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Mechanisms of enhancement of cytotoxicity in etoposide and ionising radiation-treated cells by the protein kinase inhibitor wortmannin

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Abstract

We have investigated the effects of the protein kinase inhibitor wortmannin (WM) on the cytotoxic mechanisms of etoposide and ionising radiation (IR) in the Chinese hamster ovary K1 (CHO-K1) cell line, and its radiation-sensitive derivative, xrs-6, which is defective in DNA-dependent protein kinase (DNA-PK) function. WM potentiated the cytotoxicity of etoposide and IR in CHO-K1 cells approximately 1.6 and 3-fold, respectively, and this potentiation was abolished in xrs-6 cells, which were themselves more sensitive to etoposide and IR alone. WM partially inhibited the repair of etoposide-induced DNA double-strand breaks. Etoposide treatment caused a biphasic inhibition of DNA synthesis in both cell lines, and this was abrogated by co-incubation with WM. These data suggest that WM inhibits in intact cells both DNA-PK and either or both the *ataxia telangiectasia* (*AT*) and *AT*-related gene products ATM and ATR.

Keywords: Wortmannin; DNA-dependent protein kinase; Etoposide; DNA repair

1. Introduction

Topoisomerase II (topo II) inhibitors are widely used in anticancer chemotherapeutic regimes. The cytotoxic and chemotherapeutic effects of these inhibitors reside in their ability to inhibit the enzyme action at an intermediate step following DNA scission, but before religation [1,2]. This intermediate is termed the cleavable complex, and consists of a topo II monomer which is bound covalently to the 5′ termini of a DNA double-strand break (DSB). Evidence that these potentially lethal DSBs are subject to DNA repair processes, independent of the topo II-mediated religation of the DSBs, comes from the observation that a mutant cell line (xrs-6), hypersensitive to ionising radiation (IR) and lacking a functional DNA repair enzyme, DNA-dependent protein kinase (DNA-PK), is also hypersensitive to topo II inhibitors [3,4]. DNA-PK is an enzyme, consisting of three subunits, that binds to and is activated by DSBs. Ku80, together with the Ku70 subunit, binds to DNA ends and recruits the catalytic subunit (DNA-PKcs) to the DNA, thus activating it (for reviews, see [5–7]). The xrs-6 cell line has a deletion mutation in the Ku80 subunit which destroys its ability to bind to DNA termini [8]. The xrs-6 cell line is defective in the rejoining of IR-induced DSBs [4,9–11], and has been demonstrated to be hypersensitive to a range of topo II inhibitors [12]. It is also partially defective in the rejoining of etoposide-induced DSBs [13], thus implicating DNA-PK-mediated DSB repair as a component of the cellular resistance mechanisms to topo II inhibitors.

The fungal metabolite wortmannin (WM) inhibits DSB repair and potentiates IR-induced cytotoxicity, and this correlates with an inhibition of DNA-PK [14–17]. DNA-PK activity extracted from cells pretreated with WM, at concentrations that potentiate ionising radiation cytotoxicity, is completely inhibited [16]. Prevention of DSB rejoining by WM has also been demonstrated in cell-free extracts, thus substantiating the direct effect of WM on DSB rejoining [18]. WM is not a specific inhibitor of DNA-PK, as it also inhibits phosphatidylinositol 3-kinase (PI 3-kinase, with which DNA-PK and

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the *ataxia telangiectasia* (*AT*) gene product, ATM, share homology [19]. In fact, it has recently been demonstrated that a kinase activity attributable to ATM is also inhibited by WM, both in cell extracts and in intact cells [20,21]. Like DNA-PK defective cell lines, AT cells are hypersensitive to IR, and an additional hallmark of AT cells is that they fail to undergo the ionising radiation-induced delay in S phase DNA synthesis, classically termed 'radioresistant DNA synthesis' [22].

Notwithstanding its range of known and potential enzyme targets, the use of WM has identified DNA-PK as a target for developing drugs that sensitise cells to IR and chemotherapeutic agents via an inhibition of DSB repair. The goal of this present study was to investigate the effect of WM on the cytotoxic mechanisms of etoposide and IR in CHO-K1 cells. Cell survival, DSB formation and repair and S phase DNA synthesis inhibition were selected as the endpoints for study. In order to delineate whether any effects of WM could be specifically attributed to an inhibition of DNA-PK, its effects were also studied in the xrs-6 cell line.

2. Materials and methods

2.1. Drugs

WM and etoposide were obtained from Sigma Chemical Co. (St Louis, MO, USA). They were dissolved in anhydrous DMSO at stock concentrations of 10 mM and stored at −20°C. The drugs were added to cell cultures at a final concentration of 1% DMSO, with appropriate solvent additions to control cultures.

2.2. Cell culture

The xrs-6 cell line was obtained from the European Collection of Cell Cultures, CAMR, Salisbury, UK. This cell line and the parental CHO-K1 cells were maintained as monolayers, and clonogenic survival assays were performed as previously described [15]. Briefly, cells were pre-incubated ± WM for 1 h, exposed to IR, or etoposide added, for a further 1 h, then the medium aspirated and replaced with medium containing WM alone. The plates were post-incubated for 16 h, then the cells were trypsinised and replated for colony formation in the absence of drugs. The data were averaged from at least three independent experiments ± standard error of the mean (SEM).

2.3. DNA strand break assays

DSB levels were assayed using the neutral filter elution technique [23]. Cells were prelabelled with [^{14}C] TdR (0.4 μCi/ml) for 24 h, followed by a 2 h chase in non-radioactive medium. Cells used as 'internal standards' were prelabelled with [^{3}H] TdR (1 μCi/ml), following the same time schedule described above. Internal standards were exposed to IR (100 Gy), and put immediately onto ice. The internal standards were loaded on to the same filters as the experimental samples, and eluted at pH 9.6. When used, WM was added to monolayers for a 1 h pre-incubation, and then the cells were treated with etoposide at the concentrations and for the times specified in the figure legends. DNA repair was followed by postincubating cells for the times specified in the figure legends.

To summarise the data obtained, the results were expressed using the 'relative elution' (RE) formula of Fornace and Little [24]. RE represents the amount of DNA from the drug-treated samples which was retained on the filter, as a ratio of the control (untreated). It is calculated using $(\log RR_{sample})-(\log RR_{control})$, where RR (relative retention) is the fraction of the sample DNA retained on the filter when 50% of the internal sample has eluted. RE data points represent the mean of at least four independently dosed samples derived from a minimum of two independent experiments ± SEM.

2.4. DNA synthesis inhibition

The effects of the drugs on DNA synthesis were assessed by measuring radiolabelled TdR incorporation into acid precipitable counts as previously described [25]. Briefly, cells prelabelled with [^{14}C] TdR were pretreated with WM (when used) for 1 h, then treated (in the continued presence of WM) with etoposide for a further 1 h. The medium was then aspirated and the cells incubated in drug-free medium for 30 min. [^{3}H] Tdr was added, and the cells were pulse-labelled for 10 min, then the plates were put on ice, the medium aspirated and the cells washed in ice-cold phosphate-buffered saline. The cells were then lysed and the amounts of trichloroacetic acid-insoluble radioactivity in the lysates determined. The levels of DNA synthesis in the etoposide-treated cells were expressed as a percentage of control (untreated). Because WM *per se* caused some inhibition of DNA synthesis (see Results), when WM was used in conjunction with etoposide, DNA synthesis levels were expressed as a percentage of control cells which were treated with WM alone. Note that the pulse-labelling with [^{3}H] Tdr was carried out in the absence of either drug. This was done to preclude as far as possible the possibility that the drug(s), in addition to inhibiting DNA synthesis, might inhibit the salvage of the radiolabelled TdR into dTTP, and hence its incorporation into DNA. Because WM acts irreversibly to inhibit PI 3-K by modifying a lysine residue in the active site, and evidence indicates that DNA-PK is also irreversibly inactivated, presumably by the same mechanism [16,26], its absence during the short time course of the experiment was not predicted to change

the inhibited status of DNA-PK. Data points represent the mean of two independently dosed samples from three separate experiments ± SEM.

3. Results

3.1. Clonogenic survival assays

WM treatment caused a transient growth inhibition of CHO-K1 cells; however, these cytostatic effects did not result in cytotoxicity, as even a 16 h exposure to WM at 50 μM did not cause a decrease in clonogenic survival (data not shown). The dose-dependent effects of etoposide ± WM on the clonogenic survival of the two cell lines were evaluated. WM 20 (μM) potentiated the cytotoxicity of etoposide in the CHO-K1 cell line, with the lethal dose at 10% survival (LD_{10}) value of 12.6 ± 1.1 for etoposide alone reduced to 7.5 ± 0.7 in the presence of WM (Fig. 1a). This potentiation of cytotoxicity was essentially abolished when WM was used with the xrs-6 cell line, which additionally demonstrated enhanced sensitivity to etoposide alone (LD_{10} value = 4.3 ± 0.2) compared with the CHO-K1 cells (see Fig. 1b). A similar response was obtained when the two cell lines were exposed to IR (Fig. 2). WM potentiated IR-induced cytotoxicity in CHO-K1 cells, reducing the LD_{10} value from 6.17 ± 0.25 to 2.01 ± 0.11 (Fig. 2a). The xrs-6 cell line was hypersensitive to IR alone (LD_{10} value = 0.78 ± 0.11), and no further potentiation of cytotoxicity was obtained in the presence of WM (Fig. 2b).

3.2. DSB assays

We have previously demonstrated an almost complete inhibition of DSB repair by WM in IR-treated cells [15]. Therefore, in this study we focused on the effects of etoposide on DSB formation and repair. A 1 h exposure of CHO-K1 cells to etoposide resulted in a dose-dependent increase in DSB levels, and these levels were increased at all doses by a co-incubation with 50 μM WM (Fig. 3a). For example, 5 μM etoposide alone gave a RE value of 0.165 ± 0.023; in the presence of WM, this value was increased to 0.274 ± 0.013. In the second experiment, cells were treated with etoposide (20 μM) in the presence or absence of WM for 1 h. The drugs were then washed out, WM was added back to the monolayers that had contained it before, and the cells post-incubated for 4 h. DSB levels were assayed at various time points, and the results are presented in Fig. 3b. Upon removal of the etoposide, DSB rejoining rapidly occurred, such that by 3 h the majority of DSBs had resealed. In contrast, in the presence of WM (50 μM), increased DSBs were observed at all time points, including the 1 h time point at which the etoposide was removed, but the increased levels were most marked at 3 and 4 h, indicating the persistence of a subset of unrepaired lesions.

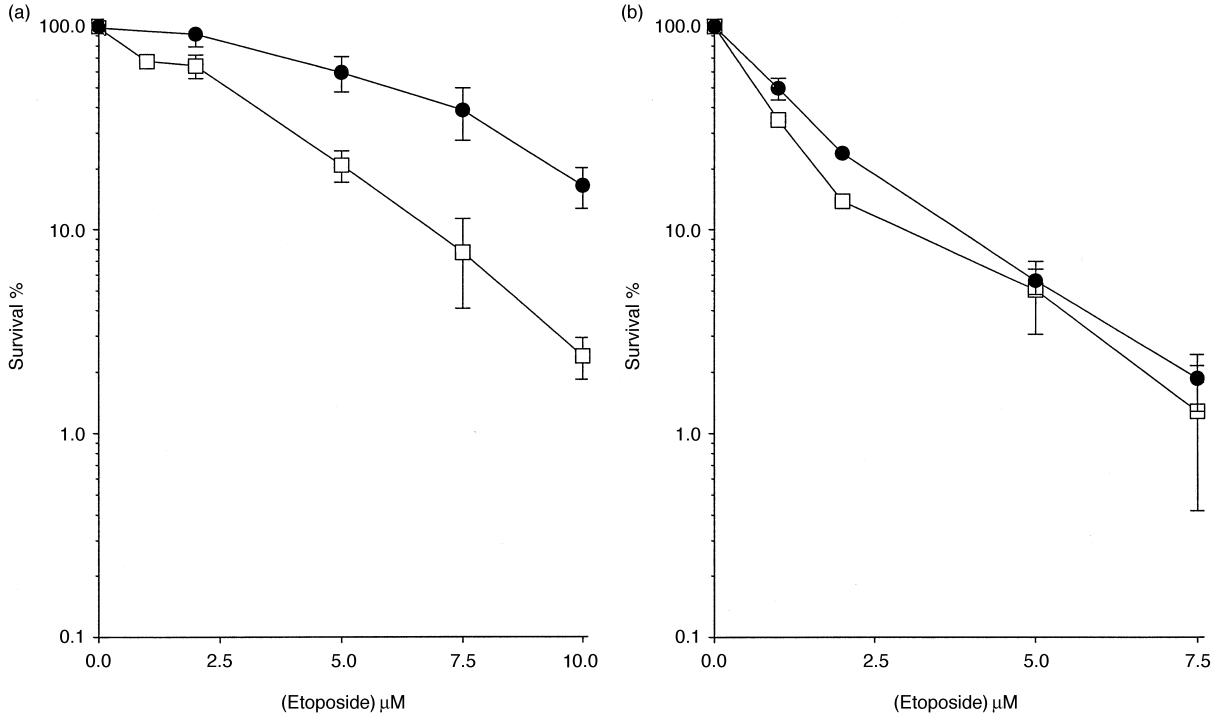

Fig. 1. Effects of exposure to increasing concentrations of etoposide ± wortmannin (WM) on the clonogenic survival of: (a) CHO-K1 cells; (b) xrs-6 cells; ● control; □ + WM (20 μM).

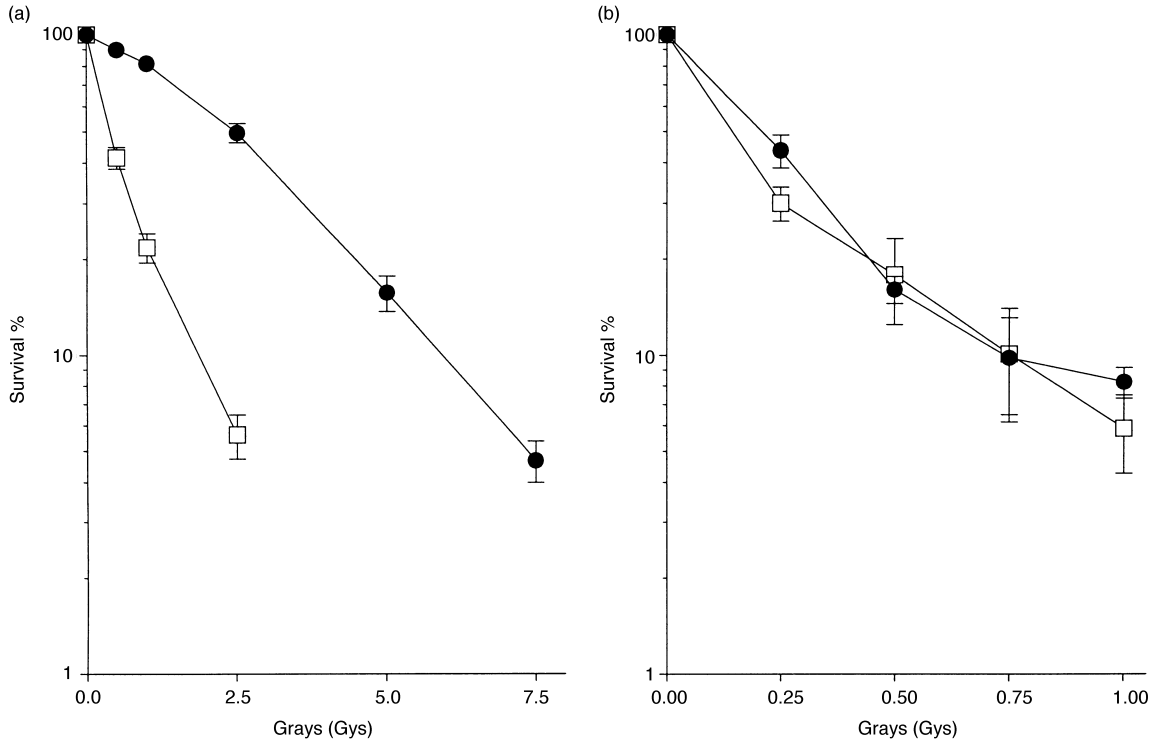

Fig. 2. Effects of exposure to increasing doses of ionising radiation (IR) ± wortmannin (WM) on clonogenic survival of: (a) CHO-K1 cells; (b) xrs-6 cells; ● control; □ + WM (20 μM).

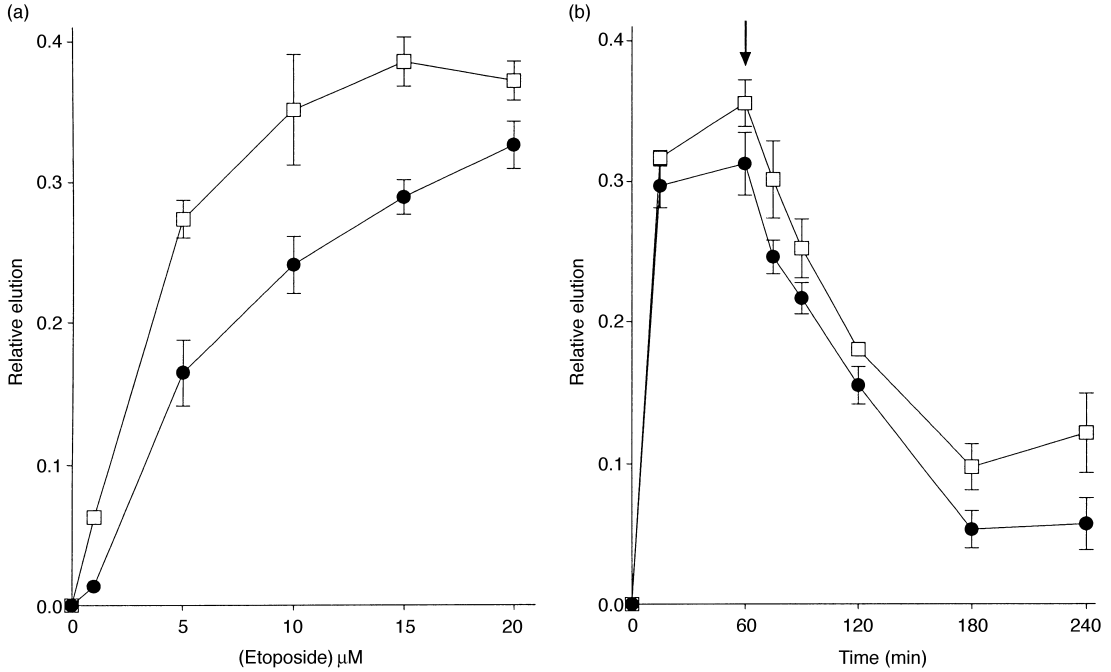

Fig. 3. (a) Concentration-dependent effects of etoposide ± wortmannin (WM) on DNA double-strand breaks (DSB) levels. CHO-K1 cells were treated with increasing concentrations of etoposide ± WM (50 μM) for 1 h, then harvested for neutral elution. ● control; □ + WM. (b) Kinetics of DSB formation and rejoining in cells treated with a fixed concentration of etoposide. Cells were pretreated ± WM (50 μM) for 1 h, then etoposide (20 μM) added for a further hour. The medium was aspirated and replaced with either medium alone or medium + WM. Incubation was continued for 3 h. Samples were removed at intervals and harvested for neutral elution. ● control; □ + WM. The arrow indicates the time of removal of etoposide.

3.3. DNA synthesis inhibition

Exposure to WM (50 μM) alone caused an approximately 46% inhibition of DNA synthesis, as measured by incorporation of [^{3}H] TdR into acid-precipitable counts. Whether this was caused by a direct inhibitory effect of WM on S phase DNA synthesis, or a consequence of the cell cycle blockade resulting from PI 3-K inhibition, cannot be determined. DNA synthetic rates were assessed following a 1 h treatment with etoposide. In CHO-K1 cells, the rate of DNA synthesis decreased with increasing etoposide concentration (Fig. 4a), demonstrating a typical biphasic response. When cells were treated with etoposide + WM, the etoposide-induced inhibition of DNA synthesis was almost completely abolished (Fig. 4a) (note that in this case DNA synthesis levels were expressed as a percentage of control cells which were treated with WM alone). When the same experiment was performed using xrs-6 cells, the etoposide-induced DNA synthesis inhibition also occurred (Fig. 4b), and the addition of WM caused a similar abrogation of the inhibition of DNA synthesis as was observed in the CHO-K1 cells (compare Fig. 4a and b). Identical results were obtained when IR was used as the DNA damaging agent (data not shown).

4. Discussion

We, and others, have previously reported on the ability of WM to potentiate IR-induced cytotoxicity, and provided evidence (correlation of potentiation of cytotoxicity and inhibition of DSB repair with inhibition of DNA-PK) to support the hypothesis that its effects are mediated via an inhibition of DNA-PK [14–18]. The results presented here show that WM potentiates the cytotoxicity of both IR and etoposide in CHO-K1 cells, and that these potentiating effects are lost in the xrs-6 cell line. These data contrast with the results of Rosenzweig and colleagues [16], who obtained a 2-fold potentiation of IR cytotoxicity by WM in the scSV3 scid cell line (also defective in DNA-PK function), and suggested that AT could be the main target of WM. However, they did not measure DNA-PK activity in the scid cell line, and it has been recently demonstrated that certain scid cell lines have as much as 15% residual DNA-PK activity, and that this activity sufficed to activate p53 binding to DNA, whereas a cell line devoid of detectable DNA-PK activity showed no p53 binding in response to DNA damage [27]. The xrs-6 cell line used here has a defined deletion mutation in the Ku80 subunit, which abolishes its ability to bind to DNA [8]; hence it is highly unlikely that it has any residual DNA-PK activity. These data thus add further weight to the hypothesis that WM exerts at least its potentiating effects via an inhibition of DNA-PK, and are also consistent with previous observations that xrs cell lines are hypersensitive to topo II inhibitors [3,12].

Jin and colleagues [28] have also reported that Ku70- and Ku80-deficient cell lines are hypersensitive to etoposide, and that these cells can be complemented to an etoposide-resistant phenotype by transfection of Ku70

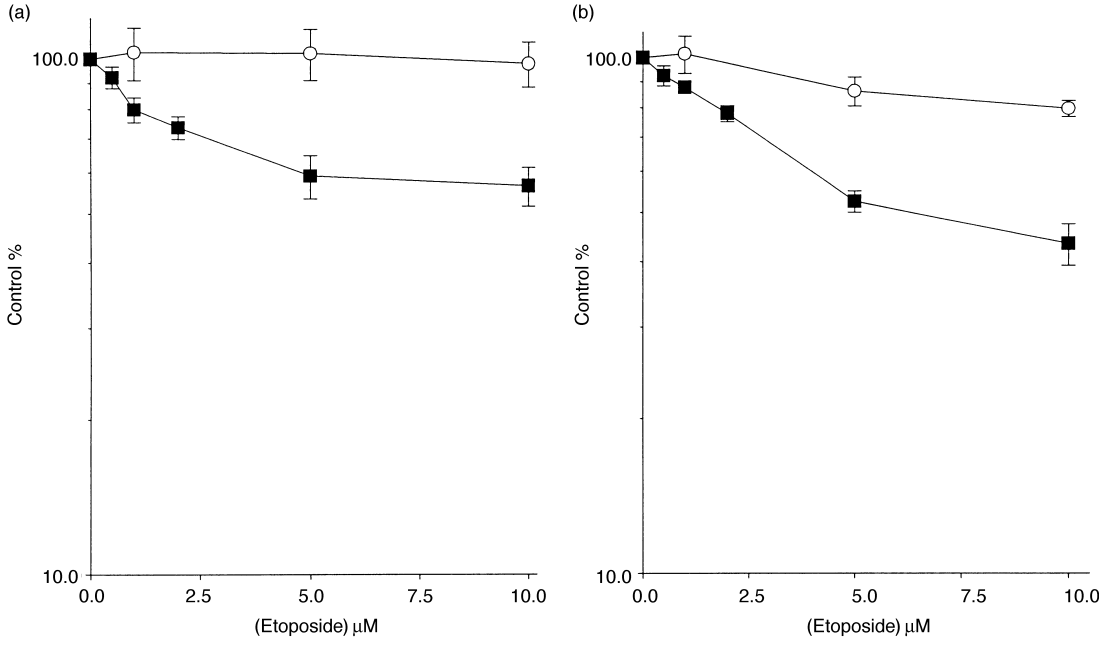

Fig. 4. Effects of wortmannin (WM) on etoposide-induced DNA synthesis inhibition. Cells were treated with increasing concentrations of etoposide ± WM pulse labelled for 10 min with [^{3}H] TdR, and then harvested and prepared for estimation of TCA-precipitable counts per min (cpm) incorporated. (a) CHO-K1 cells, ■ control; ○ + WM (20 μM). (b) xrs-6 cells, ■ control; ○ + WM (20 μM).

or Ku80 DNA, respectively. Interestingly, cell lines defective in DNA-PKcs were not hypersensitive to etoposide, suggesting that it is the Ku heterodimer, but not the DNA-PK holoenzyme that is critical for etoposide-induced DNA repair. However, although WM specifically inactivates DNA-PKcs in the phosphotransferase domain [26], without interacting with Ku70 or Ku80, it inhibits DSB repair in etoposide treated cells (see below).

Unlike our earlier results, which demonstrated an almost complete inhibition of DSB rejoining by WM in irradiated cells [15], it is apparent that WM exerted only a partial effect on DSB levels in etoposide-treated cells. Caldecott and colleagues [13] demonstrated that following removal of etoposide, the loss of protein/DNA crosslinks paralleled DSB rejoining, consistent with a direct reversal of the cleavable complex leading to topo II-mediated religation. The same authors also demonstrated in xrs-6 cells that the extent of accumulation and the rate of loss of etoposide-induced cleavable complexes were the same as in CHO-K1 cells. However, a proportion of the DSBs remained unrepaired at later times, and this was attributed to the DSB repair defect mediated by the xrs phenotype. Very similar effects on DSB levels were observed here using WM in CHO-K1 cells, again suggesting that DNA-PK-mediated DSB repair is required for a subset of the DSBs formed, and is the target inactivated both by the xrs mutation and WM. However, it should be pointed out that the neutral elution technique utilised to assay DSB levels includes SDS and proteinase K, which denature and remove the topo II from the DNA. Therefore, it does not differentiate between topo II-bridged DSBs stabilised by the action of etoposide (i.e. the cleavable complexes), and DSBs arising by e.g. the loss of topo II from the break before completion of its enzymatic function (i.e. the religation of the DSBs). The increased break levels observed at all time points in the presence of WM could therefore also be ascribed to an increased rate of formation of, or stability of, the cleavable complex. This could occur by modulation of topo II activity (e.g. by inhibition of phosphorylation of this enzyme by DNA-PK due to WM inactivation), and could account for the early increased accumulation of DSBs observed in the presence of WM during the 1 h exposure to etoposide.

Jeggo [29] showed that the xrs mutants (including xrs-6) exhibited normal DNA synthesis inhibition following irradiation. We have demonstrated here that etoposide treatment also induced DNA synthesis inhibition, and that this occurred similarly in the CHO-K1 and xrs-6 cell lines. WM abrogated this etoposide-induced immediate early DNA synthesis arrest in both cell lines. The persistence of the abrogating effect of WM on DNA synthesis inhibition in the absence of functional DNA-PK indicates that WM has other cellular targets involved in the cellular responses to DNA damage, e.g. ATM which is known to mediate the DNA synthesis inhibition [22]. Inhibition of ATM by WM would result in the loss of the intra-S phase checkpoint. Consistent with our observations and interpretation, recent evidence has demonstrated that both DNA-PK and ATM activity are inhibited by 50% in intact human A549 cells by 3.6 and 5.8 μM WM, respectively, and that 30 μM WM prevented IR-induced DNA synthesis inhibition [21]. Alternatively, WM may inhibit ATR, another recently discovered PI 3-K-related protein which also contributes to the regulation of the S phase checkpoint [30].

The dual effects of WM on etoposide-induced DSB formation and repair and the S phase checkpoint could both potentially contribute to the enhancement of cytotoxicity, since both DNA-PK defective and AT cell lines are hypersensitive to IR and topo II inhibitors [12,31], although at least in some AT cell lines, sensitivity to topo II inhibitors can be ascribed to differences in levels of topo II activity [32]. However, our results in the xrs-6 cell line, demonstrating that WM caused a complete loss of the S phase DNA synthesis arrest, but without a concomittant potentiation of cytotoxicity, suggest that the loss of the intra-S phase checkpoint regulated by ATM does not function to decrease survival of cells following DNA damage. This interpretation of our data concurs with the evidence mooted by Jeggo and associates [33] to support their hypothesis that the pleiotropic features of AT might be attributed to dual and separable functions of the *AT* gene product in DNA repair, which is responsible for the radiation sensitivity, and in cell cycle checkpoints, which contribute to maintenance of genomic stability. Furthermore, since both DNA-PK and ATM are inhibited by WM [19,20], the lack of additional potentiation of cytotoxicity by WM in the xrs-6 cells also suggests either that both these enzymes may function in a convergent downstream pathway that promotes cell survival following DNA damage, or that inhibition of the kinase activity of ATM modulates only the checkpoint function, but not the DNA repair and survival function. We favour the second interpretation as other research has demonstrated that WM potentiates IR cytotoxicity in AT cell lines [16].

The pleiotropic targets of WM in cellular DNA damage-inducible responses could elicit useful responses in chemo- and radiotherapy. For example, WM could circumvent, at least in part, the mechanisms, based on reductions in levels of intracellular drug accumulation or decreased levels of topo II enzyme, that give rise to tumour cell resistance to topo II inhibitors [1]. The effect of WM on other topo II inhibitors merits further investigation.

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