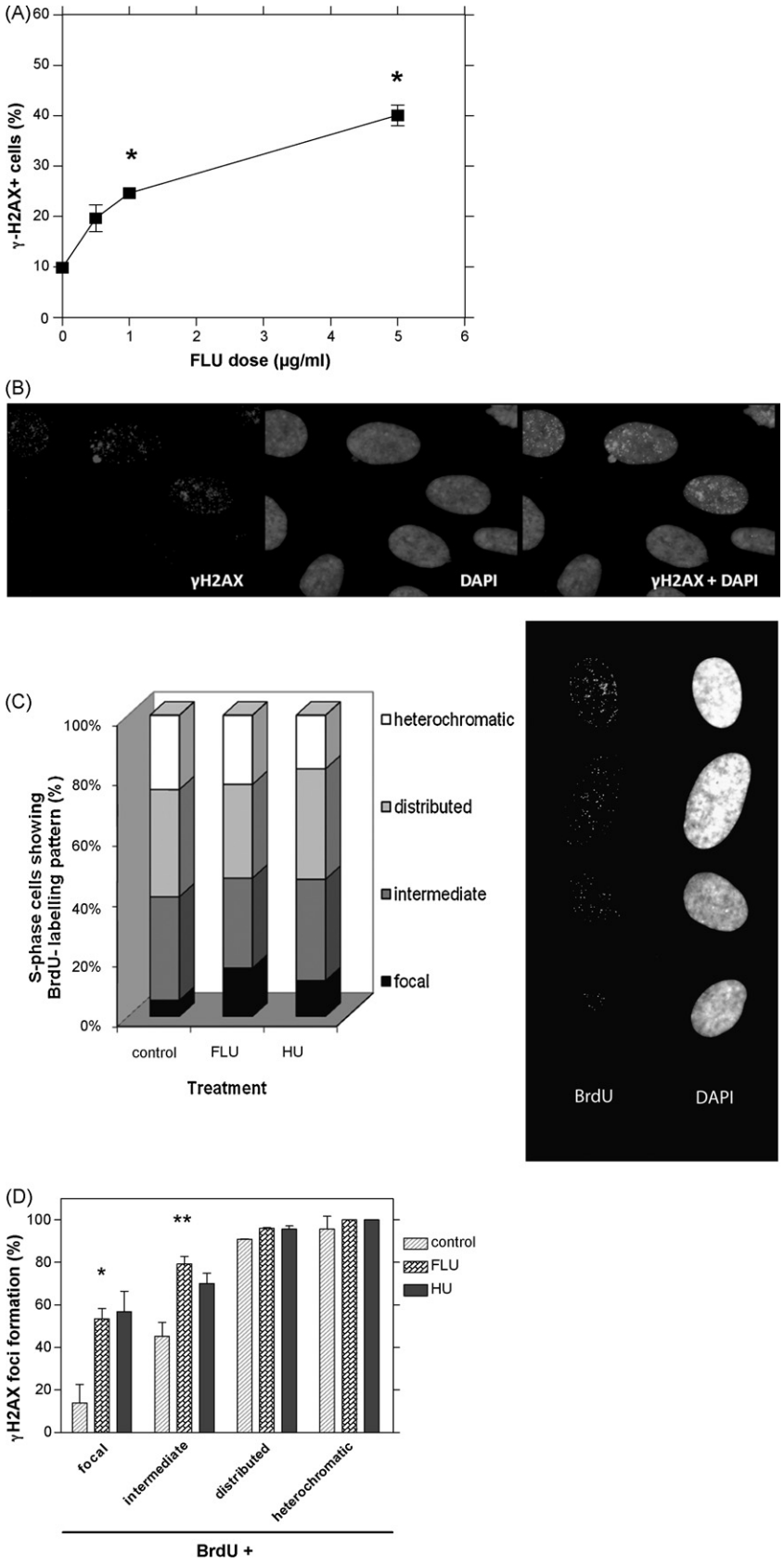

Fig. 2. γH2AX nuclear foci formation in the human fibroblast cell line. (A) Fibroblasts accumulate γH2AX foci after 16 h of treatment with FLU (*$p = 0.005$ vs. control) in a dose-dependent manner. (B) Nuclei showing γH2AX foci after treatment with FLU 1 μg/ml counterstained with DAPI (1000×). (C) Distribution of cells with different patterning of BrdU staining in the S-phase of synchronized cultures after 2 h treatment with FLU 1 μg/ml, HU 1 mM or mock treated (control). (D) γH2AX foci formation on cells showing different BrdU labelling pattern after treatments. *$p = 0.0303$ and **$p = 0.0229$. Bars represent the S.D.M.

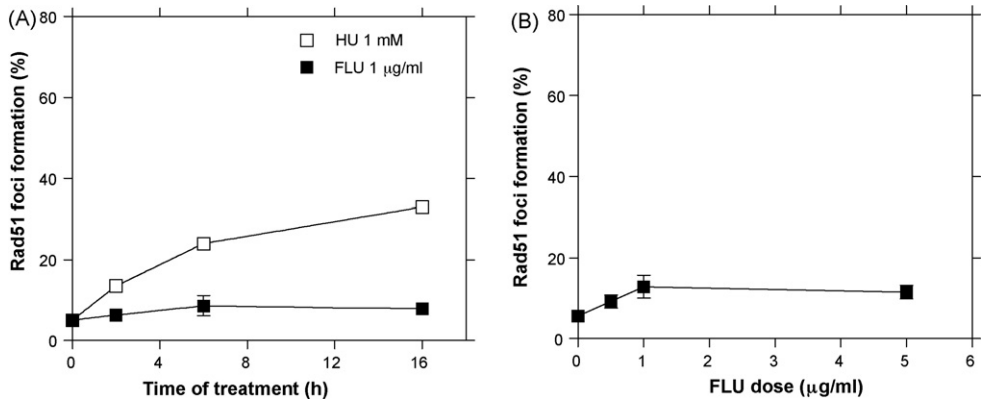

Fig. 3. Rad51 nuclear foci formation in the human fibroblast cell line. (A) Kinetics of Rad51 foci formation analyzed at 2, 6 and 16 h after treatment with FLU 1 μg/ml or HU 1 mM. (B) Induction of Rad51 foci formation with different concentrations of FLU after 16 h. Nuclei containing more than 10 foci were classified as Rad51 positive. Bars represent the S.D.M.

5 μg/ml, compared to 9.8% ± 1.3 in control cultures. The exposure to FLU 1 μg/ml for 24 and 48 h showed similar levels of DNA damaged cells (data not shown).

The sites of BrdU incorporation in mammalian normal cells reveal temporal patterns of DNA synthesis that occur during S-phase progression. To further analyze whether DSB induced by FLU are developed during the S-phase, human fibroblasts were synchronized to enrich the population in the S-phase, and then treated for 2 h with FLU 1 μg/ml before a 10 min pulse with BrdU. Fig. 2C shows that FLU increased the percentage of cells displaying focal pattern (16.1%) compared to control cultures (5.4%). Similar results were found with the synthesis inhibitor HU (11.9%). The analysis of γH2AX foci formation showed that FLU significantly increased the percentage of cells with BrdU-positive focal pattern containing γH2AX to 53.5% ± 4.9 ($p = 0.0303$) from 13.85% ± 8.7 in control cultures (Fig. 2D). Moreover, the percentage of cells showing γH2AX foci with an intermediate pattern of BrdU labelling were increased significantly after FLU treatment (79.4% ± 3.4, $p = 0.0229$) compared to controls (45.3% ± 6.6). Similar results were achieved with HU. Asynchronic cultures treated during 16 h with FLU showed similar levels of early S-phase cells containing γH2AX foci (data not shown).

3.3. Rad51-dependent HR pathway is not involved in the repair of FLU-induced DSB

To evaluate the involvement of HR in the FLU-induced DSB repair we measured the Rad51 nuclear foci formation in the fibroblast cell line by immunostaining. We considered cells with more than 10 nuclear foci as positives. The untreated fibroblasts had low levels of Rad51 foci (5.6% ± 0.4, Fig. 3A). FLU 1 μg/ml produced a slight but not statistically significant increase in the fraction of cells forming Rad51 foci at the different times analyzed. When adding HU 1 mM to the culture medium, the percentage of nuclei with Rad51 foci raised to 33% after 16 h of treatment. FLU treatment for 16 h showed that Rad51 foci did not show a dose-dependent increase (Fig. 3B).

To further confirm these results, we measured the SCE frequencies resulting from FLU treatments in both lymphocytes (Fig. 4A) and fibroblasts (Fig. 4B). FLU caused no increase of SCE on both cell types.

3.4. Lack of functional DNA-PKcs sensitizes fibroblasts to FLU-induced damage and cell death

DSB are the principal lesions in the process of chromosome aberration formation. In order to confirm the induction of DSB, the percentage of CB was scored in the fibroblast cell line. The number of CB was increased ($p = 0.013$) in fibroblast cultures treated with FLU 1 μg/ml compared to controls (Fig. 5A).

To determine whether human cells use NHEJ to repair the DNA damage induced by FLU, two different DNA-PKcs inhibitors were used. VN-pretreated cultures showed a significant increase of CB (19.3% ± 2.3, $p = 0.0034$) compared to FLU alone (10% ± 2.0). In WTM-pretreated cultures, the number of CB was 3.8-fold enhanced compared to FLU ($p = 0.0117$). Similarly, we found a significant increase ($p = 0.012$) in the frequency of SCE in the cultures exposed

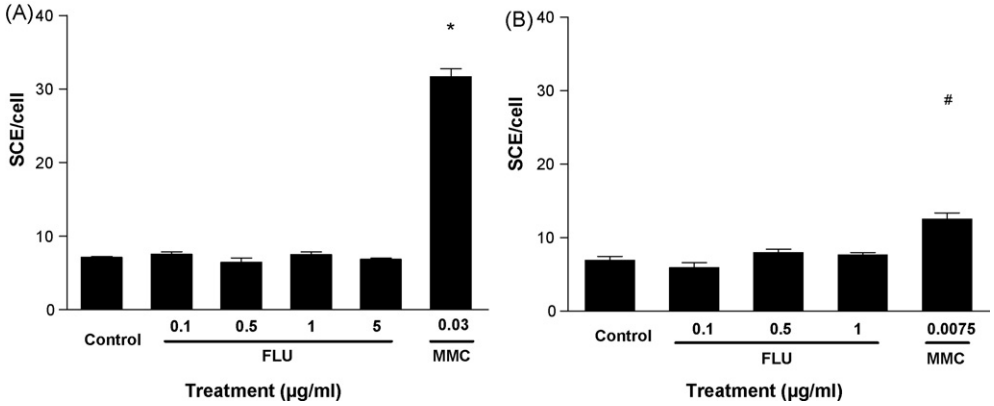

Fig. 4. Sister chromatid exchanges (SCE) in human lymphocytes (A) and fibroblasts (B) cultured in the presence of different doses of FLU during 48 h. $^{*}p = 0.0001$ and $^{\#}p = 0.005$ (vs. control). Bars represent the S.E.M.

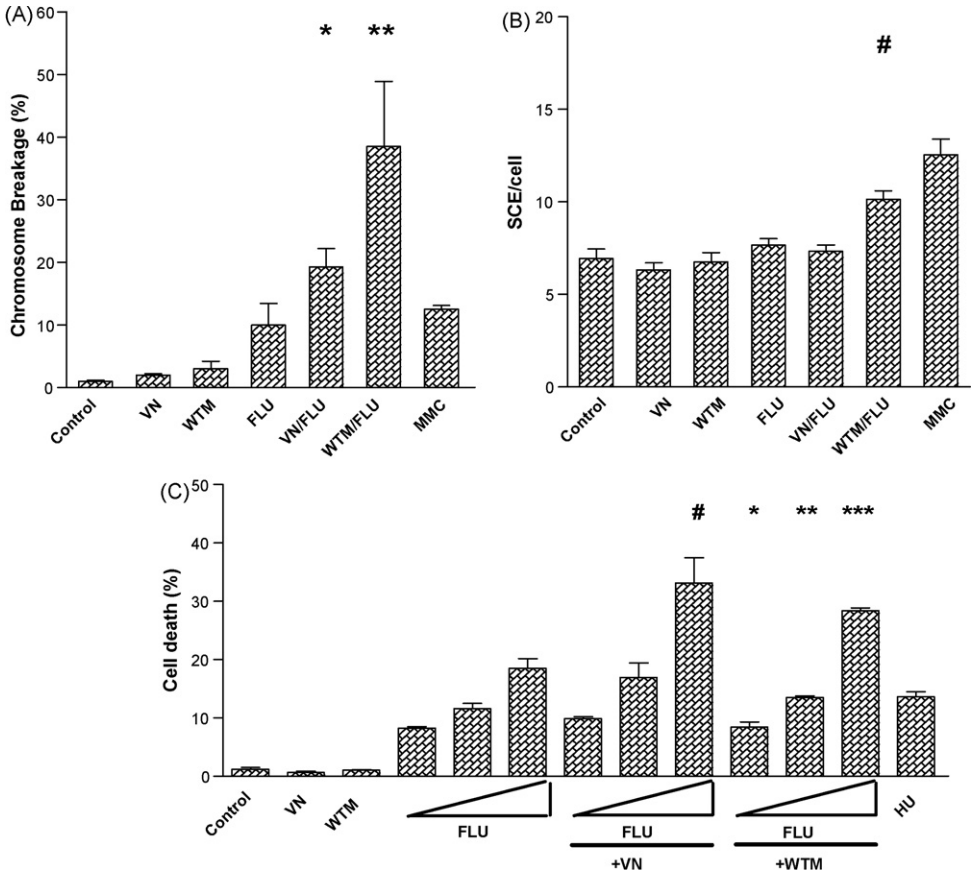

Fig. 5. Chromosome breakage (A), sister chromatid exchange (B) and cell death (C) analysis of human fibroblasts pretreated with vanillin (VN, 300 μM) or wortmannin (WTM, 8 μM) and treated with FLU. (A and B) Treatments with FLU 1 μg/ml for 48 h. Mitomycin C (MMC, 0.0075 μg/ml) was used as a positive control. $^{*}p = 0.0034$, $^{**}p = 0.0117$ and $^{\#}p = 0.012$ (vs. FLU). (C) Treatments with FLU 50, 100 or 200 μg/ml for 36 h. Hydroxyurea (HU, 1 mM) was used as a positive control. $^{\#}p = 0.0101$ (vs. FLU 200 μg/ml), $^{*}p = 0.0133$ (vs. FLU 50 μg/ml), $^{**}p = 0.0397$ (vs. FLU 100 μg/ml) and $^{***}p = 0.0179$ (vs. FLU 200 μg/ml). Bars represent the S.D.M.

to WTM plus FLU compared to FLU (Fig. 5B). Data of structural chromosome aberration induced by the different treatments are detailed in Table 1.

To test the cellular response to the damage induced by FLU, the loss of viability in cell cultures was analyzed using the trypan blue exclusion assay (Fig. 5C). As FLU is a weak inducer of cell death in human fibroblasts, the FLU concentrations used in these experiments were 50–200-fold higher than those previously employed. The cell death induced by FLU was dose-dependent ($r = 0.983$, $p = 0.017$). In pretreated cultures, the loss of cell viability induced by FLU was also linearly related to the doses (VN: $r = 0.998$, $p = 0.002$; and WTM: $r = 0.999$, $p = 0.0001$). VN pretreatment increased the cell death ($p = 0.0101$) at the highest dose of FLU compared to FLU alone. However, WTM raised cell death with all the increasing doses of FLU assayed ($p = 0.0133$, $p = 0.0397$ and $p = 0.0179$). HU 1 mM produced a similar level of cell death than FLU 100 μg/ml (Fig. 5C).

3.5. FLU induces hypersensitivity to DNA damage on NHEJ-mutant cell lines

To further determine the involvement of NHEJ in the repair of DSB induced by FLU, mutant Chinese hamster cell lines were analyzed (Fig. 6A–F). The frequency of CB induced by FLU 2.5 μg/ml in the NHEJ-deficient cell line XR-C1 (DNA-PKcs mutant) was 2.5-fold higher than CHO9, its parental cell line (Fig. 6A, $p = 0.0001$). At 1 μg/ml of FLU, XR-V15B (Ku80 mutant) showed increased frequencies of CB, being approximately 40-fold more sensitive to FLU ($p = 0.006$) than V79B. Structural chromosome aberration found in these cell lines is depicted in Table 2. Fig. 6B shows that the MI

Table 1
Chromosomal aberrations (CA) induced by fludarabine (FLU) and DNA-PKcs inhibitors (VN and WTM) in human fibroblasts

Treatment	Cells scored	Abnormal cells (%±S.D.)	Total structural CA			
			Gaps	ctb	csb	cte
Control	200	2 (1% ± 0.0)	1	1	1	0
VN 300 μM	200	4 (2% ± 0.0)	0	2	2	0
WTM 8 μM	200	6 (3% ± 1.4)	4	5	1	0
FLU 1 μg/ml	200	20 (10% ± 2.8)*	2	16	4	0
VN 300 μM + FLU 1 μg/ml	250	44 (17.6% ± 2.4)**	5	28	14	3
WTM 8 μM + FLU 1 μg/ml	250	65 (26% ± 8.5)***	8	69	21	3
MMC 0.0075 μg/ml	150	18 (12% ± 0.7)#	5	10	9	0

ctb, chromatid-type breaks; csb, chromosome-type breaks; cte, chromatid-type exchanges. $^{*}p = 0.005$, $^{**}p = 0.0001$, $^{***}p = 0.01$, $^{\#}p = 0.0001$, Student's *t*-test vs. respective controls.

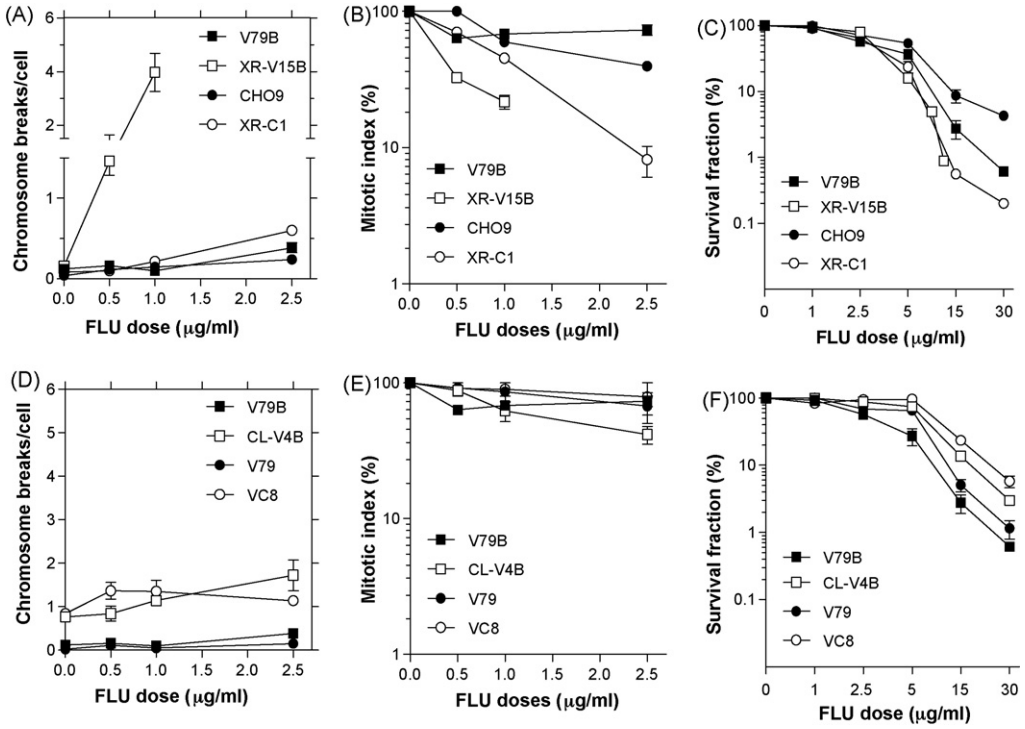

Fig. 6. Chromosome breakage (A and D) and mitotic index (B and E) in Chinese hamster cell lines treated with FLU 0.5, 1 or 2.5 μg/ml. Colony formation ability of (C) NHEJ-deficient and (F) HR-deficient cell lines exposed to different concentrations of FLU (1–30 μg/ml). Bars represent the S.E.M.

Table 2
Chromosome aberrations (CA) induced by fludarabine (FLU) in Chinese hamster cell lines

Cell line	Treatment (μg/ml)	Cells scored	Abnormal cells (%±S.D.)	Total structural CA				Highly damaged cells (≥10 breaks)
				ctb	csb	cte	cse	
CHO9	Control	151	6 (4.0 ± 0.3)	4	3	0	0	–
	FLU 0.5	150	14 (9.3 ± 2.1)	6	4	2	2	–
	FLU 1.0	150	22(14.7 ± 0.9)	14	7	1	0	–
	FLU 2.5	150	30(20.0 ± 0.9)*	28	6	1	0	–
V79	Control	150	3 (2.0 ± 1.4)	2	1	0	0	–
	FLU 0.5	150	13 (8.7 ± 1.1)	15	2	0	0	–
	FLU 1.0	150	8 (5.3 ± 3.0)	6	2	0	0	–
	FLU 2.5	150	12 (8.0 ± 2.0)#	18	4	0	0	–
V79B	Control	200	23 (11.5 ± 2.3)	21	3	0	0	–
	FLU 0.5	181	27 (14.9 ± 3.0)	27	4	0	0	–
	FLU 1.0	150	15 (10.0 ± 0.0)	11	4	0	0	–
	FLU 2.5	182	54 (29.7 ± 6.8)+	66	4	0	1	–
XR-C1	Control	206	16 (7.8 ± 1.2)	11	4	1	0	–
	FLU 0.5	220	20 (9.1 ± 2.4)	14	6	1	0	–
	FLU 1.0	106	19 (17.9 ± 1.7)	18	3	0	1	–
	FLU 2.5	20	10 (50.0 ± 0.0)*	9	3	0	0	–
XR-V15B	Control	150	17 (11.3 ± 3.1)	19	2	1	0	–
	FLU 0.5	150	78 (52.0 ± 12.5)	195	16	3	1	3
	FLU 1.0	131	109 (83.2 ± 5.3)*	490	26	1	1	16
	FLU 2.5	–	–	No mitosis				–
CL-V4B	Control	150	56 (37.3 ± 15.3)	49	6	8	22	–
	FLU 0.5	150	66 (44.0 ± 6.0)	61	3	12	19	–
	FLU 1.0	150	78 (52.0 ± 4.0)	91	6	11	26	–
	FLU 2.5	150	97 (64.7 ± 8.3)	197	7	12	15	1
V-C8	Control	150	80 (53.3 ± 9.2)	110	6	5	1	–
	FLU 0.5	175	123 (70.3 ± 12.9)	221	15	0	1	–
	FLU 1.0	160	108 (67.5 ± 10.6)	198	8	2	3	–
	FLU 2.5	175	104 (59.4 ± 8.1)	176	6	6	2	–

ctb, chromatid-type breaks; csb, chromosome-type breaks; cte, chromatid-type exchanges; cse, chromosome-type exchanges (ring and dicentric chromosomes). *$p = 0.0001$, #$p = 0.013$, +$p = 0.012$, Student's *t*-test vs. respective controls.

from both NHEJ-deficient cell lines was considerably reduced at FLU 2.5 μg/ml. This value decreased significantly in XR-C1 cells ($p = 0.003$) compared to controls. XR-V15B cells decreased its MI significantly at 1 μg/ml of FLU ($p = 0.0001$).

To confirm these data, we performed a colony formation assay (Fig. 6C). The results showed that XR-C1 ($p = 0.0172$) and XR-V15B ($p = 0.0142$) cells were hypersensitive to FLU from 5 μg/ml.

The frequencies of spontaneous CB in HR-deficient cell lines, CL-V4B (Rad51 C mutant) and V-C8 (Brca 2 mutant), revealed higher levels of damage than their parental counterparts, V79B and V79, respectively (Fig. 6D and Table 2). Although there was an increase in the chromosome damage induced with the different doses of FLU assayed, the sensitivity of HR-mutant cell lines did not differ from that of their parental cell lines. Furthermore, there were no differences in the MI between HR-mutant cell lines and their wild-type counterparts (Fig. 6E). The colony formation ability of HR-defective cell lines, CL-V15B and V-C8, revealed a FLU-resistant phenotype (Fig. 6F) showing better survival rates than their parental cell lines from 5 μg/ml of FLU ($p = 0.0152$ and $p = 0.0018$).

4. Discussion

Nucleoside analogues are effective in the clinical treatment of hematological malignances and solid tumors. FLU and other purine nucleoside analogues represent a novel group of cytotoxic agents with high activity in low-grade lymphoid malignancies [25]. FLU is cytotoxic against resting and dividing cells. In proliferating cells, cytotoxicity seems to require incorporation of this purine analogue into DNA [26].

Here we evaluated the repair pathways employed in restoring DSB that are induced by FLU during replication in mammalian cells.

We showed that a continuous exposure to FLU inhibited the human cell replication. Previous report showed that FLU blocks the progression in the S-phase [27]. We also found an accumulation of DNA DSB in response to FLU treatment, which became visible in the early and middle S-phase on synchronized cultures. Late stages of the DNA synthesis were difficult to evaluate due to the presence of endogenously originated DSB in a large proportion of cells. Our data support that cells exposed to FLU should enter the S-phase to develop DSB. In the same way, Ewald et al. [28] found an increased amount of γH2AX in human cell lines at the G1–S border in response to the nucleoside analogue gemcitabine.

Rad51-mediated HR plays an important role in DNA repair of mammalian cells [29] and replication inhibitors are potent inducers of Rad51 foci [9,30,31]. Our results show that FLU does not induce Rad51 nuclear foci at the different times and doses analyzed. Furthermore, both human lymphocytes and fibroblasts exposed to FLU showed no increased frequencies of SCE. In straight contrast, replication inhibitors were reported to be strong inducers of SCE [32–34]. Additionally, the HR-mutant cell lines did not show a FLU-sensitive phenotype. In agreement to our data, Marple et al. [35] reported a lack of sensitivity of Brca2-mutant ES cells (*brca2*$^{lex1/lex2}$ cells) to the replication inhibitors HU and aphidicolin (APH).

The DNA-PK is required for efficient repair of DSB by the classical NHEJ pathway [17]. Both VN and WTM are known inhibitors of phosphatidylinositol-3-kinase-related kinases that bind covalently to lysine 3751 of DNA-PK$_{CS}$ and irreversibly decrease the kinase activity of DNA-PK [36–38].

To test the involvement of a DNA-PK-dependent NHEJ pathway in the repair of FLU-induced DNA DSB, we assessed the induction of CB, SCE and cell death in the presence of either VN or WTM in human fibroblasts. The CB induced by FLU indicated a clastogenic effect on these cultures, which supports our previous findings on human lymphocytes [39].

Fibroblast cultures pretreated with the DNA-PK inhibitors showed increased CB levels, suggesting that more FLU-induced DSB were left unrepaired. Only WTM pretreatment was able to increase the levels of SCE, which could be explained in two different ways. It may reflect an indirect effect on ataxia-telangiectasia mutated (ATM) activity, and there is some evidence that lack or inactivation of ATM can cause high frequency of HR [40–42]. On the other hand, levels of DSB above a certain threshold can activate the HR machinery [30], consequently increasing the levels of SCE. The latter is supported by the high frequency of CB found in WTM-pretreated cultures. The cell death analysis supports that both VN and WTM sensitized human fibroblasts to FLU.

The participation of NHEJ repair pathway was further confirmed by the higher sensitivity to FLU found in NHEJ-mutant Chinese hamster cell lines, which displayed increased frequencies of CB, decreased MI and impaired survival.

Chen et al. [43] reported that cells deficient in DNA-PKcs expression were sensitive to agents that disrupt the progression of the replication fork and induce associated DSB. This suggests that DNA-PKcs may play an important role in the resolution of DNA replication-associated DSB in the S-phase of the cell cycle. An early report has demonstrated that DNA-PK and p53 are able to form a sensor complex that detects the disruption of DNA replication caused by gemcitabine [44]. The role of DNA-PK as a sensor for DNA damage after nucleoside analogue treatment might partially explain the higher sensitivity shown by fibroblasts treated with DNA-PKcs inhibitors. However, all the Chinese hamster cell lines used in this study are defective for the binding of p53 to DNA [45,46]; thus, harboring a dysfunctional pathway for sensing replication abnormalities induced by nucleoside analogues. Regarding this aspect, only NHEJ-deficient cells were affected by FLU.

Several works addressed the question of what repair pathways are responsible for repairing DSB induced by synthesis inhibitors on mammalian cells. Arnaudeau et al. [47] reported that APH, ara-C, HU and methotrexate, induce HR in Chinese hamster V79 cells. According to Lundin et al. [9], NHEJ and HR are required for the repair of damage resulting from HU-induced replication block. On the other hand, HR alone is sufficient to rescue cells from the less complete arrest induced by thymidine. As the latter does not induce detectable levels of DSB, it is considered that NHEJ is involved in the repair of blocked replication forks that have been processed into DSB.

Although the two repair pathways could act in the resolution of DSB induced by replication inhibitors, the initial binding of repair factors to the break or the levels of DSB generated may affect this choice. It was suggested that poly(ADP ribose) polymerase-1 (PARP-1) might control the balance between HR and NHEJ by decreasing the affinity of Ku to DSB [48,49], thus, favoring the access of HR factors. Other line of evidence suggests that DNA polymerase μ (pol μ) is an interacting factor with NHEJ components that may favor this pathway [50]. It would not be hard to speculate that FLU may affect the channeling to NHEJ repair through a direct effect on either PARP-1 or pol μ. Further studies should clarify this issue.

Overall, our data support that FLU induces DSB in mammalian cells, mostly in the early and middle S-phase of the cell cycle, and NHEJ plays a major role in the repair of these lesions.

Future experiments should evaluate whether combined therapies using FLU and NHEJ inhibitors could result into enhanced antitumoral activity or could be of any other clinical relevance.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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