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Both XPA and DNA polymerase eta are necessary for the repair of doxorubicin-induced DNA lesions

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ABSTRACT

Doxorubicin (DOX) is an important tumor chemotherapeutic agent, acting mainly by genotoxic action. This work focus on cell processes that help cell survival, after DOX-induced DNA damage. In fact, cells deficient for XPA or DNA polymerase eta (pol eta, XPV) proteins (involved in distinct DNA repair pathways) are highly DOX-sensitive. Moreover, LY294002, an inhibitor of PIKK kinases, showed a synergistic killing effect in cells deficient in these proteins, with a strong induction of G2/M cell cycle arrest. Taken together, these results indicate that XPA and pol eta proteins participate in cell resistance to DOX-treatment, and kinase inhibitors can selectively enhance its killing effects, probably reducing the cell ability to recover from breaks induced in DNA.

1. Introduction

Over the past 40 years or more, Doxorubicin (DOX) (tradename, Adriamycin®), an anthracycline antibiotic, has been widely used in the chemotherapeutic treatment of a broad range of malignancies, from solid tumors, as those in the breast, liver and bile ducts, to hemaetological diseases, such as non-Hodgkin's lymphoma. Nevertheless, despite its extensive clinical use, DOX mechanisms of action are, as yet, not fully understood. The most well characterized mechanism is through the inhibition of the enzyme topoisomerase-II (topo-II), with the subsequent formation of double-strand breaks (DSBs) in DNA. However, several other mechanisms are also thought to play a role in DOX induced cytotoxicity, such as DNA adduct formation, DNA cross-linking and free radical formation. Furthermore, DOX-genotoxicity is also affected by both drug concentration and cell type specificity [1]. Apart from the uncertainty regarding DOX-induced DNA lesions, the mechanisms involved in their repair are also poorly understood. Deducing how these lesions are repaired is a current challenge that, if successful, could lead to more favorable clinical outcomes for patients undergoing DOX-treatment.

It has been shown that the kinase DNA-PKcs, a component of the Non-Homologous End Joining (NHEJ) pathway, participates in the repair of DOX-induced DNA lesions, most likely by repairing the DSBs induced by the inhibition of topo-II [2]. Another pathway that seems to be relevant in their repair is Nucleotide Excision Repair (NER), one of the

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most versatile repair pathways in mammalian cells. In NER, there are two subpathways that differ only in the recognition of DNA damage, viz., transcription coupled repair (TCR), which is selective for lesions in the transcribed strand of expressed genes, and global genome repair (GGR), that acts throughout the entire genome. After DNA damage recognition (in GGR by the proteins XPC-hHR23B and DDB1/DDB2), the helicases XPB and XPD sequentially open the double helix, and the endonucleases XPG and ERCC1-XPF cleave the damaged strand a few bases away from the lesion. The proteins XPA and RPA are also important for the efficacy of this repair process, which is followed by the removal of the DNA segment containing the lesion, and the subsequent resynthesis of this strand, using the intact one as template. Defects in the NER proteins XPA–XPG give rise to the inherited disease xeroderma pigmentosum (XP) [3,4]. When NER is not impaired, a variant form of this disease (XPV) can also be caused by defects in post replication repair machinery, or more specifically, defects in the protein DNA polymerase eta (pol eta) involved in translesion synthesis of DNA (TLS) [5]. Cells derived from XP patients and defective in the proteins XPA, XPC [6] and XPD [7] are more sensitive to the treatment with DOX, suggesting a role for these proteins in the repair of DOX-induced lesions.

In order to better understand the involvement of XP proteins in the repair of DOX-induced lesions, human cells defective in the proteins XPA, XPF or pol eta (XPV) were treated with clinical doses of DOX [1], whereupon their responses to the drug were analyzed. As shown here, cells defective in XPA (XP12BE) or pol eta (XP30RO) are much more sensitive to DOX than cells either deficient in XPF (XP51RO) or proficient in DNA repair (MRC5). Cell response was also evaluated after treatment with both DOX and LY294002, an inhibitor of PIKKs, such as DNA-PKcs [8,9]. It was noted that LY294002 potentiates the cytotoxic effect of DOX only in the repair-deficient cells XP12BE and XP30RO, but not in the wild type MRC5. Moreover, in all the cells used in this study, DOX treatment induced a G2/M arrest in the cell cycle, also enhanced by LY294002.

2. Materials and methods

2.1. Cell culture conditions

The cells used were: SV40-transformed human fibroblasts derived from skin biopsies of XP patients of complementation groups A (XPA–XP12BE and XP12RO cells) and V (XPV–XP30RO cells); SV40 transformed human fibroblasts derived from lung tissue of a normal fetus (MRC5 cells); hTERT transformed human fibroblasts derived from skin biopsies of an XP patient of complementation group F (XPF–XP51RO), and those from a normal individual (C5RO). XP51RO and C5RO were kindly provided by Dr. N. Jaspers (Erasmus University, Netherlands). MRC5 and C5RO cells with normal levels of DNA repair were used as controls. Cells were routinely grown at 37 °C in a 5% CO_2 humidified atmosphere in the appropriate medium: MRC5, XP12RO and XP12BE in Dulbecco's modified Eagle medium (DMEM–Invitrogen, Life Technologies, California, USA), supplemented with 10% fetal bovine serum (FBS – Cultilab,

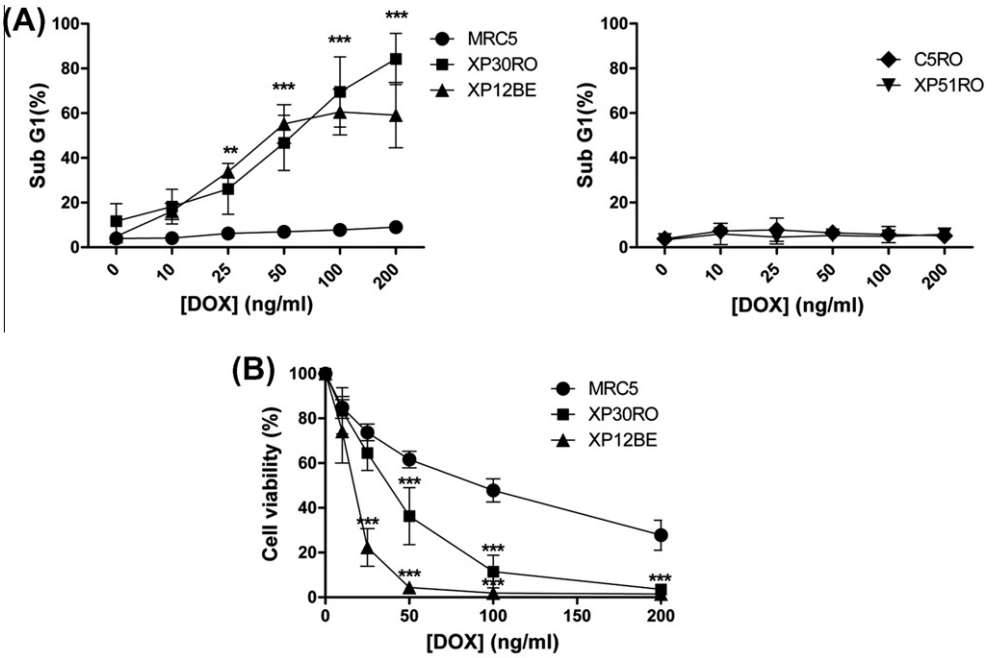

Fig. 1. DNA repair deficient and proficient cell sensitivity to DOX. Cells were exposed to the indicated doses of DOX for 48 h, whereupon either the sub-G1 fraction was detected by flow cytometry (A), or cell viability assessed by XTT assay (B). The cell lines used in these experiments were, as indicated: MRC5, C5RO, XP12BE (XPA), XP30RO (XPV) and XP51RO (XPF). Statistical analysis was applied for comparing DNA repair proficient (MRC5 and C5RO) with DNA repair deficient (XP12BE, XP30RO and XP51RO) cells. ** Significantly different $P < 0.01$ for XP12BE *versus* MRC5, and $P < 0.05$ for XP30RO *versus* MRC5; ***$P < 0.001$.

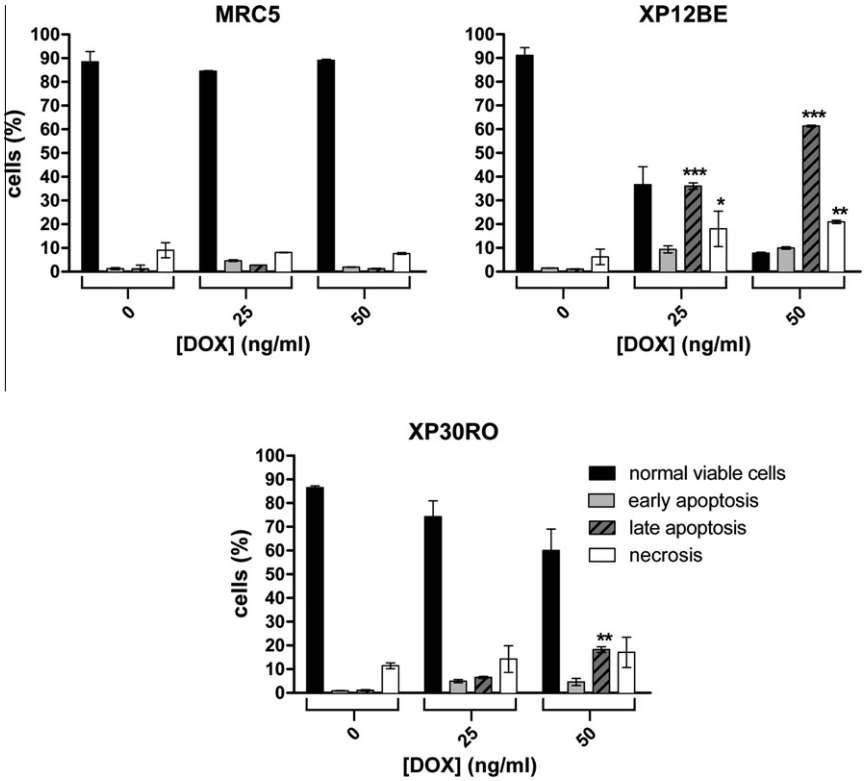

Fig. 2. DOX-induced cell death. Cells were exposed to the indicated doses of DOX for 48 h, and then analyzed with a combination of fluorescent dyes. A combination of fluorescein di-acetate (FDA), propidium iodide (PI) and Hoechst (HO) dyes was used to distinguish viable cells from those in necrosis, or early or late apoptosis. Cells were counted, and the percentages plotted on a graph. The cell lines used were: MRC5, XP12BE (XPA) and XP30RO (XPV). Statistical analysis, by cell line, was applied for comparing DOX-treated cells with non-treated. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Campinas, SP, Brazil); XP30RO in Eagle Minimum essential medium (MEM – Invitrogen, Life Technologies), supplemented with 10% FBS; and XP51RO and C5RO in HAM's medium (Invitrogen, Life Technologies), supplemented with 15% FBS.

2.2. Treatment conditions

For cytotoxicity assays, cells, in the appropriate medium, were first seeded onto 6-well plates at a concentration of 6×10^4 cells per well, and then grown for 24 h prior to treatment. The cultures were treated, in the dark, with different concentrations of DOX (up to 200 ng/mL) for 24, 48 or 72 h, as indicated. As control, cells were incubated with 0.25% ethanol for the same period of time. For co-treatments with DOX and LY294002 (modified from [8,9]), cells were incubated with 10 μM LY294002, 1 h prior to addition of DOX to the culture medium. As control, cells were incubated with 0.1% DMSO.

2.3. Cell viability

After each treatment, cell viability was assessed with a Cell Proliferation Kit II (XTT) (Roche, Basel, Switzerland), according to manufacturer's instructions. Briefly, after discarding the medium, 1 mL of XTT labeling mixture was added to the cells and incubated for approximately 2 h at 37 °C. Absorbance was measured at 492 nm (A_{492}) and 750 nm (A_{750}), the final result corresponding to A_{492}–A_{750}.

2.4. Flow cytometry analysis (sub-G1 assay, cell cycle and γH2AX analysis)

After treatment, adherent cells were trypsinized and mixed with detached dying cells, washed with PBS and then fixed with chilled 70% ethanol for at least 24 h at −20 °C. Staining was performed at room temperature for 30 min in filtered PBS containing 20 μg/mL propidium iodide (PI), 200 μg/mL RNase A and 0.1% Triton X-100. Data were collected with a Guava Flow Cytometer (GE Healthcare, United Kingdom), and the percentage of sub-G1 population determined with CytoSoft Data Acquisition and Analysis Software (GE Healthcare). For cell cycle, the collected data was analyzed with "ModFit LT" Software (Verity Software House, Maine, USA). For γH2AX immunostaining, cells were fixed with 1% formaldehyde and then 70% ethanol. After blocking and permeabilization (0.2% Triton X-100 in PBS and 1% BSA), cells were incubated with the antibody anti-γH2AX (Anti-phospho-Histone H2A.X (Ser139), clone JBW301 from Upstate, Millipore) diluted 1:500 for 1 h at room temperature. Cells were then washed and incubated with a secondary antibody (anti-mouse FITC, from Sigma) at 1:200 for 1 h at room temperature in the dark. Before collecting data, cells were stained with PI solution, as described above.

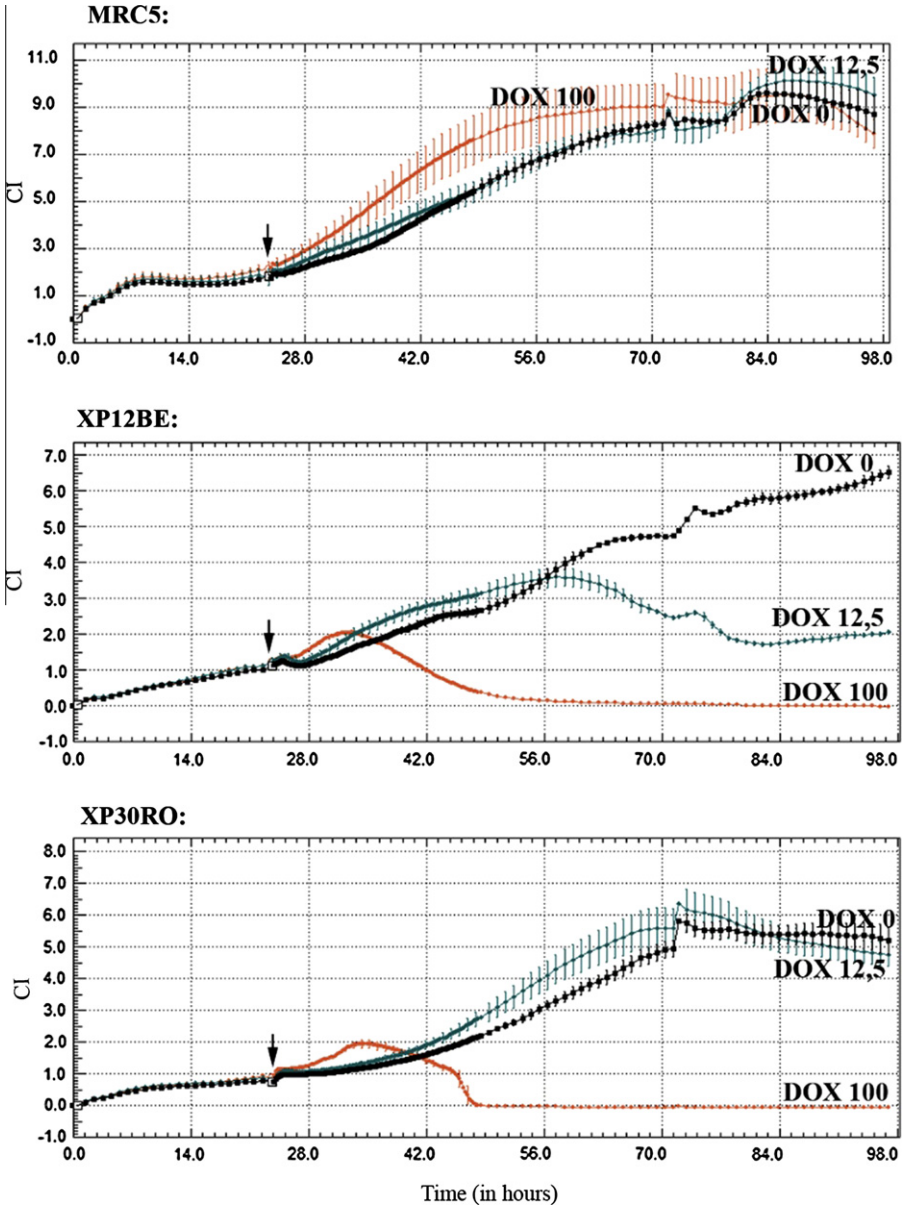

Fig. 3. Real time analysis of DOX-induced cytotoxicity. Histograms were obtained using the xCELLigence System. Cells were grown for 24 h before initiating DOX treatment (arrows), at the indicated doses (12.5 or 100 ng/mL; DOX 0 indicates the controls, incubated only with 0.25% ethanol). Cell index (CI) was recorded every hour, except for the first 48 h immediately after initiation of treatment, when recordings were every 15 min. The results are the means of at least three replicates, with displayed standard error bars.

2.5. Detection of apoptotic and necrotic cell death by fluorescence staining

Following treatment, adherent and detached cells were harvested by centrifugation, whereupon pellets were suspended in 20 μL of chilled PBS. In order to distinguish apoptotic and necrotic cells from those viable, the cellular suspension was mixed with a combination of dyes, viz., 705 μg/mL fluorescein di-acetate (FDA), 250 μg/mL PI, and 100 μg/mL Hoechst 33342 (HO). FDA and HO are vital dyes, which stain the cytoplasm and nucleus of viable cells, respectively. The necrotic and late stage of apoptotic cells are identified by PI staining. Whereas the characteristic pattern of chromatin fragmentation was noted in cells in the early (viable-HO stained) and late (dead-PI stained) phases of apoptosis, the nucleus was non-fragmented in normal and necrotic cells [10]. At least 500 cells were randomly scored by microscopic analysis.

2.6. Real-time cellular cytotoxicity assay (xCELLigence)

Assaying was according to manufacturer's instructions and as previously described [11–14]. Briefly, cells were seeded onto a 96-well E-Plate (Roche), with interdigitated microelectrodes at the bottom of each well, at a concentration of 1.2×10^4 (MRC5), 3.1×10^3 (XP12BE) or

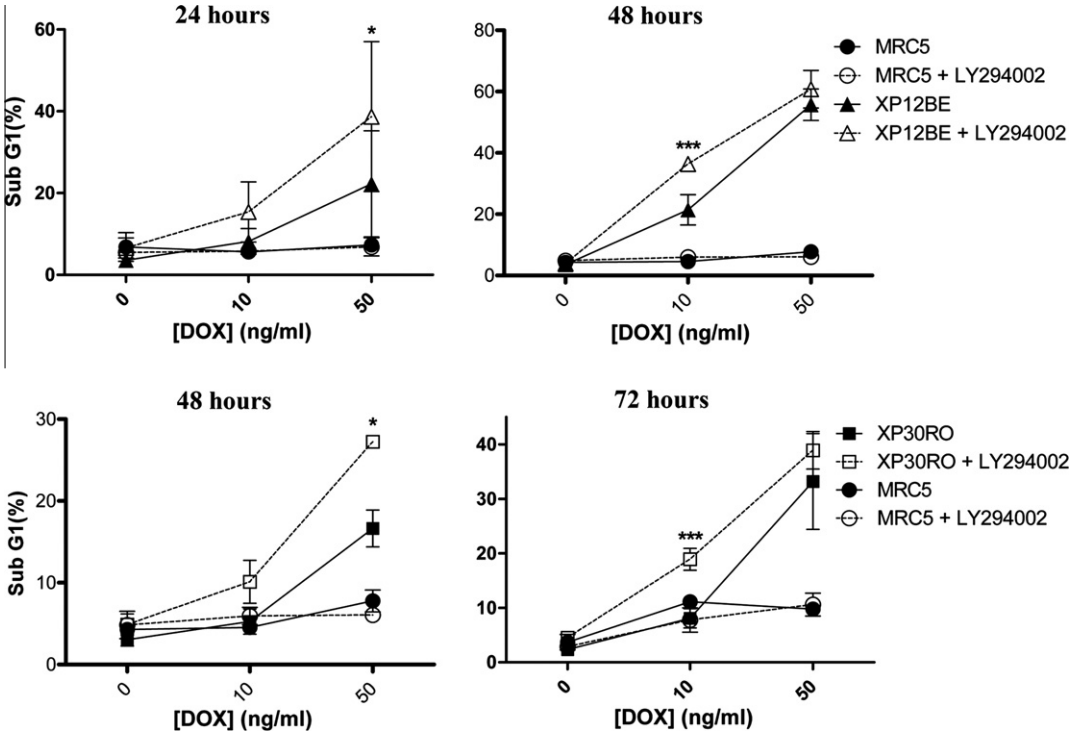

Fig. 4. LY294002 enhances the DOX cytotoxic effect. Cells were exposed to DOX (10 ng/mL and 50 ng/mL) and/or 10 μM LY294002 (LY). The sub-G1 fraction was detected by flow cytometry, at the indicated times after treatment. The cell lines used in these experiments were, as indicated: MRC5, XP12BE (XPA) and XP30RO (XPV). Statistical analysis, by cell line, was applied for comparing cells treated only with DOX with those co-treated with both DOX and LY. $^{*}P < 0.05$; $^{***}P < 0.001$.

6.2×10^3 (XP30RO) cells per well. After 30 min at room temperature, the E-Plate was placed onto an xCELLigence Real-Time Cell Analyzer (RTCA) SP System device (Roche), located inside a tissue culture incubator, where cells were left to grow for 24 h before being exposed to 0.25% ethanol or various doses of DOX (from 6.25 ng/mL to 200 ng/mL). Impedance was continuously measured over the following 72 h. The increase in the number and size of cells attached to the electrode sensors leads to increased impedance, from which derive the cell index values displayed at the plot. Hence, the cell index correlates with the number of cells attached to the bottom of the well.

2.7. Statistical analysis

Results represent the mean of two or three independent experiments, each done in triplicate, with error bars showing the standard error. Experimental differences were tested for significance using Two-way ANOVA analysis, followed by Bonferroni post-testing (Prism 5 – GraphPad Software Inc., California, USA).

3. Results

3.1. DOX-induced cytotoxicity

In order to define DOX-induced cytotoxicity in cells proficient (MRC5 and C5RO) and deficient (XP12BE, XP30RO and XP51RO) in DNA repair, cell viability and cell death induced by this chemotherapeutic drug were determined. Cells deficient in XPA (XP12BE) or in pol eta (XP30RO) proved to be more sensitive to 48-h treatments with clinical doses of DOX than those deficient in XPF (XP51RO) or the wild type (Fig. 1). Besides dying more from apoptosis, as inferred from increased percentages of fragmented DNA (sub-G1 fraction) (Fig. 1A), XP12BE and XP30RO cells are also less viable than MRC5 (Fig. 1B). XP12RO, another XPA-deficient cell line with similar higher sensitivity to DOX (data not shown; and [6]), was used for corroborating the results with XP12BE. Interestingly, XP51RO cells are not more sensitive to DOX, at least not with the doses used, as shown by sub-G1 levels (Fig. 1A). This absence of an observable phenotype could be attributed to residual NER activity retained in cells derived from XPF patients, which can sometimes reach 30% of normal NER levels [15]. Thus, it stands to reason that this residual activity could be responsible for the repair of DNA damage induced by the doses used herein. Moreover, as XPF, an endonuclease, is also involved in homologous recombination repair of mainly DNA interstrand crosslinks, the results indicated that either this repair pathway is not directly involved in the repair of DOX-induced DNA lesions, or other alternative endonucleases are. Interestingly, in these and the following experiments, XP-A cells showed to be more sensitive than XP-V cells. This may indicate that full deficiency of NER is more deleterious than the lack of pol eta, probably compensated by the repair of lesions by NER in XP-V cells.

To check whether DOX induces death by necrosis and confirm the increase in apoptosis observed earlier, treated and non-treated cells were stained with dyes that allow distinguishing normal viable cells, necrotic cells and cells in early or late apoptosis. As shown in Fig. 2, treatment with low doses of DOX for 48 h induced apoptosis in both XP12BE and XP30RO cells, but not in MRC5, thus confirming the results obtained by sub-G1 cellular fraction analysis (Fig. 1A). Although there was a slight increase in necrosis in XP12BE cells, this was not so in either XP30RO or MRC5 cells. Even though statistically significant, the occurrence was more likely due to experimental variation, since in another set of independent experiments no such increase was detected (Fig. 5).

In order to analyze DOX-induced cellular cytotoxicity in real time, thereby complementing the end-point assays already described, the xCELLigence RTCA SP System (Roche), which does not depend upon the incorporation of labels [11–14], was used. As can be seen in Fig. 3,

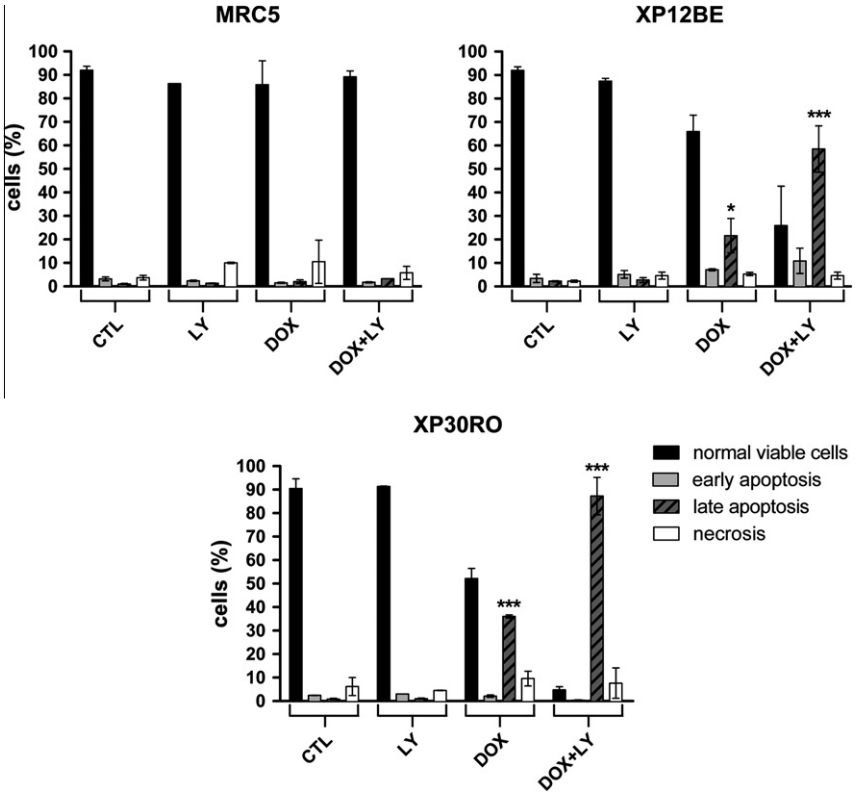

Fig. 5. Cell death induced by DOX and LY. Cells were exposed to DOX (XP12BE: 10 ng/mL; MRC5 and XP30RO: 50 ng/mL) and/or 10 μM LY, as indicated. A combination of FDA, PI and HO dyes was used to distinguish cells in early or late apoptosis from viable or necrotic cells. The cell lines used were: MRC5, XP12BE (XPA) and XP30RO (XPV). Statistical analysis, by cell line, was applied for comparing control non-treated cells with cells treated as indicated. $^{*}P < 0.05$; $^{***}P < 0.001$.

XP30RO and XP12BE are extremely sensitive to higher doses of DOX (100 ng/mL), whereas MRC5 cells presented no cytotoxicity. Confirming previous results, at lower doses (12.5 ng/mL) only XP12BE manifested DOX-induced cytotoxic effects.

Thus, it is inferred that the active involvement of XPA and pol eta proteins in the efficient repair of lesions caused by clinical doses of DOX is essential for cell survival.

3.2. LY294002 potentiates the cytotoxic effects of DOX in repair-deficient cells

In some tumor cells, the cytotoxic effects of DOX are enhanced by LY294002 [8,9], an inhibitor of phosphatidylinositol-3-kinases (PI3Ks) and related protein kinases (PIKKs) [16–20]. Since kinases involved in DNA damage response and repair, such as ATM, ATR and DNA-PKcs, are members of the PIKK family [21,22], it was decided to analyze the effects of co-treatment with DOX and LY294002 (LY) in XP12BE and XP30RO repair-deficient cells. As shown in Fig. 4, DOX-cytotoxic effects were certainly enhanced in both, since co-treatment lead to increased levels of apoptosis. As to repair-proficient MRC5 cells, the addition of LY induced no like effect. Moreover, it was noted that, although the sensitivity profile in XP30RO is the same as in XP12BE, the response of the former to both treatments (DOX alone, and DOX plus LY) was delayed.

In order to further establish the additive cytotoxic effect of LY in DNA repair-deficient cells treated with DOX, staining experiments similar to those shown in Fig. 2 were carried out. A lower dose (10 ng/mL) of DOX was used for the treatment of XP12BE cells, seeing that at higher doses, the levels of DOX-induced death were already so high in the 48-h treatment (see Fig. 4), that it would impair the detection of any potential LY-induced increase. Again, LY strongly enhanced the effects of DOX only in XP12BE and XP30RO cells, as shown by an increase in the percentage of apoptotic cells in both when compared to treatments with DOX or LY alone. In MRC5 cells, there was no increase in cell death (Fig. 5).

Thus, deficiencies in repair processes mediated by XPA and pol eta could possibly contribute to the DOX/LY synergistic cytotoxic effects observed, as the proper activity of these proteins is related to cellular resistance to this co-treatment.

3.3. Effects of DOX in the cell cycle

In several cell lines, DOX-treatment leads to cell cycle arrest, mainly in the G2/M phases [7,23,24]. As there is no mention in the literature of this in cells deficient in either XPA or pol eta, DOX-effects throughout the cell cycle progression in XP12BE, XP30RO and MRC5 cells were analyzed. Direct analysis of the cycle in treated and nontreated cells demonstrated that DOX induces G2/M arrest in MRC5, XP12BE and XP30RO cell lines, as can be observed in representative histograms (Fig. 6A) and quantitative data (Fig. 6B). It was also shown that in co-treatments DOX plus LY, the arrest was more pronounced than in treatments with DOX alone. However, treatment with LY alone was not cell cycle-arrest inductive (Fig. 6). After co-treatment, G2/M arrest in MRC5 cells seemed to be even more pronounced than in both XP12BE and XP30RO (Fig. 6), even though prolonged treatment or treatment with higher doses of DOX eventually lead to the same phenotype (data not shown). This is probably due to MRC5 cell proficiency in DNA repair, which would allow for more efficient activation of the appropriated checkpoint, hence blocking more rapidly the cell cycle and giving rise to less cell death.

Thus, after treatment with DOX, cell cycle progression response is basically similar in cells proficient (MRC5) and deficient (XP12BE and XP30RO) in DNA repair, since arrest of the cell cycle in G2/M was evident in all the three. Furthermore, growth arrest was also enhanced by LY294002.

3.4. Quantification of γH2AX in DOX-treated cells

DOX is considered a topo-II poison, and the blockage of DNA replication by this drug may lead to the formation of DSBs. In fact, cells treated with DOX show the induction of DSBs, as determined by the phosphory-

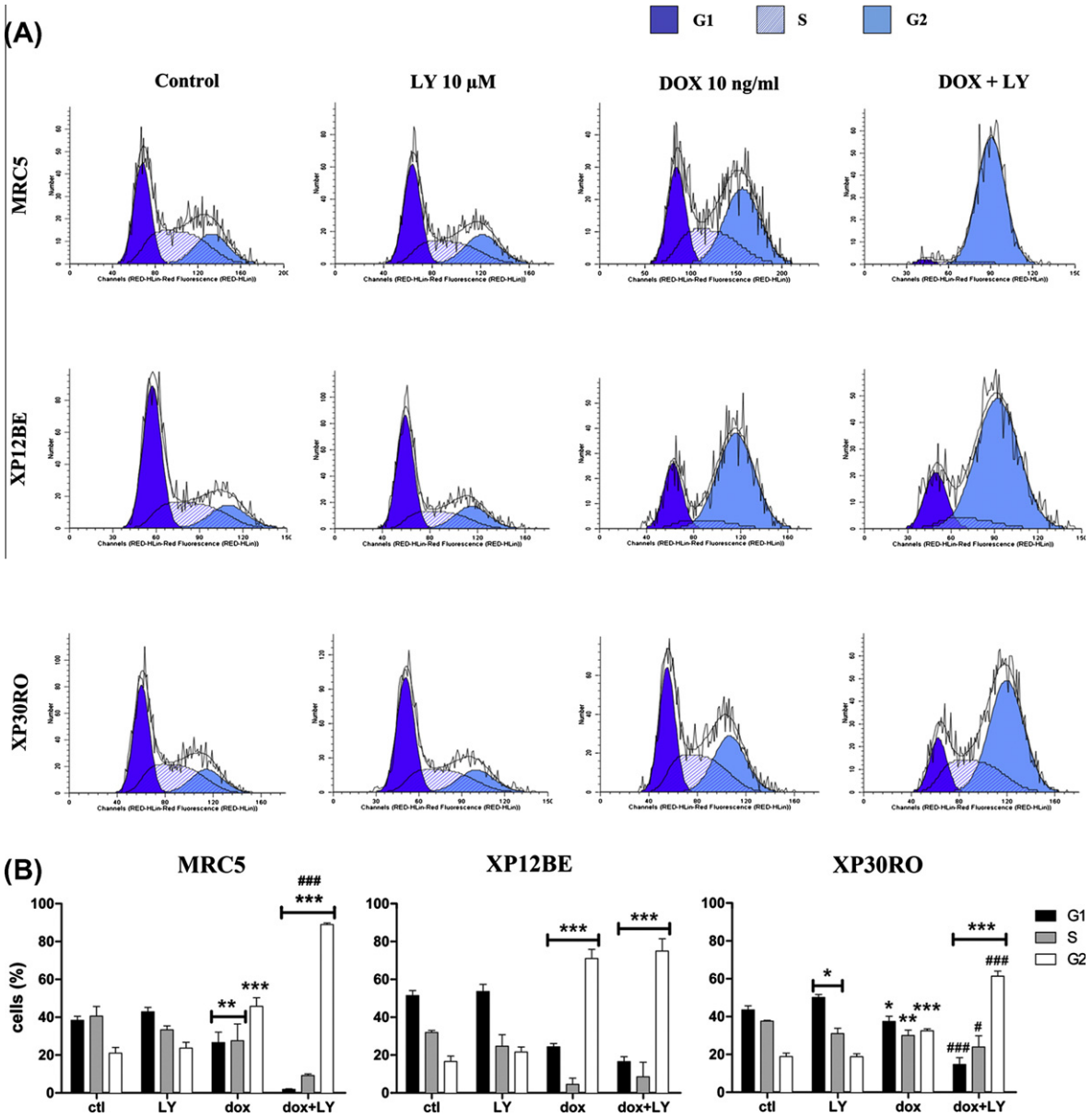

Fig. 6. DOX-induced G2/M cell cycle arrest. Cells were exposed to 10 ng/mL DOX and/or 10 μM LY for 48 h, whereupon the cell cycle of treated and non-treated cells were analyzed with "ModFiT" software. Representative histograms are shown in (A), and the quantified results in (B). The cell lines used were: MRC5, XP12BE (XPA) and XP30RO (XPV). Statistical analysis, by cell line, was applied for comparing control non-treated cells with treated cells (*), or cells treated only with DOX with cells submitted to DOX-plus-LY co-treatment (#). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

lation of H2AX histones, γH2AX [7]. This histone modification is normally responsible for chromatin remodeling at the region of DSBs, opening access of DNA repair machineries to these lesions. Although, one would not expect a participation of NER in the repair of DSBs, DNA pol eta could well be involved in protection from these types of lesion. The induction of γH2AX in treated cells was immunologically detected, through the cell cycle, by flow cytometry. The results, summarized in Fig. 7, indicate that continuous treatment of cells with low concentrations (10 ng/mL) of DOX leads to increased induction of γH2AX-labeled cells, at least up to 48 h. For higher doses of DOX (50 ng/mL, for 24 h) it seems that most of the living cells appear to be labeled. Curiously, there is no clear difference on the induction of γH2AX (in living cells) when the three cell lines are compared, although the increased induction of cell death (sub-G1) in treated XP-A and XP-V cells is confirmed. This indicates that XPA and pol eta are not involved in the processing of DSBs. However, when the inhibitor LY is added to the cells, there is a tendency to increase the frequency of γH2AX-labeled cells, indicating that the kinases targeted by LY may contribute to a reduction in these lesions, and also that kinase inhibition does not reduce phosphorylation of H2AX. Moreover, the labeled cells seem to be distributed in the different cell cycle phases, with a strong labeling of G2 cells, which seem to be arrested in G2/M, as commented above.

4. Discussion

DOX is one of the most commonly used chemotherapeutic drugs, with a wide range of activity against solid tumors, lymphomas and leukemias. Even though classified as a

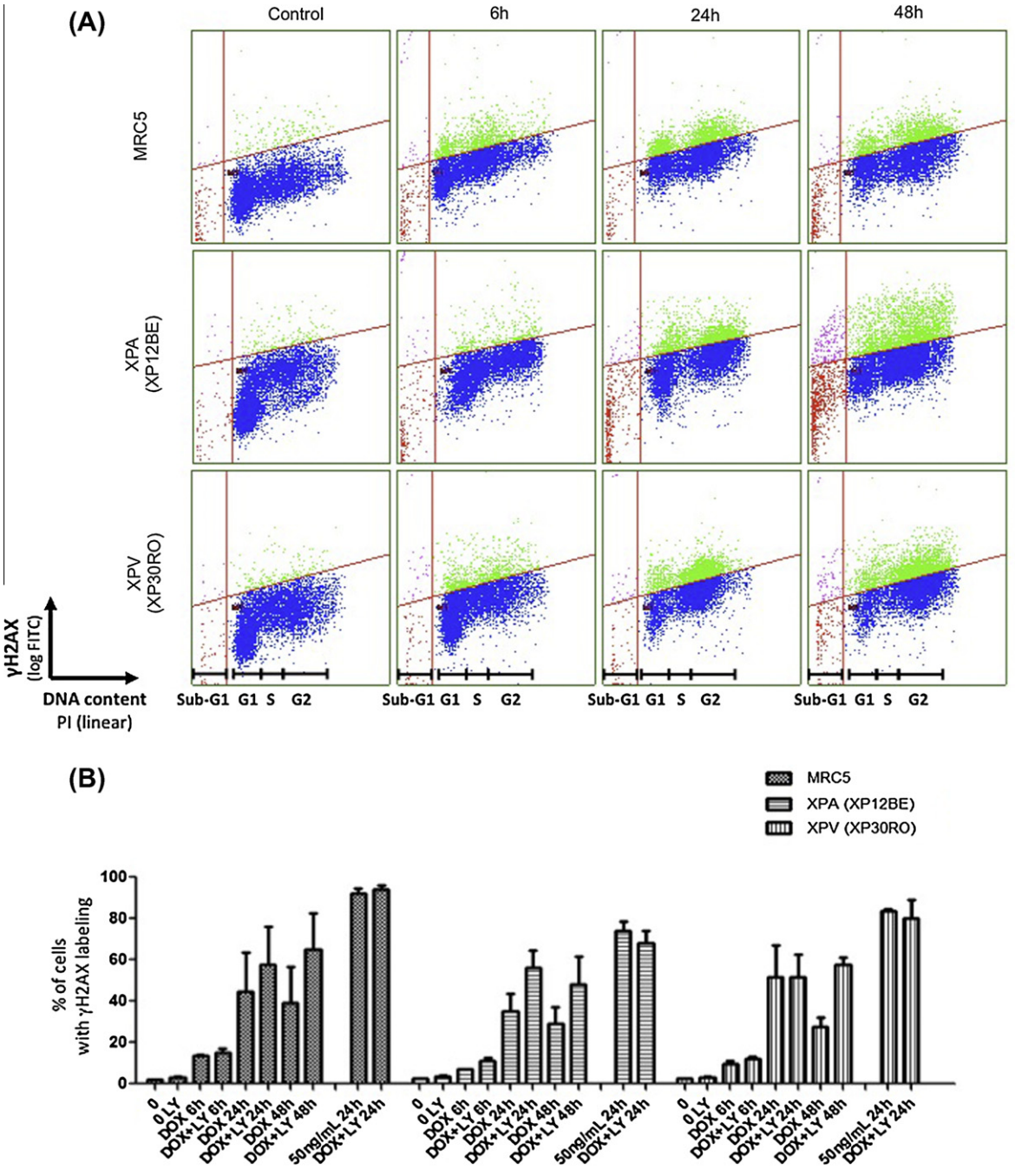

Fig. 7. Quantification, by immunodetection, of γH2AX after DOX-treatment. Cells were exposed to DOX (10 ng/mL) and/or 10 μM LY for the indicated periods of time (A), or to 50 ng/mL and/or LY for 24 h, as indicated. Cell cycle (PI) and γH2AX formation were detected by flow cytometry. Results obtained only with DOX (10 ng/mL) are illustrated in (A), where γH2AX formation is presented as a function of cell cycle phase (sub-G1, G1, S and G2 – indicated below the graphs). The quantification shown in (B) was performed only considering living cells (not considering sub-G1 cells), independently of the cell cycle phase.

topo-II inhibitor, other mechanisms of action have already been described, which relevance for specific clinical antitumoral effects remains controversial. DOX activity can generate many types of DNA damage, including DSBs, DNA cross-linking, DNA adducts and damage induced by free radical formation [1,25–30]. However, little is known about the mechanisms and proteins involved in their repair. Recently, Saffi and colleagues demonstrated that the helicase XPD, a member of the NER pathway, is involved in the repair of DOX-induced lesions, as XPD-deficient cells are more sensitive to treatments with DOX [7]. This is contrary to a previous report showing that rodent cells deficient

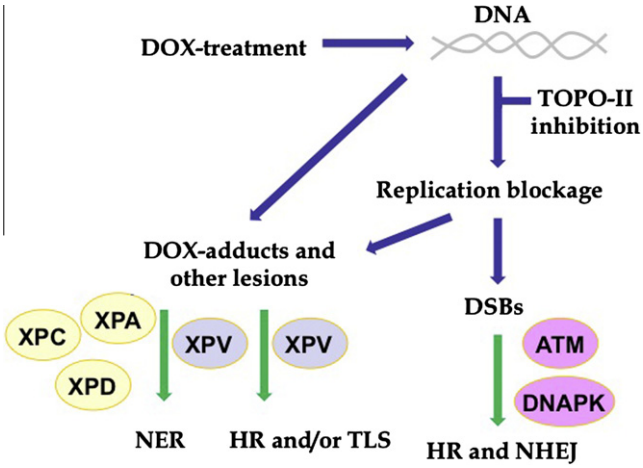

Fig. 8. Scheme indicating potential DNA repair pathways involved in DOX-induced DNA damage. Mechanisms for the induction of DNA damage by DOX are indicated by blue arrows, while DNA repair and damage response pathways are indicated in green arrows. The proteins represented within circles provide potential targets to potentiate the chemotherapy effects of DOX to induce tumor cell killing (only those discussed in the text are shown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in XPD, XPB or XPG, are more tolerant to DOX-induced DNA adducts [31]. The fact that, in the latter case, rodent cells (instead of human cells) were treated with DOX for only 4 h [31], might account for differences between the two reports. There is also evidence that XPA, XPC [6], DNA-PKcs [2] and ATM [27] are somehow involved in repairing these lesions. In order to better understand how DOX-induced DNA lesions are processed, the response of cells proficient and deficient in DNA repair to treatments with DOX was investigated.

Previously, DOX-induced DNA damage was identified through comet assays [6,7]. Herein, it is shown that both XPA and pol eta are necessary for the effective repair of DNA lesions induced by clinical doses of DOX, since cells deficient in these proteins are more sensitive to the cytotoxic effects of DOX treatment than MRC5 cells, which are proficient in DNA repair mechanisms (Figs. 1–3). On the other hand, the absence of the endonuclease XPF does not render cells more sensitive to clinical doses of DOX (Fig. 1). Since the importance of NER in the repair of DOX-induced lesions has been shown, it is possible that the residual NER activity normally observed in XPF cell lines is sufficient to avoid cell death [15], as compared to XPA cells that are >95% DNA repair deficient. In addition, cell cycle analysis demonstrated that DOX induces G2/M arrest in all the cell lines (Fig. 6). Whereas XPA is a member of the NER pathway, pol eta is traditionally considered to be a member of the TLS pathway, which bypasses unrepaired lesions by way of a series of error-prone translesion DNA polymerases [3,32]. Nevertheless, it has been shown that pol eta also participates in the process of homologous recombination (HR) [33,34]. As both processes, TLS and HR, act mainly in the S phase of the cell cycle, S phase arrest in XP30RO cells after treatment with DOX was expected. Instead, G2/M arrest was observed, with high levels of γH2AX-labeled cells. If this is so, pol eta-induced protection against DOX treatments would not involve HR, but a third undetermined pathway.

It is believed that pol eta could be necessary to allow late replication of sequences with specific structures that may be produced spontaneously or in the presence of DNA lesions, such as stalled replication forks, G4-tetraplex sequences [35] or fragile site sequences [36]. In fact, many DNA polymerases have either cooperative or redundant roles and participate in more than one cellular process. For example, Auclair and colleagues showed that functional DNA polymerase eta is important for GGR during the S phase [37], and other authors have also proposed that DNA polymerase eta might play a role in NER [38,39]. Whether this polymerase is required for the NER repair of DOX-induced DNA damage is an issue that requires further study. Preliminary work indicated that silencing of pol eta expression in XP-A cells (by shRNA knock down) do not change the levels of cell killing by DOX-treatment (Fig. 1S, Supplementary Data), indicating that pol eta and XPA could, in fact, operate in the same pathway. That would indicate that pol eta could be involved in the NER synthesis step. This, however, should be considered carefully, as these cells also did not had increased killing by UV-damage (Fig. 1S, C), and thus the reduction of XPV proteins in the cells could not be enough for an observable effect.

Finally, LY294002, an inhibitor of PI3Ks and PIKKs, was found to enhance DOX cytotoxic effects only in the DNA repair deficient cell lines, XP12BE and XP30RO (Figs. 4 and 5). Some of the kinases inhibited by LY, viz., ATM, ATR and DNA-PKcs, play important roles in DSB signaling and repair [21,22], and this is in agreement with the observation that the number of γHAX-labeled cells increases in DOX-treated cells. Together with the synergistic effect of LY in the cell killing of XP-A and XP-V cells, these results clearly indicate that these kinases signal and help to process DSBs, which are probably induced by the presence of lesions not repaired by NER or pol eta. The lack of specificity of LY, however, do not provide a direct information on the type of kinase(s) related to the repair of such lesions, when induced by DOX-treatment. The involvement of DNA-PKcs

in DSB repair by NHEJ, thereby implying the possible participation of this pathway in the repair of DOX-induced DSBs, is in accordance with previous work [2]. The activation of ATM in DOX-treated human lymphoblastoid cells have also been reported, indicating that this kinase is also involved in the cell responses triggered by this drug [27]. It is tempting to consider the possibility of a mechanistic link among these proteins, in a direct cross-talk between different repair pathways. Even though currently the existence of such a link is no more than speculation, it would be interesting to investigate this further.

Taken together, the results shed light onto how DOX-induced DNA damages are repaired. In Fig. 8, a model summarizing the data and identifying possible pathways involved in the processing of the different types of lesions induced by DOX is presented. This model facilitates the identification of the novel potential targets that can be clinically tested with DOX, so as to improve the outcome of chemotherapeutic treatment with this drug. Corroborating our model, recent data indicate that tumors from a BRCA-defective background, that should be partially deficient in recombination of DSB, appear to be more sensitive to pegylated liposomal doxorubicin [40]. Besides increasing the quality in the treatment received by thousands of patients worldwide, it is possible that in a not so distant future, the repair capacity of tumor cells will be addressed before the beginning of a treatment, and thus patients with impaired capacity will be more apt candidates for chemotherapy with DOX than patients with enhanced capacity.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2011.09.019.

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