15-Deoxyspergualin inhibits eukaryotic protein synthesis through eIF2 α phosphorylation

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DSG (15-deoxyspergualin), an immunosuppressant with tumoricidal properties, binds potently to the regulatory C-terminal 'EEVD' motif of Hsps (heat-shock proteins). In the present study we demonstrate that DSG inhibits eukaryotic protein synthesis by sequestering Hsp70 which is required for maintaining HRI (haem-regulated inhibitor), a kinase of the eIF2 α (eukaryotic initiation factor 2 α), inactive. DSG stalled initiation of protein synthesis through phosphorylation of HRI and eIF2 α . Addition of a recombinant eIF2 α (S51A) protein, which lacks the phosphorylation site, lowered the inhibitory potential of DSG in reticulocyte

lysate. The inhibitory effect of DSG was also attenuated in HRI knockdown cells. Moreover, exogenous addition of Hsp70 or the peptide 'EEVD' reversed the inhibitory effect of DSG. Interestingly, the inhibitory effect of DSG in different mammalian cancer cells was found to negatively correlate with the amount of Hsp70 expressed in the cells, emphasizing the link with Hsp70 in DSG inhibition of eukaryotic translation.

Key words: 15-deoxyspergualin, eukaryotic initiation factor 2 α , haem-regulated inhibitor, heat-shock protein, protein synthesis.

INTRODUCTION

The translation of an mRNA requires the formation of a complex of methionyl-tRNA, mRNA and the ribosome, a feat achieved in eukaryotic cells through the intervention of the GTP-bound form of the initiation factor eIF2 (eukaryotic initiation factor 2) [1]. GTP-bound eIF2 associates with methionyl-tRNA, and GTP hydrolysis facilitates the recognition of the mRNA start site. Subsequent recycling of eIF2 involves the exchange of the GDP formed for GTP and is mediated by the guanine nucleotide exchange factor, eIF2B [2]. This exchange is inhibited by phosphorylation of Ser⁵¹ of eIF2 α which promotes tight binding of eIF2B to the phosphorylated eIF2 (α -P)-GDP [3]. Therefore phosphorylation of eIF2 α blocks protein synthesis by preventing eIF2 recycling and depleting the cell of its pool of eIF2-GTP required for translation initiation [4], and is one of the major means of translational regulation.

Several conditions of stress trigger the phosphorylation of the Ser⁵¹ of eIF2 α via the autophosphorylation of the serine/threonine protein kinase, eIF2 α kinase [5]. There are at least four different eIF2 α kinases, each responding to different forms of cellular stress: PERK (PKR-like endoplasmic reticulum associated kinase) which responds to stress-induced malfunctioning of protein folding in the endoplasmic reticulum [6], GCN2 (general control non-derepressible 2) which responds to amino acid starvation, purine limitation and DNA damage [7], PKR (double-stranded RNA-activated protein kinase) and HRI (haem-regulated inhibitor) or HCR (haem-controlled repressor) which are activated during viral infection and haem deficiency respectively [8].

Hsps (heat-shock proteins) are known to play an important role in the activation of HRI [9–11]. Matts et al. [9] have demonstrated that activation of HRI is a consequence of Hsp70 titration by denatured proteins during conditions of stress. Moreover, direct evidence supports the modulation of activation of HRI by Hsp70

[10,11]. The ability of hemin to restore protein synthesis in haem-deficient reticulocyte lysates correlates roughly with the levels of Hsps in the lysate, and addition of Hsp70 reverses inhibition of protein synthesis in haem-deficient reticulocyte lysates [12,13]. It follows that sequestration of Hsps during stress could be a molecular signal for the activation of HRI and ultimately the inhibition of protein synthesis [12].

An analogue of spergualin isolated from the culture broth of *Bacillus laterosporus*, DSG (15-deoxyspergualin) has been studied for its anti-tumour and immunosuppressant properties [14,15]. The mechanism of its action however remains obscure as DSG inhibits an array of seemingly unrelated cellular activities ranging from a decrease in cellular cytochrome *c* oxidase activity [16] to depletion of polyamines [17]. Despite the uncertainty in its actual mechanism of action, DSG is known to potentially bind the cellular chaperones Hsp70 and Hsp90 [18,19]. DSG interacts with the Hsps through the C-terminal regulatory 'EEVD' motif without affecting their chaperone activity and, in fact, ATPase activity of Hsp70 is enhanced slightly in the presence of DSG [18,20]. It is therefore plausible that DSG modulates a regulatory function of Hsps. One such function could be the regulation of HRI, which is maintained in an inactive form by association with Hsp70 [9–11]. We hypothesized that sequestration of Hsps by DSG could lead to the inhibition of protein synthesis through the activation and autophosphorylation of HRI. Phosphorylated HRI would then phosphorylate eIF2 α , depleting the cell of its pool of eIF2-GTP required for translation initiation, consequently leading to protein synthesis inhibition and cell death [4].

Though only extensively investigated in rabbit reticulocytes, studies suggest that HRI might also be involved in protein synthesis regulation in several non-erythroid cells [21–24]. Further, Hsps are expressed in increased levels in many cancers, and play an important role in permitting autonomous growth [25]. This makes Hsps a tempting target for the design of cancer therapeutic

Abbreviations used: Ct, threshold cycle; DSG, 15-deoxyspergualin; eIF2, eukaryotic initiation factor 2; HRI, haem-regulated inhibitor; Hsp, heat-shock protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; RT, reverse transcriptase; siRNA, small interference RNA; TPR, trypsin-coated peptide repeat domain; TCA, trichloroacetic acid.

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agents. Given the reports of anti-tumour activity of DSG, it seems probable to us that DSG can kill cancer cells through the pathway hypothesized. In the present paper we therefore decided to elucidate the cascade of events involved in the regulation of eukaryotic protein synthesis initiation on the addition of DSG.

MATERIALS AND METHODS

Materials

DSG was a gift from Nippon Kayaku Co. The radioisotopes, [^{35}S]methionine and [γ - ^{32}P]ATP were from PerkinElmer. [^3H]thymidine was from Amersham Biosciences. The peptide 'EEVD' was custom synthesized by Genscript. Polyclonal antibodies to eIF2 α and serine-phosphorylated eIF2 α were from Cell Signaling, and anti-Hsp70, anti-Hsp90 and anti-HRI antibodies were obtained from Santa Cruz Biotechnology. All other reagents used were of the highest grade available. The Hsp70 protein (GenBank[®] accession number M19753.1, GI:309689), with and without the C-terminal 'EEVD' motif, was PCR-cloned into pET28a+, overexpressed in BL21-DE3 cells and purified. It was confirmed to possess chaperone activity by light scatter measurements (48 °C at A_{360}) of model protein substrates, alcohol dehydrogenase and glutamate dehydrogenase before use [20]. Clones expressing wild-type eIF2 α and mutant eIF2 α (S51A) proteins were a gift from Mr Rajesh and Professor K. V. A. Ramaiah (University of Hyderabad, India) and were expressed and purified following a previously reported protocol [26]. KNK437 was a gift from Dr Chandrima Shaha (National Institute of Immunology, India).

Preparation of reticulocyte lysate and *in vitro* translation of proteins in rabbit reticulocyte lysate

Reticulocyte lysate was prepared and cell-free protein synthesis with [^{35}S]methionine (10 μCi ; 800 Ci/mmol) was performed in a 25 μl assay mixture using standard protocols [27,28]. Briefly, the reaction was initiated by the addition of [^{35}S]methionine to the reaction mix containing freshly thawed reticulocyte lysate, incubated at 30 °C and terminated by spotting 6 μl aliquots at 0 min, 15 min, 30 min and 45 min on to TCA (trichloroacetic acid)-soaked Whatman 3 filter discs. The filters were immediately dried under an IR lamp source, washed in 10 % TCA at room temperature (28 °C) for 10 min, transferred to boiling 10 % TCA for 10 min, then to hot 10 % TCA with hydrogen peroxide (3 % final concentration) for 5–7 min, followed by washes in ice-cold 10 % TCA for 5 min, 1:1 absolute ethanol/diethyl ether for 5 min and finally in diethyl ether for 5 min [29]. The discs were air-dried for 10 min, placed in vials containing scintillation fluid and the radioactivity counted in a Hewlett Packard Liquid Scintillation counter.

In vitro phosphorylation of proteins in rabbit reticulocyte lysate

The status of phosphorylation of proteins in the lysates was assessed by replacing [^{35}S]methionine with non-radioactive methionine, and labelling the proteins in the *in vitro* translation reaction with [γ - ^{32}P]ATP (25 μCi , 3000 Ci/mmol). The mixture was incubated at 30 °C for 5 min before [γ - ^{32}P]ATP addition, and terminated 2–3 min after by the addition of freshly prepared reducing buffer containing SDS. The samples were analysed on polyacrylamide gels, and subjected to Western blot analysis with specific antibodies and autoradiography [29]. To examine the effect of Hsps on phosphorylation, purified and concentrated recombinant Hsp (125 μl of a 50 μM solution) was incubated on ice with or without DSG (added to a final concentration of 50 μM),

the solution was subjected to centrifugation through a protein-concentrating device [MWCO (molecular-mass cut off) 10 kDa Centricon], the filtrate was freeze-dried to a convenient small volume (2.5 μl) and added to the *in vitro* translation assay (25 μl). The peptide 'EEVD' was custom synthesized by Genscript.

Immunoprecipitation of interacting proteins from reticulocyte lysate

Lysates incubated with or without DSG were subjected to *in vitro* phosphorylation with [γ - ^{32}P]ATP (25 μCi , 3000 Ci/mmol). The samples were diluted 10 times with buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl and 5 mM EDTA, incubated with anti-HRI antibody, and processed for immunoprecipitation [29]. Prior to incubation of the lysate with Protein A-Sepharose beads complexed with anti-HRI antibody, the lysate was pre-cleared with Protein A-Sepharose beads complexed with goat anti-rabbit IgG. This was performed to rule out the possibility of any non-specific binding of the co-precipitating proteins, eIF2 α and Hsp70. Immunoprecipitates were examined by SDS/PAGE, Western blot analysis and autoradiography.

Assessment of growth inhibition of EL4, CV1, K562 and Jurkat cells

EL4, K562 and Jurkat cells were maintained in RPMI medium supplemented with 2 mM glutamine, 100 units/ml penicillin/streptomycin and heat-inactivated 10 % fetal bovine serum at 37 °C. To assess growth inhibition by DSG, EL4, CV1 and K562 cells were cultured in a 96-well plate in medium with varying concentrations of DSG, incubated at 37 °C and subsequently assayed for cell viability by incubation with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; added to a final concentration of 0.5 mg/ml] for 2 h, followed by addition of acidified propanol and measurement of A_{570} . Viability was also confirmed by microscopy using Trypan Blue exclusion of treated cells. To determine whether cells died by apoptosis or necrosis, cells were stained with Acridine Orange and ethidium bromide without fixing the cells. Apoptotic cells were identified by morphology and staining of the nucleus [30]. Viability of Jurkat cells was monitored by [^3H]thymidine (0.5 μCi /well) uptake in cells incubated with varying concentrations of DSG. Cells were harvested on to glass fibre filters using a Nunc cell harvester, the filters were washed, and the radioactivity measured using a liquid scintillation counter.

Metabolic labelling of EL4 proteins *in situ*

EL4 cells treated with varying concentrations of DSG were incubated with [^{35}S]methionine (PerkinElmer; 180 μCi /ml of culture) for 30 min. The cells were then lysed on Whatman 3 filter discs presoaked in 10 % TCA, washed with TCA and the radioactivity was measured in a liquid scintillation counter.

Assessment of growth inhibition of rabbit reticulocytes

Reticulocytes enriched in rabbit blood were cultured by a modified protocol of Tucker and Young [31]. Briefly, blood from the anaemic rabbit was filtered through glass wool aseptically to remove white blood cells and platelets, then washed thoroughly with saline and suspended in 2.5 volumes of RPMI 1640 medium supplemented with 20 % fetal calf serum. The reticulocyte cultures were incubated in the presence of [^{35}S]methionine (50 μCi /well, 800 Ci/mmol) and varying concentrations of DSG for 12 h in a 96-well plate, cells were lysed on TCA-soaked Whatman 3 filter discs, and the radioactivity measured in a liquid scintillation counter following washing with TCA as described earlier.

In vitro phosphorylation of proteins in lysates of EL4, CV1, K562 and Jurkat cells

The status of phosphorylation of proteins in the lysates was assessed by labelling the proteins in the lysates with [γ - 32 P]ATP (25 μ Ci, 3000 Ci/mmol). The samples were analysed on polyacrylamide gels, and subjected to Western blot analysis with specific antibodies and autoradiography [29].

Knocking down HRI in EL4 cells

HRI knockdown in EL4 cells was performed using annealed Silencer[®] pre-designed siRNA (small interference RNA) for the mouse HRI gene (GenBank[®] accession number NM.013557) obtained from Ambion. EL4 cells were mock transfected or transfected with negative control siRNA or the siRNAs (HRI siRNA 1: sense 5'-GCUCCAAGUGUAAAAGAGtt-3' and antisense 5'-CUCUUUUAACACUUGGAGCtc-3'; HRI siRNA 2: sense 5'-GGUCCGGAACAAUUAAGAUtt-3' and antisense 5'-AUCUAUUGUUCGACACtc-3'; HRI siRNA 3: sense 5'-GCGCAA-CUAAAACAGAUUGtt-3' and antisense 5'-CAAUCUGUUU-AGUUGCGCtc-3') designed for HRI by electroporation. EL4 cells in log phase (2×10^6 cells/ml) were suspended in siPORT[™] siRNA electroporation buffer supplied by Ambion. Electroporation was conducted in 1 mm cuvettes (containing 75 μ l cell suspension and 1–3 μ g siRNA of interest) in a BTX600 electroporator using the following parameters: low voltage mode, 60 V, 560 μ F capacitance and 13 ohms resistance timing to get a time constant of approx. 6–7 ms. Following electroporation, cells were incubated in the cuvettes for 10 min at 37 °C following which they were dispensed into vials with 1 ml of growth medium. Knockdown was assessed by mRNA and protein quantification. Total RNA was extracted from cells 48 h after transfection using a Qiagen RNA extraction kit. RT (reverse transcriptase)-PCR was carried out using MMLV (Moloney-murine-leukaemia virus)-reverse transcriptase and specific reverse primers for 18S rRNA and HRI. Real-time PCRs for 18S rRNA and HRI were performed using the BioRad SYBR Green/Fluorescein PCR master mix and primers 5'-AGAAATGGGAAAG-GAACACG-3' and 5'-ACTTGGCATCGTACTGGGAT-3', and 5'-TGATTAAGTCCCTGCCCTTT-3' and 5'-CAAGTTCGACCGTCTTCTCA-3' respectively on a BioRad iCycler real-time PCR machine. PCR conditions used were 1 cycle at 95 °C for 10 min; 35 cycles of 94 °C for 1 min, 63 °C for 1 min, 72 °C for 6 s; and 34 cycles of 60 °C–94 °C. The presence of a single amplicon following PCR was confirmed by post-PCR melt analysis on the iCycler machine as well as by agarose gel electrophoresis. The extent of HRI knockdown was determined by calculating mRNA levels using the formula: relative transcript level = $1000 \times 2^{-(\text{del Ct})}$; where $\text{del Ct} = \text{Ct (target gene HRI)} - \text{Ct (constitutive control 18S rRNA)}$, and Ct is the threshold cycle number calculated from the real-time PCR run. Protein levels in cells were determined 96 h following transfection by SDS/PAGE and Western blot analysis of cell lysates. The susceptibility of transfected cells to DSG was determined by the cell viability assays outlined earlier.

Quantification of protein levels

Quantification of protein and phosphorylation levels was carried out by analysing the intensity of bands in a Western blot or autoradiogram using the Science Lab Image Gauge software.

RESULTS

To investigate the effect of DSG on the translation initiation factor, eIF2 α , we first used a standard *in vitro* translation system, i.e. the

Figure 1 Effect of DSG on basal and haem-dependent *in vitro* translation in rabbit reticulocyte lysate determined by [35 S]methionine incorporation in TCA-precipitated proteins

DSG at concentrations greater than 500 μ M inhibited protein synthesis, in a similar manner to cycloheximide (CYL) and chloroquine (CHQ). Inset in Figure 1: the IC₅₀ of protein synthesis inhibition by DSG was between 158 μ M and 501 μ M for three preparations of rabbit reticulocyte lysate as determined by conducting *in vitro* translation in rabbit reticulocyte lysate in the presence of [35 S]methionine and varying concentrations of DSG. IC₅₀ of DSG in the presence and absence of 20 μ M hemin in this representative plot is 446 μ M and 501 μ M.

rabbit reticulocyte lysate. Prior to assaying DSG, we tested the rabbit reticulocyte lysate for its translation efficiency and hemin dependence (both of which are indices of the integrity of the system) and confirmed that the lysate was capable of haem-dependent protein synthesis. The preparation of rabbit reticulocyte lysate used in the present study had a protein synthesis efficiency of approx. 450 000 cpm incorporated [35 S]methionine/30 min/5 μ l of lysate, and shut down protein synthesis by approx. 60% in the absence of hemin, which is similar to lysates reported previously in the literature [12].

DSG decreased the incorporation of [35 S]methionine into proteins, as did cycloheximide and chloroquine (Figure 1). We studied the inhibition of protein synthesis in the lysate as a function of DSG concentration and determined the IC₅₀ of DSG for protein synthesis *in vitro* to be between 158 μ M and 501 μ M with various preparations of the lysate. We also followed the inhibition of protein synthesis by DSG in the rabbit reticulocyte lysate in the presence and absence of haem (inset in Figure 1). The IC₅₀ of DSG for protein synthesis in the presence or absence of 20 μ M haem in the representative plot was not significantly different at 446 μ M and 501 μ M respectively (inset in Figure 1). There was a time lag of approx. 30 min before protein synthesis in the reticulocyte lysate was inhibited by DSG.

We next followed the phosphorylation of HRI and eIF2 α in reticulocyte lysate using [γ - 32 P]ATP, and anti-rabbit HRI and anti-rabbit eIF2 α antibodies. Both HRI and eIF2 α were significantly phosphorylated by haem deficiency and by chloroquine as expected [29] (Figures 2A and 2B). Importantly, eIF2 α and HRI were phosphorylated in lysates treated with DSG (Figures 2A and 2B). We also confirmed by Western blot analysis with an anti-phosphorylated Ser⁵¹-specific eIF2 α antibody that DSG induced eIF2 α phosphorylation (Figure 2C). The phosphorylation of HRI and eIF2 α was DSG concentration-dependent (Figure 2D) and the time lag observed in protein synthesis inhibition by DSG (Figure 1) was reflected in the status of phosphorylation of eIF2 α

Figure 2 The status of phosphorylation of eIF2 α and HRI in the rabbit reticulocyte lysate was determined in the presence and absence of haem, and in the presence of chloroquine (CHQ), cycloheximide (CYL) and DSG by performing the *in vitro* translation in the presence of [γ - 32 P]ATP

Phosphorylated proteins of the lysate were resolved by SDS/PAGE, transferred onto PVDF membrane and visualized using autoradiography. The protein of interest was identified by probing the Western blot with a specific antibody. (A) Upper panel: autoradiogram of blot; lower panel: Western blot probed with an anti-eIF2 α antibody. (B) Upper panel: autoradiogram of blot; lower panel: Western blot probed with an anti-HRI antibody. (C) Upper panel: Western blot probed with an anti-phosphorylated-Ser⁵¹-specific eIF2 α antibody; lower panel: stripped blot re-probed with an antibody to total eIF2 α . (D) DSG concentration-dependent phosphorylation status. Upper panels: autoradiograms of blots; lower panels: Western blot analysis with anti-eIF2 α and anti-HRI antibodies. (E) Status of phosphorylation of eIF2 α in the rabbit reticulocyte lysate was determined in the presence of varying concentrations of DSG by performing *in vitro* translation in the presence of [γ - 32 P]ATP. Phosphorylated proteins of the lysate were resolved by SDS/PAGE, transferred to a PVDF membrane and visualized using autoradiography. The experiments were performed three times, and the phosphorylation status was found to be reproducible.

as determined by autoradiography following Western blot analysis with an anti-eIF2 α antibody (Figure 2E).

If DSG does indeed inhibit protein synthesis through the phosphorylation of HRI and eIF2 α , then addition of a mutant eIF2 α lacking the phosphorylation site, Ser⁵¹, should reverse protein synthesis inhibition by DSG. The exogenous addition of protein dialysis buffer or wild-type eIF2 α protein to reticulocyte lysate did not affect *in vitro* translation or inhibition by DSG (Figure 3A). However, addition of a mutant S51A eIF2 α protein could prevent inhibition of protein synthesis by DSG (Figure 3A). In S51A eIF2 α -supplemented lysates, even 2 mM DSG did not inhibit translation by even 50 %. The status of phosphorylation of eIF2 α

Figure 3 eIF2 α with phosphorylatable Ser⁵¹ is required for the inhibitory action of DSG

(A) *In vitro* translation in reticulocyte lysate in the presence of exogenously added wild-type and mutant S51A eIF2 α protein and varying concentrations of DSG. The bars represent the TCA precipitable cpm obtained which are a measure of protein synthesis at 0 min, 15 min and 45 min of the *in vitro* translation reaction. (B) Autoradiogram depicting the phosphorylation status of eIF2 α in reticulocyte lysate in the presence of exogenously added wild-type and mutant S51A eIF2 α protein and varying concentrations of DSG. The experiment was performed twice and the results were reproducible.

in these reactions also mirrored these results (Figure 3B). As expected, DSG-treated lysates, to which protein dialysis buffer or wild-type eIF2 α had been added, contained phosphorylated eIF2 α . However, when S51A eIF2 α had been added, even in the presence of 2 mM DSG, there was virtually no phosphorylated eIF2 α (Figure 3B). Our results thus confirmed that DSG did indeed inhibit protein synthesis through phosphorylation of eIF2 α and HRI in reticulocyte lysate, albeit at high micromolar concentrations.

We next ascertained the effect of DSG *in vivo* on mouse lymphoma EL4 cells. DSG inhibited EL4 cell growth after approx. 96 h with an IC₅₀ of 17 nM as assayed by the MTT cell viability assay (Figure 4A). Importantly, protein synthesis was inhibited between 84 and 96 h with an IC₅₀ of 7.6 nM, as assayed by *in situ* protein labelling, suggesting that protein synthesis was a target of DSG (Figure 4B). The phosphorylation status of eIF2 α was also followed over time. eIF2 α was found to be phosphorylated for only approx. 72–84 h (results not shown). The delay in the action of the drug seems to be typical of Hsp inhibitors. Geldanamycin, a well known inhibitor of Hsp90, takes around 48 h to block the Hsp90-dependent activation of the VEGF (vascular endothelial growth factor) receptor by VEGF, by lowering receptor levels and the ability of the receptor to undergo Hsp90-dependent maturation, and bring about apoptosis in HL-60 cells, and 72 h to inhibit phosphorylation of the RET (rearranged during transfection) tyrosine kinase and cause subsequent apoptosis in medullary

Figure 4 Effect of DSG on EL4 cells

(A) Inhibition of growth of EL4 cells monitored by the MTT assay (IC_{50} : 17 nM). (B) Inhibition of protein synthesis of EL4 cells monitored by *in situ* labelling of proteins with [35 S]methionine (IC_{50} : 7.6 nM). (C–G) Acridine Orange and ethidium bromide staining of EL4 cells untreated (C) or treated with 100 pM (D), 10 nM (E), 1 μ M (F) and 10 μ M (G) DSG. Live intact cells are only stained with Acridine Orange and have normal nuclei staining which presents as green staining of the chromatin. Necrotic cells have damaged membranes and are additionally stained with ethidium bromide and hence fluoresce orange. Apoptotic cells have condensed or fragmented chromatin which fluoresces green or orange. A few representative apoptotic cells are indicated with white arrowheads in the Figures. Growth inhibition of EL4 cells by DSG was repeated at least six times and the inhibition constant found to be in the same low nanomolar range.

thyroid carcinoma cells [32,33]. Moreover, akin to protein synthesis inhibitors, DSG, up to a concentration of 1 μ M, invoked cell death through apoptosis that could be visualized by Acridine Orange and ethidium bromide staining (Figures 4C–4F). Necrotic cells were observed upon treatment with ≥ 10 μ M DSG (Figure 4G), suggesting non-specific effects or other targets for DSG beyond this concentration.

There are several instances in the literature where the same inhibitor inhibits different cells through the same mechanism but with varying inhibitory potency. A notable example is that of methotrexate-resistant cells brought about by the increased levels of dihydrofolate reductase through gene amplification [34]. However, considering the widely differing values of IC_{50} for protein synthesis inhibition in reticulocytes and EL4 cells, we decided to confirm that DSG also acts through the same mechanism of protein synthesis inhibition in EL4 cells. If DSG does inhibit cell growth through phosphorylation of eIF2 α , then knocking out HRI, the kinase that phosphorylates eIF2 α , should make cells resistant to DSG treatment. We knocked down HRI in EL4 cells using siRNAs specific for HRI. 18S rRNA was used as the constitutive control RNA in all cases (Figure 5A). Of the three

Figure 5 Effect of DSG on HRI knockdown cells

(A) Real-time PCR plot of cycle number versus relative fluorescence units. 18S rRNA from untransfected EL4 cells, mock-transfected or cells transfected with negative control siRNA or siRNA specific to HRI, was quantified using real-time RT-PCR. (B) Real-time PCR plot of cycle number versus relative fluorescence units. HRI mRNA from untransfected EL4 cells, mock-transfected or cells transfected with negative control siRNA or siRNA specific to HRI, was quantified using real time RT-PCR. (C) Table of IC_{50} values of DSG and relative HRI transcript levels of EL4 cells, mock-transfected or cells transfected with negative control siRNA or siRNA specific to HRI. (D) Western blot analysis of cell lysates of EL4 cells transfected with negative control siRNA or siRNA specific to HRI (HRI siRNA 2) probed with an anti-HRI antibody. (E) Band intensities of HRI and actin from the Western blot in (D) have been plotted. The experiment was conducted twice and results were reproducible.

siRNAs tested, while transfection with HRI siRNA 1 brought about a modest decrease in the cellular level of HRI mRNA, HRI siRNA 2 decreased HRI transcript levels from 14.6 to 0.33 (Figures 5B and 5C). HRI siRNA 3 did not decrease the relative transcript levels of HRI at all (Figures 5B and 5C). The amount of HRI protein was analysed by Western blot analysis of cell lysates of mock-transfected and HRI siRNA 2-transfected cells with an anti-HRI antibody, and was found to be reduced in HRI siRNA 2-transfected EL4 cells (Figure 5D). Transfected cells were also subjected to treatment with varying concentrations of DSG to determine the IC_{50} for DSG. The IC_{50} for DSG increased approx. 9-fold from 42 nM in mock-transfected cells to 394 nM in cells transfected with HRI siRNA 2, confirming that HRI was indeed a player in the mechanism of action of DSG (Figure 5C). The residual activity of DSG in HRI knockdown cells could be due to the limitation of low transfection efficiency (approx. 60 % in EL4 cells using our transfection protocol) and the probable existence of secondary targets for DSG.

Figure 6 Role of Hsp70 in protein synthesis inhibition by DSG

(A) Phosphorylation of proteins in the lysate was performed in the presence of exogenously added Hsp70 with the 'EEVD' motif (Hsp70), Hsp70 lacking the 'EEVD' motif (Hsp70-EEVD), and peptide 'EEVD' with and without DSG. Upper panel: autoradiogram of blot; lower panel: blot probed with an anti-eIF2α antibody. Exogenously added 'EEVD' reversed eIF2α phosphorylation. (B and C) HRI was immunoprecipitated from reticulocyte lysates incubated with or without DSG, and the co-immunoprecipitating eIF2α and Hsp70 analysed by Western blot analysis with (B) an anti-phosphorylated-Ser⁵¹-specific eIF2α antibody and (C) an anti-Hsp70 antibody. Following DSG treatment, the amount of phosphorylated eIF2α interacting with HRI increased and the amount of interacting Hsp70 decreased. The blot was stripped and probed with an anti-HRI antibody to confirm equal loading (results not shown). The experiments were conducted twice and results found to be reproducible.

Having dissected the roles of eIF2α and HRI in the cascade triggered off by DSG, we looked at the role of Hsp70. Using rabbit reticulocyte lysate, we determined that eIF2α phosphorylation could be averted if DSG was incubated along with the tetrapeptide 'EEVD' or with purified Hsp70 protein containing the C-terminal 'EEVD' motif (Figure 6A). Incubation with Hsp70 lacking the C-terminal 'EEVD' motif could not reverse phosphorylation (Figure 6A). This suggested that the interaction of DSG with the tetrapeptide motif 'EEVD' on the Hsp is responsible for the autophosphorylation of HRI.

We used immunoprecipitation analysis to verify that DSG interferes with the interaction of Hsp70 and HRI. Using antibodies to HRI, eIF2α and Hsp70 were also pulled down, demonstrating that they interact with HRI. The amounts of co-immunoprecipitating eIF2α and Hsp70 were analysed by Western blot analysis following SDS/PAGE. Using an anti-phosphorylated Ser⁵¹-specific eIF2α antibody and an anti-Hsp70 antibody, it was observed that the presence of DSG in the lysate decreased the amount of Hsp70 interacting with HRI and increased that of phosphorylated eIF2α, confirming our proposed scheme for DSG action (Figures 6B and 6C).

Figure 7 The effect of DSG in various cell lines

(A) Effect of DSG on growth of mammalian cells, EL4 (IC₅₀: 17.1 ± 1.63 nM), CV1 (IC₅₀: 169.3 nM ± 2.2 nM), K562 (IC₅₀: 1619.9 ± 12.25 nM), Jurkat cells (IC₅₀: 42393 ± 110.1 nM) and reticulocytes (IC₅₀: 158.8 ± 0.1 μM). (B) Effect of DSG on protein synthesis of mammalian cells, EL4 (IC₅₀: 7.6 ± 2.0 nM), CV1 (IC₅₀: 67.2 ± 18.2 nM), K562 (IC₅₀: 1.1 ± 0.13 μM), Jurkat cells (IC₅₀: 4.5 ± 1.1 μM) and reticulocytes (IC₅₀: 158.8 ± 0.1 μM). (C) Western blot of cell lysates from EL4 and CV1 cells treated with varying concentrations of DSG probed with an anti-phosphorylated-Ser⁵¹-specific eIF2α antibody. eIF2α is phosphorylated by DSG in EL4 and CV1 cells. (D) Autoradiogram of Western blot of K562 and Jurkat cells treated with varying concentrations of DSG probed with an anti-eIF2α antibody. eIF2α is phosphorylated by DSG in K562 and Jurkat cells. The effect of DSG in the various cell lines was determined at least twice and results found to be reproducible.

Since exogenously added Hsp70 affected the inhibitory potency of DSG in the reticulocyte lysate system, we wondered whether there was any correlation between the inhibitory potency of DSG and the level of Hsp70 *in vivo*. We determined the IC₅₀ of growth inhibition by DSG in EL4, CV1, K562, Jurkat cells and reticulocytes. EL4 was most sensitive to DSG with an IC₅₀ in the low nanomolar range (17.1 ± 1.63 nM in the representative plot), followed by CV1 and K562 with IC₅₀ values of 169.3 nM ± 2.2 nM and 1619.9 ± 12.25 nM respectively, Jurkat cells with an IC₅₀ of 42393 ± 110.1 nM and reticulocytes with an IC₅₀ of 158.8 ± 0.1 μM (Figure 7A). Protein synthesis was also inhibited by DSG in all these cell lines with IC₅₀ values of 7.6 ± 2.0 nM for EL4 cells, 67.2 ± 18.2 nM for CV1 cells, 1.1 ± 0.13 μM for K562 cells and 4.5 ± 1.1 μM for Jurkat cells (Figure 7B). Furthermore, DSG induced phosphorylation of eIF2α in EL4, CV1, K562 and Jurkat cells, and the concentration of DSG required for eIF2α phosphorylation was in the same

Figure 8 Hsp levels in various cell lines

(A) Western blot analysis of cell lysates of EL4, K562 and Jurkat cells probed with anti-actin, anti-Hsp70, anti-Hsp90 and anti-eIF2 α antibodies. (B) Plot of normalized band intensity of Hsp70, Hsp90 and eIF2 α protein levels in EL4, K562 and Jurkat cells. The band intensities from the Western blots were normalized relative to actin levels in each of the cell lines, and plotted to give the protein levels of Hsp70, Hsp90 and eIF2 α . (C) Western blot analysis of reticulocyte lysate preparations probed with anti-actin and anti-Hsp70 antibodies. The IC₅₀ values of protein synthesis inhibition by DSG in the preparations 1, 2 and 3 were 501 μ M, 440 μ M and 158 μ M. The experiment was repeated twice and results found to be reproducible.

range as the concentration of DSG required for inhibition of cell growth in each of the cell types, suggesting that it inhibited cell growth through the same mechanism of protein synthesis inhibition in all of these cell types (Figures 7C and 7D). The concentration of DSG required for inducing eIF2 α phosphorylation correlated with that required for inhibition of cell growth. We also determined the levels of Hsp70, Hsp90, eIF2 α and actin in EL4, K562 and Jurkat cells, and found that Hsp70 and Hsp90 levels (normalized with respect to actin) were significantly less in EL4 as compared with K562 and Jurkat cells (Figures 8A and 8B). An alternate reason for the differential susceptibility of cell lines to DSG could be a difference in the level of eIF2 α in the cells. However, the level of eIF2 α (normalized with respect to actin) was constant in the three cell lines, ruling out this possibility (Figure 8B). The level of Hsp70 in three different preparations of reticulocyte lysate was also correlated with the IC₅₀ of protein synthesis inhibition by DSG in these lysates (Figure 8C). Moreover, the binding affinity of DSG for Hsps is known to be in the nanomolar range [35]. Thus the Hsp70 and Hsp90 protein levels could be considered to be negatively correlated with the DSG sensitivity of the cells.

If the correlation of Hsp70 levels with the DSG sensitivity of the cell lines is not a mere coincidence, it should, in principle, be possible to modulate the sensitivity of a given cell line to DSG by altering the cellular Hsp70 level. We achieved this by

Figure 9 Role of Hsp70 in DSG inhibition

(A) Inhibition of EL4 cells untreated or treated with KNK437 by DSG. The IC₅₀ of growth inhibition assayed by the MTT assay was reduced from 66.06 nM to 1.158 nM upon KNK437 treatment. (B) Inhibition of K562 cells untreated or treated with KNK437 by DSG. The IC₅₀ of growth inhibition assayed by the MTT assay was reduced from 3090 nM to 524.8 nM upon KNK437 treatment. (C) Western blot analysis of EL4 cell lysate prepared from cells untreated or treated with KNK437 probed with anti-Hsp70 and anti-actin antibody. (D) Western blot analysis of K562 cell lysate prepared from cells untreated or treated with KNK437 probed with anti-Hsp70 and anti-actin antibody. (E) Inhibition of EL4 cells untreated or treated with 10 μ M hydrogen peroxide for 12 h. The IC₅₀ of growth inhibition assayed by the MTT assay increased from 25.53 nM to 7389 nM upon hydrogen peroxide treatment. (F) Western blot analysis of EL4 cell lysate prepared from cells untreated or treated with 10 μ M hydrogen peroxide probed with antibodies specific to actin and Hsp70. The experiments were repeated twice and results were reproducible.

employing either an inhibitor of Hsp70 induction, KNK437, or hydrogen peroxide, which is known to enhance Hsp70 levels. While treatment with 100 μ M KNK437 for a period of 96 h itself caused loss of cell viability, and treatment with 100 μ M KNK437 for a period of 24 h or less did not bring about any change in the sensitivity of EL4 or K562 cells to DSG (results not shown), we found that incubation of the cell cultures with 100 μ M KNK437 from 60 h to 96 h, after addition of DSG, resulted in a heightened sensitivity of the cells to DSG. Following KNK437 treatment, the IC₅₀ values of DSG in EL4 and K562 cells decreased from 66.06 nM and 3090 nM to 1.16 nM and 524.8 nM respectively (Figures 9A and 9B). Hsp70 protein levels determined by Western blot analysis were reduced upon KNK437 treatment (Figures 9C and 9D). In the absence of any heat shock or other stress which is required for Hsp70 induction, the basal level of Hsp70 expression is expected to be low, and this is probably why prolonged

treatment with KNK437 was required to diminish Hsp70 levels in the cells. In contrast with KNK437 treatment, treatment of EL4 cells with 10 μ M hydrogen peroxide decreased the sensitivity of the cells to DSG. The IC₅₀ of DSG increased from 25.53 nM to 7389 nM upon hydrogen peroxide treatment (Figure 9E). An increase in Hsp70 levels was observed concomitant with this increased DSG tolerance (Figure 9F).

DISCUSSION

Organisms owe their cellular differentiation and functions to differential gene expression. Gene expression is regulated at various levels: transcriptional, post-transcriptional, translational and post-translational, and one of the major means of translational regulation is through phosphorylation of eIF2 α kinase, the protein kinase which governs the phosphorylation status of the initiation factor, eIF2 α , and hence the decision to continue or abort protein synthesis. Previous studies have indicated a role for Hsp70 in suppressing activation of HRI, a ubiquitous eIF2 α kinase, which is activated by phosphorylation during haem deficiency and various forms of cellular stress [9–11]. In the present paper we have explored the possibility that the tumoricidal agent, DSG, inhibits cell growth by sequestering Hsp70 and inhibiting protein synthesis through activation of eIF2 α kinase.

Our initial findings demonstrated that DSG not only inhibits protein synthesis in the rabbit reticulocyte lysate through phosphorylation of HRI and eIF2 α , but also that the inhibition is independent of the presence of haem. It is known that unlike regulation by haem, which involves the haem-binding domains of HRI, activation of HRI by other stresses such as arsenite, heat shock and oxidative stress is independent of haem binding, since phosphorylation of HRI during such stress is not reversed by the addition of haem, and requires living, intact cells. Presumably, these kinds of stress release reactive oxygen species either directly act on cysteine residues of HRI, thus altering its conformation, or bind cysteine-rich, redox-sensitive proteins such as thioredoxin or glutathione which in turn mediate HRI activation [36]. Our findings indicate a regulation by DSG which mediates HRI activation in lysates as well as in living, intact cells, yet activation of HRI is independent of haem binding.

As previously described, phosphorylation of the Ser⁵¹ residue on eIF2 α by an eIF2 α kinase is a prerequisite for protein synthesis regulation. In the present study, experiments employing either an eIF2 α lacking its Ser⁵¹ phosphorylation site or the tool of siRNA to knockdown cellular HRI confirmed the indispensability of HRI and phosphorylatable eIF2 α in the inhibition of cells by DSG.

Also, we demonstrated through immunoprecipitation and *in vitro* translation experiments with exogenously added Hsps or the peptide 'EEVD' that the DSG-induced eIF2 α kinase phosphorylation could be reversed by the 'EEVD' motif of Hsp70. *In lieu* of previous observations that DSG binds the 'EEVD' motif of Hsp70 [18,19] and that Hsp70 interacts with HRI [9–11], our results suggest that the interaction of HRI with Hsp70 involves the 'EEVD' motif. This is a distinct possibility as an anti-HRI antibody indeed pulls down Hsp70, and the amount of Hsp70 in the immunoprecipitate is compromised by the presence of DSG. This interaction could directly involve the C-terminal 'EEVD' motif of Hsp70, so that DSG and HRI compete to bind to the 'EEVD' motif. Alternatively, HRI could interact with the substrate-binding site of Hsp70 which in turn is regulated by DSG binding to the 'EEVD' motif. However, Nadler et al. [18] have previously demonstrated that Hsp70 chaperone activity is not affected by DSG binding, suggesting that the substrate-binding motif of Hsp70 is structurally distinct from the 'EEVD' motif. In fact,

Thulasiraman et al. [10] have demonstrated that peptides that bind Hsp70 can have differential effects on its chaperoning and regulatory activities. Thus while DSG may not affect Hsp70 'chaperone' activity it could directly affect its ability to interact with HRI. It seems therefore, that HRI directly binds to the 'EEVD' motif of Hsp70. While the 'EEVD' motif is typically thought to interact with a groove with basic residues such as formed by the TPR (tetratricopeptide repeat) domain of proteins [37], HRI is not known to have any TPR domains. It is also possible that a TPR is not required for this interaction. Takenaka et al. [38] have demonstrated that the 'EEVD' motif is also involved in binding some hydrophilic peptide substrates, not part of a TPR domain. Another alternative that should be noted is that DSG could block the interaction of Hsp70 with a co-chaperone that is needed for Hsp70 to bind and suppress HRI activity, as was the case in [38]. We have recently demonstrated that the Hsp70 interaction with its co-chaperone Hsp70-interacting protein, Hip, is also not affected by DSG [20]. Nevertheless, it is possible that the co-chaperone employed by Hsp70 for HRI binding is not Hip.

The actual mode of interaction between Hsp70 and eIF2 α kinase notwithstanding, our studies thus reveal that DSG targets protein synthesis in mammalian cells by compromising the cellular chaperone, Hsp70. Hsps are known to play an important role in tumour biology [25]. While Hsp70 expression is low and largely stress inducible in non-transformed cells, it is expressed abundantly in a wide range of tumours and is associated with poor prognosis and resistance to most anti-cancer therapies [39,40]. Earlier reports have established that endogenous or ectopic expression of Hsp70 can inhibit apoptosis due to a variety of anti-leukaemic agents [41,42]. Hsp70 has also been reported to abrogate apoptosis by interfering with nuclear DNA fragmentation, and by inhibiting lysosomal membrane permeabilization [43,44]. Attenuation of Hsp70 levels, in contrast, sensitizes human leukaemia cells to apoptosis by several anti-cancer agents as well as to radiation [45,46]. For instance, abrogation of Hsp70 induction in human leukaemia cells, K562, by siRNA or the agent KNK437, has been associated with increased 17-AAG-induced apoptosis [47]. Clearly, Hsp70 is an ideal target for the rational design of anti-cancer drugs.

Although efforts are being made, Hsps other than Hsp90 have not as yet been effectively targeted. While several small molecules, such as non steroidal anti-inflammatory agents, flavonoid kinase inhibitors and serine/threonine phosphatase inhibitors such as okadaic acid are known to modulate the expression of Hsp70 molecules, only derivatives of spargualin have been shown to directly alter Hsp70 function [18,48–50]. Several studies have focused on the immunosuppressant action of DSG in the past. However, the mechanism of the anti-tumour action of DSG has remained an enigma. The present study has not only shed light on the elusive cascade of events triggered by DSG, but has also served to highlight the anti-cancer potential of DSG across different cancer cells. We have established the negative correlation that exists between the cellular Hsp70 protein levels and the sensitivity of the given cell line to DSG. Taking this a step further, we also demonstrate how the sensitivity of cancer cells to DSG can be modulated by altering the levels of Hsp70 or one of its key downstream partners, HRI, involved in regulating protein synthesis and thereby permitting cell death or survival. Future endeavours in this direction could very well pave the way for more efficacious anti-cancer therapy that caters to both sensitive and resilient cancer cells.

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