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A. J. McIlwrath · V. G. Brunton · R. Brown

Cell-cycle arrests and p53 accumulation induced by geldanamycin in human ovarian tumour cells

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Abstract We have analysed the cell-cycle arrests and cytotoxicity of the A2780 human ovarian cell line in response to geldanamycin, a benzoquinoid ansamycin that can inhibit tyrosine kinases. Geldanamycin causes a dose-dependent G2 arrest and reversible inhibition of entry into the S phase in A2780 cells. After a 3-h exposure to 0.1 μ*M* geldanamycin, the cells show an increase in accumulation of p53 protein that is maximal at 24 h after drug exposure. Increased p53 levels can be induced in cells by DNA-damaging agents; however, using alkaline elution and sister chromatid exchange assays we detect no DNA damage induced by geldanamycin. Using dominant negative mutant TP53 transfectants of A2780 we have analysed the possible dependence of geldanamycin-induced cell-cycle arrests on the presence of functional p53. We observe no difference in cell-cycle arrests in mutant p53 transfectants known to have the p53-DNA damage-response pathway inactivated as compared with vector-alone controls. Similarly, we observe no difference in clonogenic resistance to the cytotoxicity of geldanamycin in these cells. These results suggest that geldanamycin can induce increased p53 protein by a mechanism not involving DNA damage. Furthermore, the cell-cycle arrests and cytotoxic effects of geldanamycin in these cells are not mediated by p53-dependent pathways.

Key words Geldanamycin · Cell-cycle arrest · P53 accumlation · Human ovarian tumour cells

A.J. McIlwrath · R. Brown (✉)
CRC Department of Medical Oncology, CRC Beatson Laboratories, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, UK

V.G. Brunton
Beatson Institute, CRC Beatson Laboratories, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, UK

Introduction

Geldanamycin is an ansamycin antibiotic related to herbimycin A and has been shown to be a tyrosine phosphorylation inhibitor [25]. Both agents have been shown to reverse the oncogenic phenotype of *v-src* transformed fibroblasts [23, 24] and to inhibit *v-src* kinase activity in vitro [25]. Herbimycin A is known to inhibit *c-src* activity in human colon tumour cell lines [7], which correlates with growth inhibition. However, in neuronal tumour cell lines at concentrations of herbimycin A and geldanamycin that cause extensive cell death, inhibition of *c-src* activity does not occur [22]. Furthermore, these authors have suggested that metabolism of geldanamycin may lead to metabolites that could be capable of causing DNA damage.

Cytotoxic drugs can induce reversible cell-cycle arrests in proliferating cells at the G1- to S-phase transit and at the G2 phase of the cell cycle [19]. An important component of the pathway from DNA damage to arrest in the G1 phase of the cell cycle is wild-type p53 function [13, 16]. We have investigated the induction of cell-cycle arrests by geldanamycin in a human ovarian tumour cell line by flow cytometric analysis and evaluated their possible dependence on functional p53. Furthermore, we show that the level of p53 protein is increased after treatment of cells with geldanamycin. DNA damage is generally regarded as an important signal for the induction of increased p53 protein in cells [18]. Since it has been suggested that geldanamycin can directly or indirectly induce DNA damage [3, 27], we have examined the ability of geldanamycin to induce detectable DNA damage as measured by alkaline elution and sister chromatid exchange assays. If the toxicity of geldanamycin is being mediated by DNA damage-induced p53 response pathways, then inactivation of this pathway should lead to resistance to geldanamycin. This we have examined using clonogenic survival assays of drug sensitivity in A2780 cells with

functional p53 and in mutant p53 transfectant cells that have the p53 pathway inactivated [16].

Materials and methods

Cell culture, geldanamycin treatment and drug sensitivity assays

A2780 is a human ovarian carcinoma cell line derived from an untreated patient [2]. The plasmid pC53SCX3 [1] containing mutant TP53 complementary DNA (codon 143, Val to Ala) expressed from a CMV promoter (A2780/mp53) and vector alone (A2780/v) without insert were transfected into the A2780 cell line [4]. Confirmation of mutant or wild-type p53 expression was done by enzyme-linked imunosorbent assay (ELISA), and mutant p53-expressing cell lines were previously shown to have an abrogated radiation-induced G1 arrest [16]. The cell lines were maintained as monolayer cultures in RPMI 1640 medium with 10% fetal calf serum and were grown at 37° C in 95% air/5% CO_2. All cell lines were routinely checked for *Mycoplasma* contamination. Exponentially growing cells were treated with geldanamycin in fresh RPMI 1640 for 3 h, after which the medium was removed and replaced with fresh medium. Drug sensitivity was measured by clonogenic assay; 10^3 cells were seeded into 10-cm plates and, 24 h later, were treated with geldanamycin for 3 h as described above. After a 10-day period of incubation, colonies were stained and those with greater than 200 cells were counted.

p53 Protein detection

Whole-cell extracts were prepared by lysing exponentially growing cells in 1% Nonidet P-40, 500 mM NaCl, 50 mM TRIS (pH 7.5), and 1 mM dithiothreitol in the presence of protease inhibitors. Protein concentrations were determined by the Bio-Rad (Richmond, Calif.) protein assay. Immunoblotting was carried out as described previously [4] using the p53 antibody pAB2 (Oncogene Sciences, Manhasset, N.Y.) and was visualized by enhanced chemiluminescence; the intensity of the autoradiographic signal was quantified by laser densitometry.

Flow cytometry

Cells were treated as described above and at various times DNA synthesis was assessed by incorporation of bromodeoxyuridine (BrdUrd) and flow cytometric analysis [4]. No marked difference in total cell number was noted during this time course for treated and untreated cultures. BrdUrd (10 μ*M*) was incorporated over a 4-h incubation period at 37° C and cells were harvested and fixed in 70% ethanol. After denaturation of the DNA with 2 N HCl, cells were incubated with an anti-BrdUrd mouse monoclonal antibody (Dako) followed by a fluorescein isothiocyanate (FITC)-linked goat anti-mouse IgG (Sigma Ltd., Poole, UK). Cells were stained for 30 min at room temperature with propidium iodide and analysed by flow cytometry using a Coulter (Hialeah, Fla.) EPICS Profile Analyzer.

Alkaline Elution Assay

DNA single-strand breaks were measured by alkaline filter elution as previously described [11]. In brief, control cells and cells treated for 3 h with geldanamycin were incubated with 0.03 μCi [^{14}C]-thymidine/ml (sp. act., 51 mCi/mmol; Amersham Ltd., Amersham, UK) for 24 h so as to label their DNA. γ-Irradiated cells labeled with 0.17 μCi [^{3}H]-thymidine/ml (sp. act., 5 Ci/mmol; Amersham Ltd., Amersham, UK) were used as an internal control. Cells were loaded onto 2-μm (pore size) polycarbonate filters and lysed. DNA was eluted at pH 12.2 with 0.1 *M* tetrapropylammonium hydroxide containing 0.1% sodium dodecyl sulfate and 0.02 *M* ethylenediaminetetraacetic acid (EDTA) over a 15-h period at an elution rate of 0.01 ml/min. The radioactivity was determined in the final fraction and the results were expressed as the fraction of ^{14}C retained versus fraction ^{3}H (internal standard) retained.

Sister chromatid exchange assay

Non-specific DNA damage was assessed using the fluorescence plus Giemsa (FPG, 'harlequin') staining method [20]. Cells growing exponentially in flasks were exposed either to 10 μ*M* cisplatin for 1 h, to 1 μ*M* geldanamycin for 3 h or to no treatment, after which the medium was replaced with fresh medium containing 10 μ*M* BrdUrd and the cells were allowed to grow for a further 48 h (two complete cell cycles). For the last hour prior to harvesting the cells were treated with 0.01 μg colcemid/ml and, after trypsinising, were treated with hypotonic solution (75 m*M* KCl, 30 m*M* tri-sodium citrate). The cells were then fixed at 4° C with 3 parts methanol: 1 part glacial acetic acid, once for 10 min and again in fresh fixative overnight. The cells were dropped onto glass slides and air-dried to produce chromosome spreads. After staining with 20 μg Hoechst 33258/ml for 10 min the slides were washed with 2 × SSC and placed on a UV transilluminator for 2 h. The slides were washed three times in distilled water, air dried and stained using 3.5% Giemsa solution in Sorensen's buffer (pH 6) for 3–5 min. After rinsing in tap water and air-drying, the slides were mounted in DPX mountant (BDH). The frequency of sister chromatid exchanges per chromosome was scored using light microscopy in 20 metaphase spreads for each treatment.

Results

Effect of geldanamycin on cell-cycle distribution of A2780/v cells

When wild-type p53-expressing A2780/v cells were exposed to geldanamycin, cell-cycle arrests occurred at the G1 to S-phase transition and at the G2 phase (Fig. 1). At 0.1 μ*M*, geldanamycin resulted in an inhibition of entry of cells into the S phase at 8 h after treatment. This inhibition was reversible, since the relative numbers of BrdUrd-positive cells had returned to control (untreated) levels by 24 h. At 1 μ*M*, geldanamycin produced a more prolonged reduction in entry of cells into the S phase, which remained apparent at 24 h after treatment but reverted to control levels by 48 h.

After the application of 0.1 μ*M* geldanamycin the proportion of cells in the G2 phase reached a maximum of 184% of the control level at 8 h and returned to levels equivalent to those seen in untreated controls by 24 h. The extent of the G2 arrest was increased and prolonged by an increased dose of geldanamycin. A 1 μ*M* dose of the drug elevated the proportion of cells in the G2 phase to 279% at 24 h and extended by a further 24 h the return to control levels. Thus, the ability of A2780/v cells to arrest, either in the G2 phase

or at the G1 to S-phase transition, in response to geldanamycin is both reversible and dose-dependent.

Geldanamycin-induced p53 protein accumulation

A2780 cells show an increase in accumulation of p53 protein after treatment with ionising radiation [16] and cisplatin [4]. Figure 2 shows a Western blot obtained for p53 protein in whole-cell extracts of A2780 human ovarian adenocarcinoma cells at various times after either no treatment or the application of 0.1 or 1 μM geldanamycin. We have previously determined that A2780 cells express the wild-type TP53 gene sequence and that the protein expressed is in wild-type conformation [4]. Untreated cells consistently showed increased p53 accumulation during growth of the cell cultures; however, geldanamycin reproducibly induced a further increase in accumulation of p53. The accumulation was apparent at 8 h remained so at 48 h and was observed in repeat independent experiments. The maximal magnitude of increase over control levels, as measured by laser densitometry of an autoradiographic image of a Western blot, was approximately a factor of 2 after both the low dose and the high dose of geldanamycin at 24 h post-treatment. This accumulation of wild-type p53 protein suggests that p53 may be involved in the response of A2780 cells to geldanamycin.

Lack of dependence on wild-type p53 expression for cell-cycle effects and drug sensitivity

To determine the dependence on p53 expression for the cell-cycle effects of geldanamycin, we used stabile transfectants of A2780 cells containing a dominant negative mutant p53 (codon 143, Val to Ala) as described by us previously [16]. We have shown that transfection of a mutant p53 into these cells does not affect the ionising radiation-induced G2 arrest. After treatment of mutant p53-expressing A2780/mp53 cells with 0.1 or 1 μM geldanamycin the number of cells in the G2 phase of the cell cycle was increased (Fig. 1A). Wild-type p53- and mutant p53-expressing cells displayed similar kinetics of G2 cell-cycle arrest in response to geldanamycin. Therefore, the ability of geldanamycin to arrest cells in the G2 phase did not appear to be dependent on the status of the p53 protein expressed within the cells.

A2780/mp53 cells have lost the G1 arrest in response to an acute dose of ionising radiation as compared with the vector-alone control cells A2780/v [16]. However, after treatment with geldanamycin the cell-cycle arrest at the G1- to S-phase transition of A2780/mp53 cells was intact. After the addition of 0.1 μM geldanamycin the proportion of A2780/mp53 cells in the S phase of the cell cycle was reduced to 60% at 8 h (Fig. 1B). After the application of 1 μM geldanamycin a greater and prolonged reduction in entry of cells into the S phase was observed. Only 53% of cells were entering the S phase at 24 h after treatment. The wild-type and mutant p53-expressing cells displayed similar kinetics of inhibition of entry into the S-phase (Fig. 1), Thus, as in the case of the G2 arrest, the ability of geldanamycin to inhibit entry of cells into the S phase was independent of the status of p53. During these experiments we confirmed that the A2780/mp53 cells continued to express mutant p53 and had lost an ionising radiation-induced G1 arrest as compared with A2780/v cells. The A2780/v cells showed a 53% decrease in cells entering the S phase at 24 h after irradiation with 2 Gy, whereas the A2780/mp53 cells showed no decrease (the percentage of A2780/mp53 cells in the S phase 24 h after 2 Gy was 101%). Thus, although the introduction of a dominant negative mutant of TP53 abrogates the G1- to S-phase arrest in response to ionising radiation, it does not abrogate the inhibition of entry of cells into the S phase in response to geldanamycin.

In addition to the loss of the G1 arrest in response to ionising radiation, A2780/mp53 cells also become more resistant to ionising radiation [16]. Treatment of two independent A2780/v cell lines with geldanamycin in the dose range of 0.001–1 μM produced clonogenic survival curves at doses of geldanamycin that produce 50% reductions in the surviving fraction (LD_{50} values) of 0.033 ± 0.006 and 0.022 ± 0.002 μM (Fig. 3, dotted lines). Transfection of a dominant negative mutant TP53 (codon 143, Val to Ala) did not result in any significant change in the sensitivity of the cells to geldanamycin (Fig. 3, solid lines). The LD_{50} values recorded for two independent A2780/mp53 cell lines after geldanamycin treatment were 0.033 ± 0.002 and 0.023 ± 0.005 μM. Since the cells are only exposed to drug for 3 h and then allowed to form colonies without replating, cytotoxicity is being measured rather than reversible cytostasis. There is no statistically significant difference between the LD_{50} values noted for the two cell types. It would therefore appear that the introduction of a dominant negative mutant TP53 into A2780 cells does not render them more resistant to geldanamycin.

Lack of geldanamycin-induced DNA damage

Alkaline elution of DNA was used to investigate the possibility that geldanamycin treatment of A2780 cells resulted in the formation of single strand breaks in DNA. In cells treated with geldanamycin for 3 h at concentrations in excess of that required for cytotoxicity (50 and 100 μM) there was no significant difference in the ratio of [^{14}C]-thymidine to [^{3}H]-thymidine (γ-irradiated internal control cells) retained on the filter (2.09 ± 0.03 in control cells as compared with 1.98 ± 0.08 and 2.11 ± 0.05 in cells treated with 50 and 100 μM geldanamycin, respectively). This corresponded

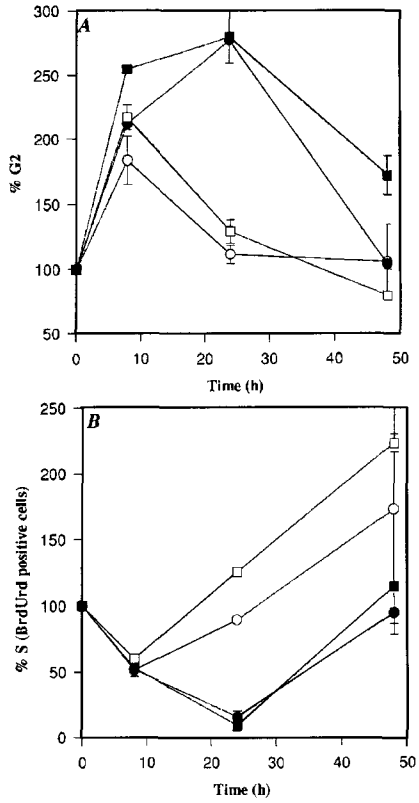

Fig. 1A, B Cell-cycle effects of 0.1 μ*M* geldanamycin (*white symbols*) and 1 μ*M* geldanamycin (*black symbols*) on A2780/v (○, ●) and A2780/mp53 (□, ■) transfectant A2780 cells. **A** Proportion of cells in the G2 phase. **B** Proportion of cells in the S phase. Proportions were determined by BrdUrd incorporation and fluorescence-linked immuno-detection followed by propidium iodide staining and flow cytometric analysis.Values are expressed as a percentage of untreated cells and are the average of at least two experiments using independent mutant p53 and vector-alone transfectant cell lines. A minimum of 20,000 events were recorded in each cell sample. Bars represent the standard errors of the means from repeat experiments

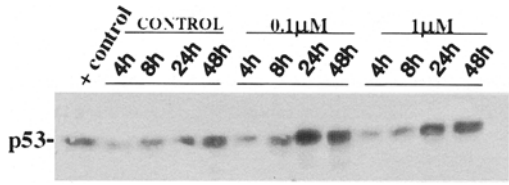

Fig. 2 p53 protein levels detected after SDS-PAGE by immunoblotting using anti-p53 antibody pAb2 (Oncogene Science). Whole-cell extracts were prepared in parallel from untreated (control) and 0.1 and 1-μ*M* geldanamycin-treated A2780 cells at the times shown (+*control* An extract of A2780 cells known to be positive for p53 protein)

to 44% of the [^{3}H]-thymidine being retained on the filters loaded with cells irradiated with 5 Gy, as compared with 92% of the [^{14}C]-thymidine being retained on the filters loaded with cells treated with 100 μ*M* geldanamycin.

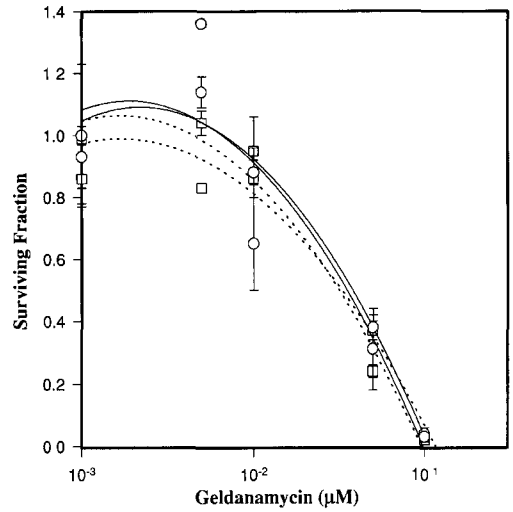

Fig. 3 Geldanamycin sensitivities of A2780 p53 transfectants. The surviving fraction of clonogenic cells of A2780/v (— ○; LD_{50} values, 0.033 and 0.022 μ*M*) and A2780mp53 (···· □; LD_{50} values 0.033 and 0.023 μ*M*). The *curves* represent second-order regressions of the mean of two experiments and the bars are the standard errors of the means

We also looked for evidence of an increase in sister chromatid exchange (SCE) frequency as a measure of DNA damage in geldanamycin-treated cells. Exposure of A2780 cells to 1 μ*M* geldanamycin for 3 h, a dose that is cytotoxic, produced an SCE frequency of 0.146 ±0.08, which was not significantly different from that found in control cells (0.135 ±0.07). In contrast, treatment of the cells with the DNA-intercalating agent cisplatin (10 μ*M* for 1 h) resulted in an SCE frequency of 1.173 ±0.45, which was significantly higher than that found in control cells ($P < 0.05$).

Discussion

We have shown that geldanamycin induces cell-cycle arrests at the G1- to S-phase transit and at the G2 phase in A2780 human ovarian adenocarcinoma-derived cells. We have previously shown that these cells express wild-type p53 [4] and that the dominant negative (codon 143, Val to Ala) mutant of TP53 can cause loss of an ionising radiation-induced G1 arrest [16]. The p53 protein is known to be involved in the DNA-damage signaling pathway leading to cell-cycle arrest in a variety of cell types [10, 13, 14]. Since geldanamycin can induce cell-cycle arrests with equivalent kinetics in the A2780 cells expressing wild-type or mutant p53, then p53 does not mediate geldanamycin-induced cell-cycle arrests even though increased accumulation of p53 is observed after geldanamycin treatment. The p53-dependent G1 arrest has been suggested to be at the restriction point in the late G1 phase. The reversible inhibition of entry into the S phase that we have observed may be due to an arrest at an earlier

point in the G1 phase, or even to reversible entry into a G0 phase. Furthermore, it is possible that this kinase inhibitor is inducing cell-cycle arrest by inhibition of cell-cycle-associated kinases [21]. Indeed, the *src* kinase, which is inhibited by geldanamycin, has a potential role in cell-cycle arrest at both the G1 and G2 phases of the cell cycle [26].

The dominant negative mutant p53 transfectants of A2780 cells used in the present study have previously been shown to have increased resistance to ionising radiation [16]. Genetic inactivation of the TP53 gene in mice has been shown to cause reduced DNA damage-induced apoptosis and increased resistance to anticancer drugs [5, 15]. Tyrosine kinase inhibitors have been shown to induce apoptosis in cells without a nucleus and therefore, independent of DNA damage [9]. The lack of difference in the clonogenic sensitivity to geldanamycin we observe in cells with or without a functional p53-dependent DNA damage-response pathway would support tyrosine kinase inhibitors inducing cell death in a manner independent of DNA damage.

A2780 cells accumulate increased levels of p53 protein after DNA damage [16]. This increased p53 accumulation has been observed in many cell types in response to various DNA-damaging agents [6], and it has been suggested that one of the crucial signals in this DNA damage-response pathway is DNA double-strand break induction [18]. Geldanamycin may induce DNA damage indirectly via the inhibition of DNA polymerase [27] or more directly by the formation of free radicals [3]. However, we have not observed induction of any DNA strand breaks as measured by alkaline elution or by the induction of sister chromatid exchanges, but we cannot exclude the induction by geldanamycin of DNA damage that could not be detected due to the limitations in the sensitivities of the two assays. Indeed, it has previously been shown that p53 levels can be increased by treating cells with the kinase inhibitor genistein, again in the absence of DNA damage [8]. Increased levels of p53 are often due to stabilization of the protein as a result of phosphorylation [17]. However, it has been observed that herbimycin A is capable of increasing p53 protein levels in human breast carcinoma cells with a concomitant reduction in p53 tyrosine phosphorylation [22]. Increased p53 levels induced by ionising radiation can be inhibited by protein kinase C inhibitors and phosphatase inhibitors [12]. It is possible that the increased levels of p53 are due to stabilization of the protein as a result of changes in p53 phosphorylation due to the action of geldanamycin on other cellular kinases. Consistent with the increased p53 levels not being due to DNA damage are the observations that the geldanamycin-induced G1 arrest and the drug sensitivities are not dependent on functional p53 activity. Together these results suggest a cellular pathway leading to p53 protein stabilization induced by geldanamycin that is independent of DNA damage. Furthermore, the increased p53 levels do not appear to mediate cell-cycle arrest or drug sensitivity.

Loss of p53 function in A2780 cells results in an increased resistance to ionising radiation [16]. We have shown that the introduction of a dominant negative mutant TP53 into A2780 cells does not render them more resistant to geldanamycin. Since loss of p53 function in certain cell types is associated with resistance to anticancer drugs [5, 15, 16], geldanamycin may be of particular interest for the treatment of tumours that have becme resistant due to loss of p53 function.

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