Wortmannin is a potent inhibitor of DNA double strand break but not single strand break repair in Chinese hamster ovary cells

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Wortmannin, an inhibitor of p110 PI 3-kinase, also inhibits DNA-dependent protein kinase, which is known to mediate DNA double strand break repair. It was recently demonstrated that wortmannin sensitized cells to ionizing radiation (IR) (Price and Youmell, Cancer Res., 56, 246-250, 1996). Wortmannin was used to determine if the potentiation of IR-induced cytotoxicity in Chinese hamster ovary cells could be accounted for by an inhibition of DNA double strand break (DSB) repair. Wortmannin, at concentrations which were non-toxic per se (5 and 20 μM), increased IR cytotoxicity with dose enhancement factors at 10% survival of 2.7±0.28 (5 μM) and 5.3±0.86 (20 μM). The effects of wortmannin on DSB levels were assessed by neutral elution. The effects of wortmannin on the kinetics of DSB repair were evaluated over a 3 h time course. Wortmannin (50 μM) completely inhibited DSB repair over this period, without having any effect on DSB levels itself. The concentration-dependent effects of wortmannin on DSB levels showed that inhibition of DSB repair was significant at 1 μM, and near-maximal at 20 μM. In marked contrast, it exerted no effect on the kinetics of single strand break (SSB) repair as assessed by alkaline elution, even at concentrations as high as 50 μM. There was an excellent correlation between the concentration-dependence and exposure time of wortmannin required to enhance IR cytotoxicity and inhibit DSB repair. These data implicate inhibition of DNA-dependent protein kinase, and the consequent inhibition of DSB repair, as the mechanism whereby wortmannin potentiates the cytotoxicity of IR.

Introduction

It is well established that DNA repair processes contribute largely to the intrinsic resistance of tumour cells to anticancer drugs and ionizing radiation (IR*). The use of DNA repair inhibitors, in conjunction with drugs that damage DNA, is currently being evaluated as a potential chemotherapeutic strategy. For example, potent inhibitors of poly(ADP-ribose) polymerase have been developed which inhibit base excision repair (BER) and potentiate the cytotoxicity of alkylating agents such as temozolomide (1,2). O6-methylguanine methyl-transferase removes the O6-methyl group from guanine in cells treated with alkylating agents. Inhibition of this activity by O6-benzylguanine sensitizes cells to chemotherapeutic alkylating agents which produce this lesion (3). However, these enzyme targets have a limited range of DNA-reactive anticancer drugs with which they can be used, and poly(ADP-ribose) polymerase inhibitors demonstrate only very modest responses with IR (4). The identification of DNA repair inhibitors which selectively target double strand break(s) (DSB) repair processes would considerably widen the repertoire of anticancer drugs with which such compounds could be used.

The molecular mechanisms of DSB repair in mammalian cells have not been fully elucidated. However, a new enzyme implicated specifically in the repair of DSB has recently been identified. The double strand DNA-dependent protein kinase (DNA-PK) was identified as a member of the phosphatidylinositol-3-kinase (PI 3-K) family, by virtue of its high sequence homology with p110 PI 3-K in the catalytic domain of the protein (5). DNA-PK consists of a large catalytic subunit of 350 kDa, which is inactive on its own. However, when bound to the autoimmune antigen, Ku, which comprises a heterodimer of two polypeptides of 70 and 86 kDa, it can bind specifically to DSB in the DNA, and is thereby activated (6). Although a number of protein substrates for this enzyme, including p53 (7), have been identified in cell extracts, its in vivo acceptors are not known. Analyses of X-ray sensitive cell lines, and of cell lines derived from mice with severe combined immune deficiency (which are unable to carry out V(DJ) recombination), have revealed that they are defective in the Ku86 and p350 subunits respectively (8,9). Both of these cell lines have established defects in DSB repair, but are normal with respect to single strand break(s) (SSB) repair (10,11).

p110 PI 3-K is a key enzyme involved in transducing growth factor responses mediated by tyrosine kinases (12,13). A powerful tool in the understanding of the biological function of PI 3-K has been the fungal metabolite, wortmannin, that was initially identified as an inhibitor of myosin light chain kinase (14). Recently, wortmannin was shown to be a more potent inhibitor of PI 3-K (15). It is non-competitive and irreversible in action, with an IC50 in vitro of 2–4 nM. DNA-PK is also inhibited by wortmannin in vitro, but 500 nM is required to completely abolish its activity (5).

Yet another kinase, the AT gene product, defects which sensitize cells to IR and abolish the increase in p53 protein levels, shares sequence homology with PI 3-K (16). This suggested another possible cellular target for wortmannin. Price and Youmell (17) demonstrated that wortmannin increased the radiosensitivity of cells and inhibited the induction of p53 DNA-binding activity by IR or actinomycin D, and also inhibited the transcriptional activation of a p53 CAT reporter gene by actinomycin D. However, the enhancement of IR-induced cytotoxicity that was obtained by wortmannin proved independent of these effects, since it occurred in cell lines
which did not possess functional wtp53 as well as cell lines which did. Thus, they were unable to identify conclusively the cellular target of wortmannin which resulted in radiosensitization.

In the body of work described here, we have determined the effects of wortmannin on DSB and SSB repair as well as survival in IR-treated cells. The results demonstrate an excellent correlation between inhibition of DSB repair by wortmannin and its effects on enhancement of IR-induced cytotoxicity.

Materials and methods

Drugs
Wortmannin was obtained from Sigma Chemical Co. (St. Louis, MO). It was dissolved in DMEM at a stock concentration of 10 mM, stored at −20°C, and added to cell cultures at a final concentration of ≤ 1% DMEM, with appropriate solvent additions to control cultures.

Cell culture
CHO-K1 cells were maintained as monolayers as previously described (18), except that the culture medium was RPMI 1640 medium (supplemented with 10% serum, glutamine and antibiotics). HEPES and sodium bicarbonate were added at final concentrations of 18 and 11 mM respectively. Clonogenic assays were performed as previously described (18). Briefly, cells were pre-incubated ± wortmannin for 1 h, exposed as monolayers to IR, and then post-incubated at 37°C for the times stated in the continued presence of wortmannin, when used. Cells were then trypsinized and replated for survivors in the absence of wortmannin. The details of the IR doses, wortmannin concentrations, and times of exposure are given in the figure legends. The data are averaged from at least three independent experiments ± standard error (SE).

DNA strand break assays
SSBs were assessed using the technique of Kohn et al. (19), and DSB levels according to Bradley and Kohn (20). Cells were prelabelled with [3H]dThD (0.4 μCi/ml) for 24 h, followed by a 2 h chase in radio-inactive medium. Cells were trypsinized, centrifuged and resuspended in medium in bijou flasks. When used, wortmannin was added for a 1 h pre-incubation, and then the cell suspensions were exposed to IR. IR exposure was carried out at room temperature. DNA repair was followed by post-incubating the cell suspensions at 37°C following IR. Cells used as 'internal standards' (19), were prelabelled with [3H]dThD (1 μCi/ml), following the same time schedule described above. Internal standards for alkaline elution experiments were exposed to 3 Gy, and those for neutral elution were exposed to 100 Gy, and put immediately on ice. The internal standards were loaded on to the same filters as the experimental samples, and eluted at pH 12.2 (alkaline elution) or pH 9.6 (neutral elution). Note that wortmannin effects on either DSB or SSB levels cannot cross-interfere with either assay because SSB are not detectable by neutral elution, and DSB are a minor component of IR-induced strand breaks (compare IR assay doses).

To summarize the data obtained, the results were expressed using the 'relative elution' (RE) formula of Fomace and Little (21). RE represents the amount of DNA from the treated samples retained on the filter as a ratio of the control (untreated). It is calculated using (log RRsignal) − (log RRonpated), where RR (relative retention) is the fraction of the sample DNA retained on the filter when 50% of the internal sample has eluted. In certain experiments, as described in the figure legends and Results section, the control sample is defined differently, either being the unirradiated sample, or the sample treated with IR in the absence of wortmannin. RE data points represent the mean of at least four independently dosed samples derived from a minimum of two independent experiments ± SE. Where shown, elution profiles are calculated from the data of a single, representative experiment.

Results

Clonogenic survival assays
The dose-dependent effect of IR ± wortmannin was evaluated. CHO-K1 cells were pre-exposed to wortmannin for 1 h, and then exposed to increasing doses of IR. Following a 16 h post-incubation in the continued presence of wortmannin, cells were trypsinized and plated for survivors. Consistent with previous results using NIH-3T3 cells (17), inclusion of wortmannin in the culture medium, at concentrations which were non-toxic per se (5 and 20 μM), resulted in a synergistic enhancement of IR-induced cytotoxicity (Fig. 1a). The DEFₙ values were calculated using the Hill equation of GraphPad Inplot (San Diego, CA, USA software) and were derived from four independent experiments. DEFₙ values of 2.7 ± 0.3 and 5.3 ± 0.9 were obtained for 5 μM and 20 μM wortmannin respectively.

The cytotoxicity of increasing concentrations of wortmannin treatment per se, and in conjunction with a fixed dose of IR (2.5 Gy), over a 16 h exposure was then assessed. Figure 1b shows that during this time period there was no significant decrease in clonogenic survival when cells were exposed to wortmannin alone, even at concentrations as high as 50 μM. 2.5 Gy itself reduced survival to 42%, normalized to 100% in the figure. Inclusion of wortmannin caused a rapid dose-dependent enhancement of cytotoxicity, such that by 10 μM, survival had dropped to 10%.

Finally, experiments were carried out to determine the length of exposure time to wortmannin required to obtain maximum potentiation of IR-induced cytotoxicity. Briefly, cells were irradiated in the presence or absence of a fixed concentration of wortmannin (20 μM), and post-incubated for 24 h before plating out for survivors. At different times during this period, either the wortmannin-containing medium was removed and replaced with drug-free medium, or in the obverse experiment, wortmannin was added to the medium either immediately before IR, or at successively later times. The experiments were thus designed to allow for the fact that wortmannin acts as an irreversible inhibitor, and so altering the removal time schedule may have no effect if the binding of the wortmannin to the enzyme is rapid. In the obverse experiment, where wortmannin is added at different times, the irreversible action of the inhibitor will not pose a problem. The results are shown in Figure 1c. It can be seen that by the end of a 1 h post-irradiation period, the potentiation of cytotoxicity was near maximal in the drug removal experiment. However, in the drug addition experiment, although the increased cell killing was rapidly lost in the first 2 h, the time of addition of wortmannin had to be delayed for at least 4 h to regain >80% survival. These data are consistent with the irreversible action of wortmannin on DNA-PK, resulting in a complete response at 1 h in the drug removal experiment, but portraying its true schedule-dependency in the drug addition experiment. The >4 h time of exposure to wortmannin required to potentiate cytotoxicity reflects the time course of DSB repair (see below).

DNA strand break assays

The kinetics of DSB repair was assessed over a 3 h period following exposure of cells to 100 Gy ± wortmannin (50 μM), and the results are shown in Figure 2a. Repair of DSB followed biphasic kinetics, with ~63% of the DSB rejoined within the first 30 min. DSB rejoining continued slowly, with ~85% repaired by 3 h. In marked contrast, there was no significant change in DSB levels over the 3 h period in the presence of 50 μM wortmannin, although this concentration of wortmannin per se had no effect on DSB levels (results not shown).

The dose-dependent effects of wortmannin on DSB levels was assessed 2 h post-irradiation (100 Gy). The neutral elution profiles are presented in Figure 2b. Immediately following IR, the DNA eluted very rapidly, but after 2 h the DNA eluted only slightly more rapidly than the control DNA, indicating that substantial rejoicing of DSB had occurred. Between 1 and
Wortmannin is a potent inhibitor of DNA.

Fig. 1. (a) The effect of increasing doses of IR in the presence or absence of fixed concentrations of wortmannin. Monolayers were preincubated for 1 h ± wortmannin, irradiated and post-incubated for 16 h before plating out in the absence of drug for survivors. (●), control; (□), + 5 μM wortmannin; (▲) + 20 μM wortmannin. (b) The effect of a 16 h exposure of cells to increasing concentrations of wortmannin in the presence (▲) or absence (●) of a fixed dose of IR (2.5 Gy). (c) The effect of varying the exposure time to a fixed concentration (20 μM) of wortmannin following IR. Monolayers were pre-incubated in the presence or absence of wortmannin for 1 h, irradiated with 2.5 Gy, then post-incubated for 24 h before trypsinizing and plating out for survivors. The post-irradiation exposure time to wortmannin was varied either by: (1) aspirating the medium off at different times, and replacing with drug-free medium (●), or (2) adding wortmannin to the medium at different times (▲).

Fig. 2. (a) The effect of 100 Gy ± 50 μM wortmannin on DSB levels assessed by neutral elution over a 3 h time course. RE values have been plotted against time. (●), IR alone; (□), IR + wortmannin. (b) The effect of increasing concentrations of wortmannin on DSB levels as assessed by neutral elution. Cell suspensions were preincubated for 1 h ± wortmannin, then exposed to 100 Gy in the continued presence or absence of wortmannin, and post-incubated for 2 h before harvesting cells for neutral elution. (●), control (untreated); (O), wortmannin alone (20 μM); (■) IR alone, no post-incubation; (▲) IR alone; (□) IR + 1 μM wortmannin; (▲) IR + 2 μM wortmannin; (△), IR + 5 μM wortmannin; (★), IR + 10 μM wortmannin.

10 μM wortmannin there was a dose-dependent increase in the rates of elution, with a significant increase observed already by 1 μM.

Alkaline elution experiments were carried out to assess the effect of wortmannin on SSB repair. Cells were irradiated with 6 Gy and post-incubated for 15 or 30 min. The results are shown in Figure 3. In the absence of wortmannin, it can be seen that SSB levels decreased rapidly over the first 30 min. When 50 μM wortmannin was present, the elution profiles were identical, indicating that wortmannin has no effect whatsoever on SSB repair.

Correlation between wortmannin effects on survival and DSB levels

The dose-dependent ability of wortmannin per se to cause cytotoxicity, to potentiate IR-induced cytotoxicity, and to increase DSB levels by inhibiting repair was compared (in both latter cases, using a single fixed dose of IR: see figure legend). Figure 4 shows the RE value and % increase in cell kill as the two Y axes plotted against increasing wortmannin concentration. It can be seen that the RE and the wortmannin + IR curves almost superimpose over the dose range 0–10 μM, suggesting that wortmannin is affecting the
In cell culture, wortmannin can elicit complete responses in signal transduction pathways involved, for example, in differentiation or insulin-induced glucose transport mediated by PI 3-K at concentrations of $\leq 100$ nM (22,23).

At least two other possible PI 3-kinase-like activities involved in the cellular response to IR may be modulated by wortmannin. IR activates a stress activated protein kinase (p54 SAP kinase). p54 SAP kinase forms a complex with PI 3-kinase and an adaptor protein termed Grb2 (growth factor binding protein) in irradiated cells (24). Wortmannin (250 nM) stimulates p54 SAP kinase, suggesting that PI 3-kinase regulates this stress-activated kinase. Also, the AT gene product, another PI 3-kinase-like protein involved in the cellular response to IR, has been identified as a putative substrate for wortmannin (5). AT cells are extremely sensitive to IR and deficient in the p53 DNA damage-response pathway (25,26).

Notwithstanding the several possible targets for wortmannin, the data presented here are consistent with a direct cause–effect relationship of wortmannin in inhibiting DSB repair and hence enhancing IR-induced cytotoxicity. We have demonstrated that wortmannin caused a dose-dependent inhibition of DSB repair in cells exposed to IR between 1–20 μM. Moreover, there was an excellent correspondence between the concentrations required to inhibit DSB repair and enhance cytotoxicity. It should be pointed out that the intracellular concentrations of wortmannin achieved are not known, and there is good evidence that it loses activity after about 3 h in culture medium (22). The 10–50 fold higher concentrations of wortmannin required to produce effects in our experiments in vivo, compared to concentrations required to inhibit DNA-PK in vitro, are very similar to the in vivo/in vitro concentration range differences observed when PI 3-kinase is the assumed target for wortmannin (see above).

p53 is an allosterically regulated protein with latent DNA-binding activity. A large body of evidence implicates phosphorylation by protein kinase(s) as a prerequisite for activating the sequence-specific DNA-binding function of p53 (27). DNA-PK phosphorylates p53 in vitro (7), and therefore wortmannin could act to inhibit the function of p53 by inhibition of DNA-PK in intact cells, as was observed by Price and Younell (17). However, their data indicated that the radiosensitizing effect of wortmannin was independent of the existence of functional p53. Evidence indicates that the CHO-K1 cell line used in this paper contains mutated or functionally inactivated p53 (28). Huang et al. (29) have demonstrated that the p53-induced arrest mechanism in cells can be activated by very few DSB, and that only one may be sufficient. DNA-PK is thus a prime candidate for an upstream monitor of DSB repair in intact cells of defined p53 status.

There are additional observations which support the contention that wortmannin is targeting DSB repair to increase cell kill. Firstly, the concentration ranges and times of exposure of wortmannin (5–20 μM) used in the survival experiments were completely non-toxic per se. Secondly, although DSB repair was severely inhibited e.g. at 20 μM wortmannin, SSB repair was completely unaffected, even at concentrations as high as 50 μM. These data rule out any general metabolic perturbations as contributing to its effects. Thirdly, there was a good correspondence between the time interval of exposure to wortmannin required to maximize cell kill (the first 4 h) and
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the time required to complete the majority of DSB rejoining (at least 3 h).

IR damage, under oxic conditions as performed here, results in a variety of free-radical induced lesions repaired mainly by the base excision repair (BER) pathway (e.g. thymine glycols, 8-oxoguanine residues) (30). IR-induced DSB form a small component of these lesions, but are widely considered to be important contributors to the cytotoxicity. For example, the xrs mutants of CHO cells, which are highly radiosensitive, are specifically defective in DSB repair (10). Note, however, that the radiosensitive irs mutants in the Chinese hamster cell line V79-4 are normal with respect to DSB and SSB repair (31), as are AT cells, indicating that there are other cellular responses to IR that mediate resistance. The totally exclusive inhibition by wortmannin of DSB repair, but not BER, in our experiments, and the enhanced radiosensitivity, mimics exactly the xrs phenotype, known now to be due to a defect in DNA-PK activity (8).

Núñez et al. (32) demonstrated an excellent correlation between the radiosensitivity of different cell lines and the half-life of the fast rejoicing component of DSB repair. The biphasic kinetics of DSB repair include a fast component which is complete within about 30 min, and a slower component which takes several hours (32). Our data indicate that wortmannin completely inhibits the fast repair observed over a 30 min time period. Because of the low levels of DSB repair involved in the slow component, it is not possible to conclude from our data whether wortmannin also inhibits the slow repair.

In conclusion, we cannot completely exclude the possibility that wortmannin may modulate two completely independent processes (viz: DSB repair as well as cell survival), and also target different kinases. However, we deem it highly unlikely that such effective and specific inhibition of IR-induced DSB repair would be unrelated both to an inhibition of DNA-PK, and to the observed enhancement of IR-induced cell killing, based on the arguments stated above.

These findings point to new possibilities for the development of drugs to improve the effectiveness of several major anticancer drugs by overcoming intrinsic or acquired drug resistance, in an analogous manner to the application of poly(ADP-ribose) polymerase inhibitors which retard base excision repair (1). In this context, 3-cyano-5-(4-pyridyl)-6-hydrizonomethyl-2-pyridone (OK-1035) has recently been identified as a DNA-PK inhibitor with an IC₅₀ value of 8 µM (33). Although considerably less potent than wortmannin, it was far more selective, the IC₅₀ values for seven other kinases being at least 50-fold higher.

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References


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