Cancer Chemother Pharmacol (1998) 41: 417–422

SHORT COMMUNICATION

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Geldanamycin-induced cytotoxicity in human colon-cancer cell lines: evidence against the involvement of c-Src or DT-diaphorase

Received: 20 April 1997 / Accepted: 5 September 1997

Abstract We investigated two of the major proposed modes of action of the benzoquinoid ansamycin geldanamycin using a pair of human colon-carcinoma cell lines, BE and HT29. One potential mechanism of action in colorectal cancer is the inhibition of c-Src kinase activity, since this proto-oncogene is hyperexpressed in human large-bowel tumours. Our results show that despite the 9-fold higher level of c-Src kinase activity found in HT29 cells, there was only a 1.4-fold difference in cytotoxicity as compared with BE cells, the latter being the most sensitive. Moreover, even at concentrations of geldanamycin that resulted in cell kill of 80% or more after a 24-h period of exposure, there was no effect on c-Src kinase activity in HT29 cells, although c-Src protein was depleted at supralethal levels of exposure. We also investigated the metabolism of the quinone moiety of geldanamycin by DT-diaphorase, an enzyme that activates certain quinone antibiotics such as mitomycin C and is hyperexpressed in colorectal cancer cells. Geldanamycin was shown to be a substrate for DT-diaphorase present in HT29 cells. However, the lack of a major differential in cytotoxicity between HT29 and BE cells indicates that this is unlikely to be pharmacologically significant, since the former contains high levels of enzyme activity, whereas BE cells have no significant activity due to a point mutation in the DT-diaphorase (NQO1) gene. Although reduction of geldanamycin was also catalysed by non-DT-diaphorase reductases in HT29 and BE cells, providing the potential for free radical induction, this is unlikely to be significant since studies previously reported by us elsewhere showed that cells exposed to geldanamycin exhibited no evidence of DNA damage. Thus, as far as the mode of action of geldanamycin in human colon-carcinoma cells is concerned, the present results rule out two major possibilities, namely, the involvement of c-Src tyrosine kinase inhibition and DT-diaphorase metabolism.

Key words Geldanamycin · Colon cancer · c-Src · DT-diaphorase

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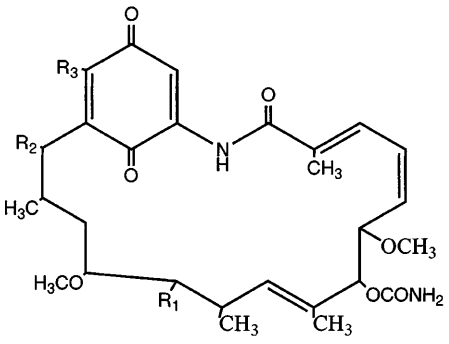
Introduction

Activation of the proto-oncogene product $p60^{c\text{-}src}$ (c-Src), a non-receptor tyrosine kinase and the cellular counterpart of the transforming protein of Rous sarcoma virus (v-Src), occurs with high frequency during colon tumour development. In support of a causal involvement, increased c-Src kinase activity in colonic adenomas has been reported to correlate with malignant potential [5, 23]. A complete understanding of the mechanism of c-Src activation and its role in the cellular changes associated with colorectal tumour progression and development remains to be established. Nevertheless, inhibitors directed against c-Src activity could possibly serve as valuable pharmacological tools in this respect as well as having potential therapeutic benefits in a tumour type that yields a 5-year survival of less than 30% in the United States following current treatments [3].

The ansamycin benzoquinonoid group of antibiotics include two closely related compounds, herbimycin A and geldanamycin (Fig. 1), which have been reported to reverse v-Src transformed morphology in fibroblasts [25–27]. There are, however, opposing reports in the literature on the relationship between the antiproliferative and c-Src inhibitory effects of both herbimycin A and geldanamycin. For example, direct inhibition of c-Src activity by herbimycin A has been demonstrated in colorectal cancer cell lines, the extent of which appears to correlate with inhibition of cell growth [10]. However,



	R_1	R_2	R_3
Geldanamycin	OH	H	OCH_3
Herbimycin A	OCH_3	OCH_3	H

Fig. 1 Structure of geldanamycin and herbimycin A

in a range of tumour lines of neuronal origin, where c-Src may also play a role in oncogenesis, herbimycin A and geldanamycin were shown to exert cell-killing activity at concentrations that did not inhibit c-Src activity [31].

Because of its potent antitumour activity, geldanamycin has been under consideration for clinical trials [22]. In view of this and in the light of the previous uncertainties concerning the mode of action of these compounds, we felt that it was important to evaluate the proposed mechanistic hypotheses concerning the antitumour action of geldanamycin in human colon-carcinoma cell lines. The cell lines used, HT29 and BE, were selected for the following reasons. Firstly, our preliminary studies showed that these two lines exhibited marked differences in their c-Src levels. As based on previous observations [10], differences in sensitivity between these cell lines might be expected if a c-Src related mechanism were operative. Secondly, these compounds possess a quinone moiety (Fig. 1) that may potentially undergo reductive metabolism to reactive species [16], and the two cell lines display major differences with respect to the expression of DT-diaphorase, which is a quinone-reducing enzyme that can either activate or detoxify various quinone-containing drugs and toxins [17, 18, 33]. Interestingly, like c-Src, DT-diaphorase has been shown to be elevated in colorectal cancer as compared with normal mucosa [19]. If DT-diaphorase-mediated metabolism of geldanamycin were pharmacologically significant, then we would again expect to see major differences in sensitivity to geldanamycin in HT29 and BE cells because HT29 cells exhibit high levels of the enzyme, whereas BE cells have a mutation in the DT-diaphorase gene (NQO1) and lack enzyme activity [24]. For example, such differences in sensitivity are seen with a number of bioreductive drugs, including mitomycin C and the indoloquinone EO9 [20, 29].

The results of the studies reported herein argue against the involvement of c-Src inhibition and metabolic reduction by DT-diaphorase in the in vitro antitumour effects of geldanamycin in human colon-carcinoma cell lines.

Materials and methods

Clonogenic assay

HT29 (from the American Type Culture Collection, Rockville, Md.) and BE cells (from Dr. N. Gibson, University of Southern California, Los Angeles) [7] were grown for 48 h in DMEM supplemented with 10% (v/v) fetal bovine serum. The medium was then removed and fresh medium containing a range of concentrations of geldanamycin (a kind gift from Dr. V. Narayanan, Division of Cancer Treatment, NCI, Bethesda, Md.) was added. Stock solutions of geldanamycin were prepared in dimethylsulfoxide at a concentration of 10 m*M* and the final concentration of dimethylsulfoxide in the medium was 0.01% (v/v). After 24 h the drug was removed and the control cells were counted and plated at 1×10^3/60-mm plate. The drug-treated cells were diluted to the same extent. Triplicate plates were incubated for 10 days, after which the clones were fixed in methanol and stained with 0.1% (w/v) crystal violet. Colonies of more than 50 cells were counted and IC_{50} values, determined from log-concentration response curves. The cloning efficiency of control cells was between 40% and 60% for both cell lines.

Immune complex c-Src kinase assay

c-Src kinase activity was measured in immunoprecipitates prepared from HT29 and BE cells as previously described [4]. In brief, c-Src was immunoprecipitated using monoclonal antibody mAb327 (Oncogene Science) and immune complexes incubated with [γ-^{32}P]-ATP (1 μM, 5μCi: Amersham sp.act. 3,000 Ci/mmol) and 10 μg acid-denatured enolase for 10 min at 20 °C. The reaction was terminated by the addition of 2× Laemmli sample buffer and the proteins were then resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by autoradiography and densitometry.

Immunoblotting

Total cell lysate proteins (50 μg) were resolved by 10% SDS-PAGE and immunoblotted using Src mAb327 (1 μg/ml) as described elsewhere [4].

DT-diaphorase assay

Cell pellets were resuspended in 500 μl 10 m*M* sodium phosphate buffer (pH 7.4) containing 0.1 m*M* ethylenediaminetetraaceticacid (EDTA) and 1% aprotinin before being sonicated on ice (3 × 10 s). Sonicated cell preparations were centrifuged at 13,000 *g* for 20 min and the supernatants were retained and stored at −70 °C before use. Measurement of substrate reduction by purified rat DT-diaphorase (a kind gift from Dr. Richard Knox, Institute of Cancer Research, Sutton, UK) or human colonic tumour cell-line sonicates was determined by the method of Ernster [8]. In brief, this involves monitoring of the reduction of the terminal electron acceptor cytochrome c at 550 nm in the presence of NADH as a cofactor, with either the benchmark quinone, menadione, or geldanamycin serving as the substrate and intermediate electron acceptor. Specific reduction of the substrates by DT-diaphorase was calculated as the fraction that was inhibited by dicoumarol (100 μM), a potent inhibitor of DT-diaphorase. The reactions contained 50 m*M* TRIS-HCl (pH 7.5), 77 μM cytochrome c, 2 m*M* NADH, 50 μM menadione or geldanamycin and 0.14% bovine serum albumin in a final volume of 1 ml. All reactions were carried out at 37 °C and were initiated by the addition of

cofactor. Rates of reduction were calculated from the linear portion of the progress curves over a 1-min period and were expressed as micromoles of cytochrome c reduced per minute per milligram of protein using an extinction coefficient of 21.1 mM cm^{-1}. Appropriate controls such as minus drug, cofactor, or enzyme were also taken into account in the calculations, in the standard fashion for DT-diaphorase measurements.

Results

Cytotoxicity of geldanamycin in HT29 and BE cells

Figure 2 shows a typical experiment in which there was a dose-dependent decrease in cell survival in HT29 and BE cells following 24-h period of exposure to geldanamycin. The mean IC_{50} values ±SD derived from three independent experiments were 68.7 ± 25.7 and 48.7 ± 24.5 nM for HT29 and BE respectively. Although this difference was statistically significant ($P = 0.0107$, Student's t-test), it represented only a 1.4-fold disparity in IC_{50} values between the two cell lines. No reduction in colony diameter was seen in the geldanamycin-treated cells.

Involvement of c-Src kinase inhibition in the cytotoxicity of geldanamycin in BE and HT29 cells

The c-Src kinase activities were assayed in immunoprecipitates by measurement of the phosphorylation of enolase. Similar results were obtained when c-Src autophosphorylation was used as the end point (data not shown). The activity in HT29 cells was found to be significantly higher than that in BE cells (9-fold; Fig. 3a). This could be attributed to the low levels of c-Src protein in the BE cells as determined by Western immunoblotting (Fig. 3b). The low levels of c-Src kinase activity in the BE cells were just above the limit of detection. For this reason the effects of geldanamycin are presented for c-Src kinase activity immunoprecipitated from HT29 cells. Pre-treatment of HT29 cells with geldanamycin for 24 h prior to preparation of the immunoprecipitates, at concentrations that resulted in a marked reduction in cell survival after this period of exposure, had no effect on c-Src kinase activity (results not shown). At the highest concentration used (5 μM) there was an 80% reduction in cell survival in the HT29 cells (Fig. 2) but no significant reduction in c-Src kinase activity. Increasing the exposure time to up to 96 h did result in inhibition of c-Src kinase activity in cells treated with 0.5 and 0.1 μM geldanamycin, but there was a reduction in protein levels at the longer periods of exposure to high concentrations of geldanamycin (Fig. 3d), which resulted in no decrease in specific c-Src kinase activity (Fig. 3c).

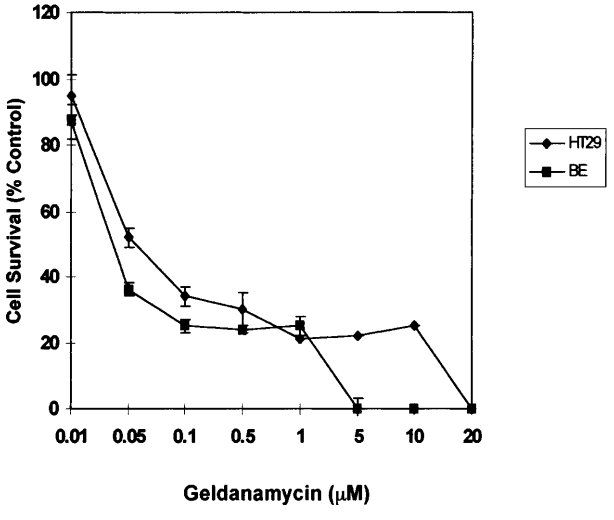


Fig. 2 Cell-survival curves generated for geldanamycin in BE and HT29 cells. Data represent mean values ± SD for triplicate plates. The results derive from a representative experiment in a series of three

Metabolism of geldanamycin by DT-diaphorase

The ability of purified DT-diaphorase and HT29 and BE sonicates to metabolise menadione and geldanamycin is shown in Table 1. The data demonstrate that purified rat DT-diaphorase and a dicoumarol-inhibited fraction of HT29 sonicates could both reduce geldanamycin. The reduction of geldanamycin by both purified DT-diaphorase and HT29 sonicates occurred at a rate that was 20- to 30-fold lower than that observed for the benchmark substrate menadione. As expected, BE cells, which lack a functional DT-diaphorase enzyme, did not show any measurable dicoumarol-inhibited reduction of geldanamycin. It should also be noted that in both HT29 and BE sonicates a significant proportion of the reduction of geldanamycin was not dicoumarol-inhibited (145 ± 23 and 57 ± 7 nmol cytochrome c reduced min^{-1} mg protein^{-1}, respectively).

Discussion

The ansamycin antibiotic geldanamycin has been shown to exhibit an interesting profile of both in vitro and in vivo antitumour activity. It is therefore a potential candidate for clinical development, particularly for the treatment of colorectal tumours because of the expression of high levels of both c-Src kinase and DT-diaphorase activities. In this study our first priority was to determine whether the reported effects of geldanamycin on Src kinase activity were related to its cytotoxicity profile in human colon-carcinoma cell lines. In a clonogenic assay a dose-dependent decrease in cell survival was seen in both BE and HT29 cells following a 24-h period of exposure to geldanamycin. This implies a cytotoxic rather than a cytostatic action of geldanamycin.

Our studies indicate that the effect of geldanamycin on cell survival in the present study could not be attributed to a reduction in c-Src kinase activity. Firstly, despite a

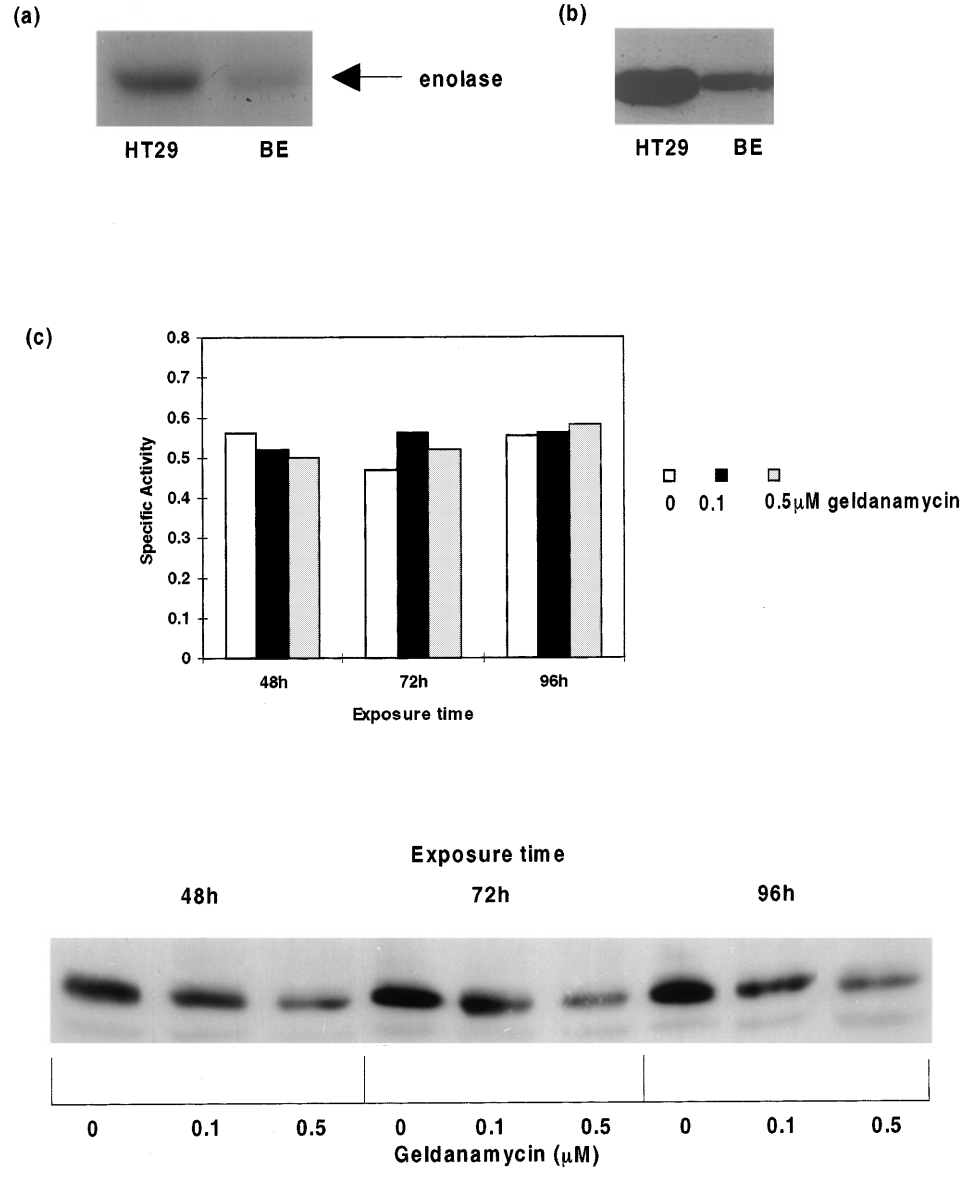


Fig. 3a–d c-Src kinase activity and protein levels detected in BE and HT29 cells. **a** c-Src kinase activity determined using enolase as a substrate. **b** c-Src protein levels. Time course of geldanamycin effects on **c** c-Src kinase activity and **d** c-Src protein levels in HT29 cells. Cells were treated with geldanamycin for the indicated times, after which the c-Src kinase activity and protein content were determined. The c-Src kinase activity is expressed as specific activity, whereby the activity has been adjusted for the level of c-Src protein present in the immunoprecipitate

9-fold difference in c-Src kinase activity and protein levels, we observed only a 1.4-fold difference in sensitivity between the BE and HT29 cell lines, with the lower-expressing BE cell line being the most sensitive. Although a simple relationship between c-Src expression and sensitivity to a c-Src inhibitor would not necessarily be expected, Garcia et al. [10] demonstrated that colon-tumour cell lines with elevated c-Src kinase activity were more sensitive to herbimycin A than a "normal" colon mucosal cell line that did not have high levels of the enzyme. Secondly, at concentrations that yielded an 80% reduction in cell survival, geldanamycin had no effect on

Table 1 Measurement of menadione and geldanamycin reduction by purified DT-diaphorase and BE and HT29 sonicates. Reduction of substrate represents the activity that was inhibited by 100 μ*M* dicoumarol and, therefore, was due to the DT-diaphorase enzyme as described in Materials and methods. The data represent mean values ± SD for at least three experiments carried out in triplicate (*ND* Not detected)

Substrate	Reduction of substrate (μmol cytochrome c reduced min^{-1} mg $protein^{-1}$)		
	Purified DT-diaphorase	HT29 sonicates	BE sonicates
Menadione	1,786.6 ± 404.9	0.906 ± 0.095	ND
Geldanamycin	60.5 ± 13.1	0.052 ± 0.008	ND

c-Src kinase activity in HT29 cells treated with the drug. Inhibition of c-Src kinase activity was seen, but only following more prolonged exposure of the HT29 cells to high and supratoxic concentrations of geldanamycin. Moreover, this observed decrease in kinase activity was also accompanied by a similar reduction in c-Src protein levels, resulting in no decrease in specific c-Src kinase activity. This indicates an effect on the c-Src protein content of cells rather an inhibitory effect on catalytic activity. Our observations contrast with the results reported by Garcia et al. [10] for the related drug herbimycin A. They reported a growth-inhibitory but non-cytotoxic effect of herbimycin A in HT29 cells and a time-dependent decrease in c-Src autophosphorylation that could be observed as early as 2 h after the addition of herbimycin A. However, although this decrease correlated with the growth-inhibitory effect of herbimycin A in HT29 cells, the kinase reductions were also mirrored by a fall in the steady-state c-Src protein levels [10].

Other studies have also shown that non-cytotoxic concentrations of both geldanamycin and herbimycin A are capable of decreasing v-Src kinase activity, an effect that could be attributed to accelerated degradation of the v-Src protein [27]. This effect was very rapid (within 2 h of treatment), whereas in the present study a decrease in c-Src protein levels was observed only following prolonged exposure to geldanamycin for 48 h or more, suggesting that it may be a consequence of the toxicity of the high concentration of geldanamycin (0.5 μM) used.

Our results are in agreement with the work of Whitesell and co-workers [31], who studied the effects of geldanamycin and herbimycin A in a panel of human tumours of neuronal origin, for which c-Src has also been implicated in oncogenesis. In that study the antibiotics exerted an irreversible cytotoxic effect that was unrelated to c-Src kinase inhibition, and circumstantial evidence was presented for induction by herbimycin A of an active program of cell death, although not strictly apoptosis. There was substantial DNA degradation in herbimycin A-treated cells but no nucleosomal cleavage, which is a characteristic often associated with apoptosis.

Both herbimycin A and geldanamycin contain benzoquinone moieties that could undergo metabolic reduction by enzymes such as DT-diaphorase, a reductase that is up-regulated in many tumours, including colorectal cancers [33]. The products of quinone reduction may be reactive oxygen species or drug metabolites that could induce DNA damage. The two colon-tumour cell lines used in this study provide an excellent model for determination of the role of DT-diaphorase in the biological activation of quinone-containing drugs because HT29 cells express high levels of DT-diaphorase, whereas BE cells have a mutation in the DT-diaphorase (NQO1) gene and, hence, lack significant enzyme activity [18, 24]. In particular this pair of cell lines have previously been used to determine the role of DT-diaphorase in the bioactivation of a number of bioreductive quinoid drugs such as mitomycin C and the novel indoloquinone EO9 [20, 29].

Geldanamycin was clearly shown to be a substrate for DT-diaphorase purified from rat Walker tumour cells as well as for that present in sonicates of HT29 cells. The rate of reduction of geldanamycin was 20- to 30-fold lower than that observed for the benchmark quinone menadione. The indoloquinone anticancer drug EO9 is a somewhat better substrate for DT-diaphorase than is geldanamycin, as its reduction rate is only 6-fold lower than that reported for menadione [28]. DT-diaphorase is believed to catalyse a two-electron reduction of EO9 to a DNA-damaging alkylating species, and HT29 cells are 15- to 30-fold more sensitive to EO9 than are BE cells, which lack DT-diaphorase activity [24]. Mitomycin C is a much poorer substrate for DT-diaphorase than is either EO9 or geldanamycin, yet it shows a clear differential towards HT29 versus BE cells [15, 20].

In the present study there was only a 1.4-fold difference in the sensitivity of the HT29 and BE cells to geldanamycin, suggesting that although this benzoquinoid ansamycin antibiotic can be reduced by DT-diaphorase, this does not represent an important activation (or detoxification) step. This conclusion is supported by the observation that, in contrast to our previous experience with mitomycin C and EO9 [9], there was no correlation between DT-diaphorase expression and sensitivity to either geldanamycin or herbimycin A across the NCI human tumour cell-line panel (Paull, personal communication). Interestingly, in the HT29 and BE cell sonicates a significant proportion of the reduction of geldanamycin was not inhibited by the potent DT-diaphorase inhibitor dicoumarol, indicating that other enzyme(s) were present in the sonicates that could metabolise the drug. Such enzymes may include cytochrome b5 reductase and cytochrome P450 reductase, which are present in these and other human tumour cell lines [9, 15, 20]. Studies by other investigators have shown that cytochrome P450 reductase is capable of reducing both geldanamycin and herbimycin A to reactive hydroxyl radicals [1], and this may have contributed to the dicoumarol-insensitive geldanamycin reduction seen in the HT29 sonicates. Although radical products were seen in cells, the role of these in drug sensitivity was not clear [1, 2]. Moreover, we saw no DNA damage in human tumour cells exposed to pharmacological and supralethal concentrations of geldanamycin as measured by alkaline elution and sister chromatid exchange [12].

In conclusion, the present results rule out two of the previously proposed hypotheses for the mode of action of geldanamycin as far as human colon-carcinoma cell lines are concerned. With respect to the cellular oncoprotein c-Src, the effects of geldanamycin could not be explained by inhibition of c-Src tyrosine kinase activity, degradation of c-Src protein or inhibition of the putative association with PI3 kinase (results not shown). Moreover, unlike the case for quinone bioreductive agents such as mitomycin C and indoloquinone EO9, the evidence rules out

reductive metabolism by DT-diaphorase and indicates that DNA damage is not involved [12]. Recent results have highlighted the potential importance of degradation of oncoproteins, including v-Src, c-Lck and erbB2 (as well as the progesterone/glucocorticoid receptors), and point to a mode of action involving binding of geldanamycin to chaperonin proteins such as the heat-shock protein hsp90 and the glucose-regulated protein grp94, leading to disruption of stable heterocomplexes and degradation via polyubiquitinylation and proteosomal degradation [6, 11, 13, 14, 21, 30, 32]. In this model it appears that geldanamycin targets other than c-Src are important for the effects on cell survival, which may include disruption of such complexes.

Acknowledgements This work was funded by the UK Cancer Research Campaign, and Paul Workman acknowledges the receipt of a Cancer Research Campaign Life Fellowship.

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