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Geldanamycin, a potent and specific inhibitor of Hsp90, inhibits gene expression and replication of human cytomegalovirus

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The effect of geldanamycin (GA), a specific inhibitor of heat shock protein 90 (Hsp90), on gene expression and replication of human cytomegalovirus (HCMV) was studied in human embryonic lung (HEL) fibroblasts. Kinetic analysis indicated that GA delayed synthesis of major immediate early (MIE), early and late viral proteins, and blocked a second tier of the synthesis of these proteins that occurred in untreated cells after 48 h post-infection (pi). Moreover, when HCMV-infected HEL cells were maintained with medium containing 40 nM GA for 6 days, with medium changes at 2-day intervals, the virus yield was reduced to an undetectable level. On a molecular level, the cellular kinase Akt and the transcription factor NFκB were activated in HCMV-infected cells within 30 min pi. NFκB was shown to be essential for MIE gene expression. However, in GA-treated cells, activation of both Akt and NFκB was greatly inhibited. Because LY294002, an inhibitor of cellular phosphatidylinositol 3-kinase (PI3-K), also prohibited HCMV-mediated activation of Akt and NFκB and synthesis of the MIE proteins, PI3-K signalling was necessary for expressing the MIE genes. These results suggest that the inhibitory effect of GA on HCMV replication is primarily caused by the disruption of the PI3-K signalling pathway, leading to the activation of NFκB, which plays a crucial role in expression of the critical MIE genes.

Keywords: human cytomegalovirus, heat shock protein 90, geldanamycin, gene expression, virus replication

Introduction

Human cytomegalovirus (HCMV), a betaherpesvirus, is capable of establishing lifelong latent infection after primary infection *in vivo* (Mocarski *et al.*, 1990). The latent virus is reactivated during immunosuppression and sometimes causes devastating diseases, such as congenital malformation in newborns and interstitial pneumonia in immunocompromised hosts. Although a variety of stimuli may lead to reactivation *in vivo*, the molecular mechanisms controlling HCMV latency and reactivation are not well understood.

The immediate early (IE) genes of HCMV are the first genes expressed after infection without *de novo* viral protein synthesis. Transcription of the major IE (MIE) genes, IE1 (UL123) and IE2 (UL122), is regulated by a strong and complex enhancer located upstream of the MIE transcription unit. This enhancer contains several different non-repeat and repeat elements, with a binding site for known eukaryotic transcriptional factors (Chan *et al.*, 1996; Meier & Stinski, 1996). In addition to the cellular factors interacting with these elements, a structural component of the virion also indirectly transactivates the enhancer (Cherrington & Mocarski, 1989; Liu & Stinski, 1992; Malone *et al.*, 1990). Since the products of the IE1 and IE2 genes are required for efficiently activating promoters of the next temporal class (Fortunato & Spector, 1999; Mocarski & Courcelle, 2001; White *et al.*, 2004), these proteins are considered to play a key role in HCMV lytic cycle progression. Therefore, a better understanding of the nature of cellular factors involved in the regulation of MIE gene expression, and the molecular mechanisms by which these cellular factors regulate it, is important for controlling viral infection and clarifying the mechanism involved in HCMV latency and reactivation.

The heat shock proteins (Hsps) are a set of cellular proteins that are synthesized following various stresses, including heat shock and virus infection (Morimoto & Milarski, 1991). For example, expression of the 70 kDa Hsp (Hsp70) is induced (Santomenna & Colberg-Poley, 1990); biphasic translocation of this protein from cytoplasm to nucleus at early stages, and from nucleus to cytoplasm at late stages, after infection is observed in HCMV-infected human fibroblasts (Ohgitani *et al.*, 1999),

although little is known about the function of induced Hsp70 in virus replication. Hsp90 is one of the most abundant and highly conserved chaperone proteins. Unlike the more general Hsp70 and Hsp60 chaperones, Hsp90 appears to have substrate-specific folding activity. Recent studies have demonstrated an Hsp90 requirement for the proper function of, firstly, the mitogen-activated protein kinase pathway (Stancato *et al*., 1993; Wartmann & Davis, 1994; Lovric *et al*., 1994; Schulte *et al*., 1995; Jaiswal *et al*., 1996); secondly, the activity of several tyrosine kinases (Brugge, 1986; Hartson & Matts, 1994; Chavany *et al*., 1996); thirdly, the activity of several transcription factors, including p53 (Blagosklonny *et al*., 1995), retinoid receptors (Nair *et al*., 1996; Pratt & Toft, 1977) and hypoxia-inducible factor-1α (Gradin *et al*., 1996); and fourthly, the activity of the cyclin-dependent kinase CDK4 (Stepanova *et al*., 1996). These data indicate that Hsp90 plays an important role in the signal transduction pathway required for cell growth and survival. Therefore, the possibility has been suggested that Hsp90 may be involved in the regulation of HCMV gene expression. To understand the molecular mechanism by which cellular factors regulate MIE gene expression, in the present study we investigate whether Hsp90 has any effect on gene expression and replication of HCMV by using geldanamycin (GA), a specific inhibitor of Hsp90, in human embryonic lung (HEL) fibroblasts which are fully permissive for HCMV replication. The results indicate that the treatment of HCMV-infected HEL cells with GA preferentially blocks viral IE2 protein synthesis, thereby inhibiting synthesis of early and late proteins and, consequently, infectious progeny virus production. HCMV-mediated activation of Akt and induction of NFκB, essential for MIE gene expression in HEL cells, was blocked in GA-treated cells.

Materials and methods

Cells and virus

HEL cells, prepared from the lung of a 4-month-old female fetus, were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum and 2 mM L-glutamine in 5% CO_2 incubators at 37°C. All experiments were done using confluent HEL cells between passages 16 and 22. To reduce the basal levels of cellular gene expression, HEL cells were serum-starved before HCMV infection. The Towne strain of HCMV was propagated in HEL cells by infection at a multiplicity below 0.001 plaque-forming units (PFUs) per cell. Two HCMV stocks containing 1.3×10^7 and 2.5×10^7 PFU/ml were used in the present experiments.

Compounds and antisera

GA was purchased from Wako, Japan, and both LY294002 and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were obtained from Sigma-Aldrich (St Louis, MO, USA). The stock solutions of GA, LY294002 and TPCK, were made by dissolving them in dimethyl sulphoxide at the concentrations of 4, 20 and 100 mM, respectively. SN50 was obtained from Calbiochem (Darmstadt, Germany) and 0.2 mM stock solution was made by dissolving it in water. These compounds were further diluted in DMEM prior to use. The mouse monoclonal antibody (mAb) MAB810, which recognizes HCMV MIE (IE1 and IE2) gene products (Mazeron *et al*., 1992), and mouse anti-actin mAb C4 (MAB1501) were obtained from Chemicon (Temecula, CA, USA). The mouse mAbs which react with HCMV early protein (pUL44) or late protein pp65 (pUL83) were purchased from Fitzgerald (Concord, MA, USA) and ViroGen (Watertown, MA, USA), respectively. The rabbit polyclonal antibodies which can recognize Akt or only Akt that is phosphorylated on Ser473 were purchased from Cell Signaling Technology (Beverly, MA, USA).

Determination of cell viability

The cytotoxic effect of GA was tested by the methylene blue assay, as previously described (Dimanche-Boitrel *et al*., 1992). Briefly, confluent HEL cells in 35 mm plates were incubated with medium containing DMEM (mock treatment) or 40 nM GA with or without medium changes at 48 h intervals. At 6 days after treatment, cells were fixed with absolute methanol and stained by methylene blue (1% in borate buffer) for 30 min. The cell-fixed dye was eluted with 0.1 N HCl and absorbance of the eluted dye was measured at a wavelength of 630 nm. Each experimental point was determined in triplicate. The percentage of cell survival was calculated as shown below.

Percentage=(absorbance in GA-treated cells/absorbance in DMEM-treated cells)×100.

Virus infection and treatment of the infected cells with inhibitors

HEL cells were grown to confluent state in a growth medium and then serum-starved for 48 h in DMEM. The cells were infected with HCMV at a multiplicity of 1 PFU/cell, unless otherwise indicated. After a 1 h adsorption period, the infected cells were washed with Hanks' balanced salt solution (HBSS) and incubated in growth medium for the indicated times after infection. When cells were treated with GA or TPCK, these drugs were present during the 1 h adsorption period and from 1 h to the times after infection indicated in each experiment. When cells were treated with LY294002 or TPCK, these compounds were present 2 h before infection, during the 1 h viral adsorption period and from 1 h to the times after infection indicated in each experiment.

Virus growth study

Confluent, serum-starved HEL cells, grown in 25 cm^2 culture flasks, were infected with HCMV at various multiplicities of infection as indicated in each experiment. After a 1 h viral adsorption period, cells were washed with HBSS and maintained in growth medium containing various concentrations of GA. At 1–6 days after infection, the total amount of infectious HCMV (the virus released into the medium plus the cell-associated virus) was determined after disrupting the infected cells by freezing and thawing once, and by sonic treatment by plaque assay on HEL cells.

Western blotting analysis

For preparation of total cell lysates, mock- and HCMV-infected cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in sodium dodecyl sulphate (SDS) lysis buffer [50 mM Tris (pH6.8), 2% SDS, 3% 2-mercaptoethanol, 10% glycerol]. The lysates were sonically treated briefly, boiled for 3 min, and clarified by centrifugation at 15 000 rpm for 15 min. The supernatants were stored at −85°C until use. Proteins (50–80 μg) were separated on SDS-7.5% polyacrylamide gels and transferred to Hybond-ECL nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS). HCMV-specific MIE, early and late proteins and actin protein (used as an internal marker) were detected by the reaction with MAB810 (diluted 1:400 in TBS plus 5% skim milk), anti-pUL44 (diluted 1:800 in the same), anti-pp65 (diluted 1:500 in the same), or anti-actin antibody (diluted 1:1 000 in the same), respectively, followed by reaction with horseradish peroxidase (HRP)-labelled goat secondary antibody to mouse IgG (Santa Cruz Biotech, Santa Cruz, CA, USA; diluted 1: 5 000 in the same). Cellular Akt and phosphorylated Akt proteins were detected by incubation with rabbit polyclonal antibodies specific to Akt (diluted 1:1 000 in the same) or phosphorylated Akt (diluted 1:1 000 in the same), followed by the reaction with HRP-labelled goat antibody to rabbit IgG (diluted 1: 2 000 in the same). The visualization of signals was ECL (Amersham, UK).

Preparation of nuclear extracts

Mock- or HCMV-infected HEL cells were washed three times with ice-cold PBS, scraped off using a rubber cell scraper, and collected by centrifugation. Two packed volumes of buffer A [10 mM HEPES (pH 7.9),1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT), and proteinase inhibitor mixture (Sigma-Aldrich)] were added to the cells and kept on ice for 5 min to swell the cells. Vortex was then used to rupture the cell membranes, and nuclei were collected by centrifugation at 5000 rpm for 1 min at 4°C. The pellet was resuspended in a high salt buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl] and kept at 4°C for 30 min before centrifugation at 5 000 rpm for 15 min. The supernatants were stored at −85°C as nuclear extracts in small aliquots until use.

Electrophoretic mobility shift assay (EMSA)

Using Dig Gel Shift Kit (Hoffmann-LaRoche, Basel, Switzerland) EMSA was performed according to the manufacturer's protocol. In brief, the nuclear extracts (10 μg) were incubated with 20 μl of reaction mixture [20 mM HEPES (pH 7.6), 30 mM KCl, 1 mM EDTA, 10mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2% Tween 20, 0.1 μg of poly-L-lysine, 1 μg of poly [d (IC)] DNA and digoxigenin-labelled oligonucleotide probe specific to NFκB (Santa Cruz Biotech)] for 15 min at room temperature. DNA protein complexes were electrophoretically resolved on 8% polyacrylamide gels. After blotting on to a positive nylon membrane, signals were detected by reaction with sheep anti-digoxigenin polyclonal antibody conjugated with alkaline phosphatase.

Reverse transcription (RT)-PCR

Total RNA was prepared from mock- and HCMV-infected cells using ISOGEN (Nippon Gene, Toyama, Japan). RT-PCR was performed using the Superscript One-Step RT-PCR system (Invitrogen, Carlsbad, CA, USA). Total RNA (0.1 μg) was used for a single reaction. Nucleotide sequences of oligonucleotide primers for the IE1 and IE2 DNA used for RT-PCR were described elsewhere (Shirakata *et al.*, 2002). The reverse transcriptase reaction was performed at 55°C for 30 min. To amplify the IE1 and IE2 cDNAs, each sample was denatured at 94°C for 30 s, annealed at 50°C for 30 s and extended at 72°C for 2 min. The RT-PCR products were subjected to agarose gel electrophoresis and stained by ethidium bromide.

Results

Effect of GA on HCMV replication

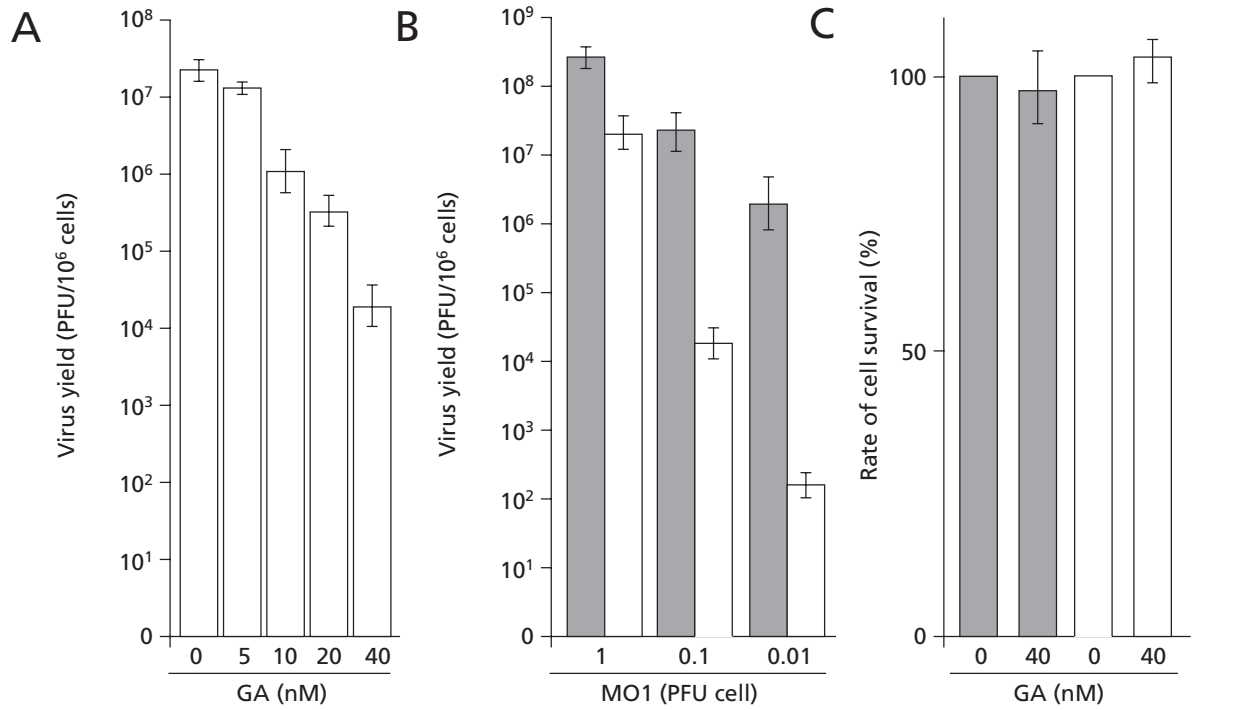
In the first experiment we asked whether GA has any effect on HCMV replication. For this purpose, confluent, serum-starved HEL cells were infected with HCMV and maintained with medium containing GA before the HCMV yield was analysed at 5 days post-infection (pi). Treatment with 10–40 nM GA inhibited HCMV replication in a concentration-dependent manner (Figure 1A). There was an approximately 1-log_{10} to 3-log_{10} reduction in the yield compared to untreated cells. The magnitude of inhibition caused by the GA treatment was markedly affected by the inoculum size of HCMV (Figure 1B). In HEL cells infected with HCMV at multiplicities of infection (MOI) of 1, 0.1 and 0.01, 40 nM GA caused about 1-log_{10}, 3-log_{10}

and 4-$\log_{10}$ reduction in the yield, respectively. To rule out the possibility that non-specific cytotoxicity caused by GA was responsible for the block to HCMV replication, cell viability was tested by the methylene blue assay after treating HEL cells for 6 days. Although GA at the concentration of 80 nM or higher had a cytocidal effect on HEL cells, GA at concentrations below 40 nM did not cause morphological changes, nor significantly reduce the viability of the cells maintained with or without medium changes (Figure 3C). This indicated that 40 nM GA, used in the experiments described below, is not cytotoxic for HEL cells.

GA inhibits synthesis of HCMV-specific proteins

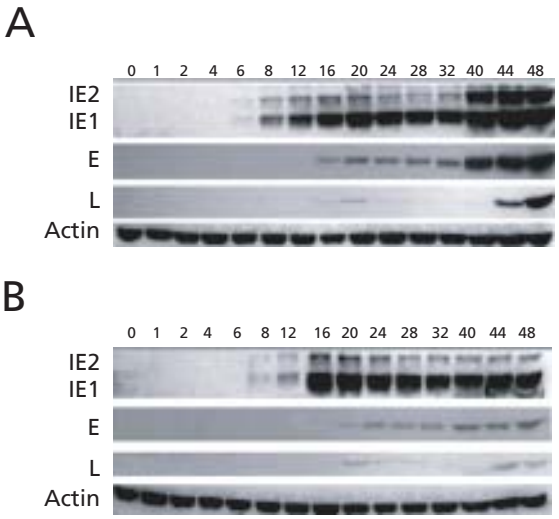
The effect of GA on the synthesis of viral proteins was examined next. In untreated cells, synthesis of both the IE1 and IE2 proteins was first detected at 6 h pi and the levels continued to increase until 20 h pi (Figure 2A). Although a slight decrease in the expression levels of IE1 and IE2 proteins was found from 24–32 h pi, a second tier of increase in MIE, especially in IE2, protein synthesis was observed after 40 h pi. Synthesis of the early pUL44 protein started at 16 h and greatly increased in amount after 40 h pi (Figure 2A). Similarly, the expression level of the late protein pp65 which was first synthesized at 40 h pi elevated after 44 h pi (Figure 2A). In GA-treated cells, on the other hand, the IE1 and IE2 proteins were first synthesized at 8 h pi. There was a 2 h delay in the appearance of these proteins, relative to that in untreated cells (Figure 2B). Although the expression levels of the MIE proteins, especially IE1, were greatly promoted after 16 h pi, the second tier of MIE protein synthesis, found after 40 h pi in untreated cells, could not be induced. Similarly, there was a 4 h delay in the first appearance of both pUL44 and pp65 compared to that in untreated cells, and an increase in the levels of these proteins, induced in control cells after 40 h pi, was greatly diminished by GA (Figure 2B). We had the same results when cells were infected with HCMV at a multiplicity of 5 PFU/cell. This excluded the possibility that the second tier was due to secondary viral spread (data not shown). These results indicate that GA delayed commencement of MIE protein synthesis and blocked the second tier of MIE (mainly IE2) protein synthesis, thereby inhibiting synthesis of both early and late viral proteins.

Figure 1. Effect of GA on HCMV replication and cell viability



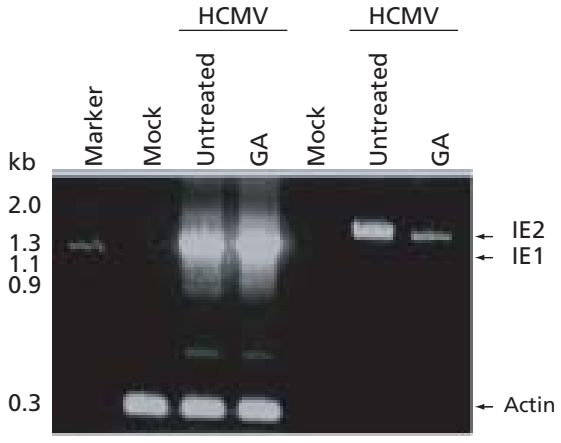
A, HEL cells were infected with HCMV at a multiplicity of 0.1 PFU/cell and maintained in the presence of the indicated concentrations of GA for 5 days before virus yield was determined. B, HEL cells were infected with HCMV at multiplicities of 0.01, 0.1 and 1 PFU/cell. They were maintained for 5 days in the absence (dark box) or presence (clear box) of 40 nM GA before virus yield was determined. C, confluent HEL cells in 35 mm plates were treated with 0 or 40 nM GA, without (dark box) or with (clear box) medium changes at 2-day intervals. At 6 days after treatment the rates of cell survival were analysed by methylene blue assay, as described in Materials and methods.

Figure 2. Effect of GA on synthesis of MIE, early and late viral proteins



HEL cells were infected with HCMV at a multiplicity of 1 PFU/cell and maintained in medium containing either A, 0 or B, 40 nM GA. At the indicated times (h on the top) pi expression levels of the MIE (upper), early (middle) and late (lower) proteins were analysed.

Figure 3. Effect of GA on synthesis of MIE mRNAs



HEL cells were infected with HCMV at a multiplicity of 1 PFU/cell or mock-infected with DMEM (labelled mock) and maintained in medium without or with 40 nM GA. At 36 h pi the total cellular RNA was isolated and expression levels of the IE1 and IE2 mRNAs were examined by RT-PCR as described in Materials and methods.

Synthesis of IE1 and IE2 mRNAs in HEL cells treated with GA

To investigate the mechanisms by which GA down-regulates MIE protein synthesis, we first examined whether the inhibitory effect of GA on MIE gene expression occurs at the transcriptional or translational level. HCMV-infected HEL fibroblasts were maintained in the presence or absence of GA and total RNA was isolated at 36 h pi, 4 h before the induction of the second tier of increase in the level of the MIE proteins (see Figure 2A). Synthesis of the MIE mRNAs was evaluated by RT-PCR. The results indicated that there is little, if any, difference in the level of the IE1 mRNA synthesized in untreated and GA-treated cells (Figure 3). On the contrary, the expression level of the IE2 mRNA in GA-treated cells was markedly reduced compared to that in the untreated cells. This indicated that IE2 gene expression in GA-treated cells is preferentially reduced at the transcriptional level.

Synthesis of viral proteins in HEL cells treated with GA at various times pi for various periods

We tried to investigate the stage in HCMV replication cycle that is affected by GA action. For this experiment, infected HEL cells were treated with GA for various periods at different times pi and the expression levels of viral proteins were analysed at 48 h pi. When cells were incubated in the presence of GA from 0–8 h and then maintained in the medium lacking GA from 8–48 h pi, synthesis of a relatively high level of the MIE, early and late proteins was allowed (Figure 4A). However, when GA was present in the medium from 0–12 h and absent from 12–48 h pi, synthesis of IE2, but not IE1, early and late proteins was efficiently blocked. Moreover, when GA was added to the medium within 8 h pi and cells were incubated in the presence of the drug until 48 h pi, synthesis of IE2, but not IE1, early and late proteins was most efficiently inhibited (Figure 4B). Taken together, these results suggest that the early event(s) that must be induced within 12 h after infection is probably affected by GA action and this event(s) is required for the second tier of MIE protein synthesis that occurs after 40 h pi.

Effect of PI3-K inhibitor on synthesis of the MIE proteins and activation of Akt

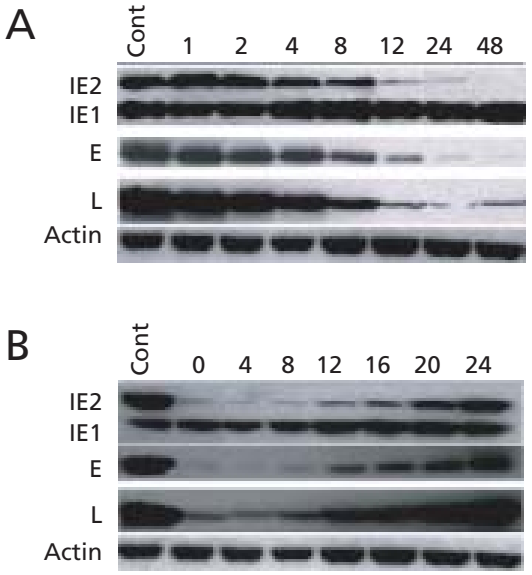
PI3-K is strongly activated immediately after HCMV infection of quiescent human fibroblasts and PI3-K activity is required for activation of Akt and the transcription factor NFκB. Activation of NFκB is thought to be important for the expression of the MIE promoters which encode the IE1 and IE2 proteins. Therefore, we examined the effect of LY294002, a specific inhibitor of PI3-K kinase activity, on the expression of MIE proteins and the activation of Akt to see whether PI3-K activation

pathway might be a target of GA action. The results indicated that synthesis of the IE1 and IE2 proteins at 6–8 h and synthesis of the IE2 protein at 36–48 h pi seen in untreated cells (Figure 5A) was markedly blocked in cells treated with LY294002 (Figure 5B) or GA (Figure 5C). In untreated cells the phosphorylated Akt, which has been shown to correlate well with Akt kinase activity (Alessi *et al*., 1996; Atlas & Alder, 1981), was induced as early as 0.5 h pi and continuously detectable during the 48 h incubation period of this experiment although the level decreased 24 h after infection (Figure 5A). However, in LY294002- and GA-treated cells the activated Akt could be detected for only a very limited period, that is, from 0.5–1 h pi (Figure 5B and 5C). The kinetics of the MIE protein synthesis and the activation of Akt in LY294002-treated cells basically appear to be consistent with patterns of the MIE protein synthesis and Akt activation in GA-treated cells (compare Figure 5B to Figure 5C). The amount of total Akt protein in mock- and LY294002-treated cells remained constant, indicating that changes in Akt phosphorylation in these cells were not due to changes in protein levels (Figure 5A and 5B). On the other hand, in GA-treated cells Akt protein showed a tendency to degrade as early as 2 h pi, in agreement with the result by Basso *et al*. (2002) who showed that Hsp90 inhibition by its specific inhibitor resulted in a marked reduction in the half-life of Akt (Figure 5C). These results suggest that GA may disturb the PI3-K signalling pathway which leads to the activation of the MIE genes.

LY294002 and GA inhibit HCMV-mediated NFκB activation

As shown in the previous section, HCMV infection activates Akt and the activation of Akt is inhibited by both LY294002 and GA. Since activated Akt mediates induction of the transcription factor NFκB (Kane *et al*., 1999; Madrid *et al*., 2000), we next examined whether NFκB could be induced in HCMV-infected HEL cells and whether LY294002 and GA might block the HCMV-mediated NFκB activation in these cells. For this, infected HEL cells were maintained in the presence or absence of these inhibitors for various times, nuclear extracts were prepared, and the NFκB activation was assessed by DNA binding activity to the NFκB probe using electrophoretic mobility shift assay (EMSA). Consistent with earlier reports (Johnson *et al*., 2001; Yurochko *et al*., 1995), the infection of HEL cells with HCMV induced NFκB activity within 20 min pi and this activity was continuously detectable during the 48 h incubation period of this experiment (Figure 6). On the other hand, in the cells maintained in the presence of LY294002 or GA a lower level of DNA binding activity was induced only from 30 min to 4 h pi (Figure 6). The time course of the NFκB activation in GA-treated cells mirrored that in LY294002-treated cells. Thus, Akt and NFκB are activated in a PI3-K-dependent manner during HCMV infection.

Figure 4. Synthesis of viral proteins in HEL cells treated with GA at various times pi for various periods

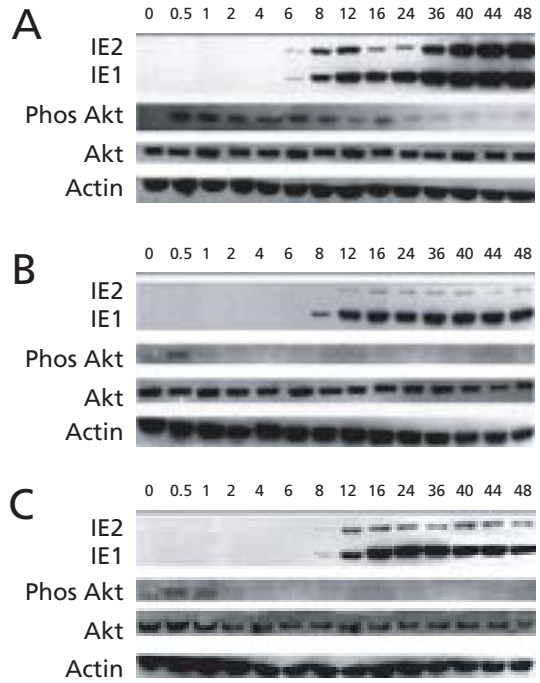


A, HEL cells were left untreated ('cont') or treated with 40 nM GA 1 h before infection, during the 1 h adsorption period, and from 1–48 h after infection with HCMV at a multiplicity of 1 PFU/cell. At the indicated times (h on the top) pi, GA was removed from the cultures by washing the cells with HBSS and maintained in medium without GA before cell lysates were prepared at 48 h pi. B, HEL cells were infected with HCMV at a multiplicity of 1 PFU/cell. After a 1 h adsorption period, cells were maintained in medium without GA. At the indicated times (h on the top) pi, GA was added to the cultures at a final concentration of 40 nM and cells were incubated before cell lysates were prepared at 48 h pi. The time of virus infection is taken as 0.

Induction of NFκB is required for expression of the MIE proteins in HEL cells

To determine whether the synthesis of the MIE proteins in HEL cells is actually dependent on the induction of NFκB activity, the effect of SN50, a selective inhibitor of NFκB (Lin *et al*., 1995), on MIE protein synthesis was examined. The results, shown in Figure 7A, indicated that synthesis of both IE1 and IE2 proteins at 12, 24 and 48 h pi was blocked in the presence of 10 and 20 μM SN50 in a concentration-dependent manner. In the cells treated with 20 μM SN50, detectable levels of IE1 and IE2 proteins could not be synthesized at all times examined. To confirm the results

Figure 5. Synthesis of MIE proteins and phosphorylation of Akt in HCMV-infected HEL cells treated with LY294002 or GA



HEL cells were infected with HCMV at a multiplicity of 1 PFU/cell and maintained in medium A, without, or with either B, 20 μM LY294002 or C, 40 nM GA. At the indicated times (h on the top) pi the total cell lysates were prepared and expression levels of the MIE (upper), phosphorylated Akt (Phos.Akt; middle) and Akt (lower) proteins were analysed.

obtained using SN50, we used TPCK, another commonly used inhibitor of NFκB (Henkel *et al.*, 1993; Wu *et al.*, 1996; Phillips & Ghosh, 1977). As shown in Figure 7B, 10 μM TPCK inhibited synthesis of the IE2 protein and 20 μM TPCK effectively blocked the synthesis of both the IE1 and IE2 proteins in HEL cells at 48 h pi. Moreover, the same concentrations of TPCK also blocked the induction of NFκB activity by HCMV in a concentration-dependent manner (Figure 7C). The methylene blue assay revealed that at 20 μM neither SN50 nor TPCK significantly alter the cell viability when judged at 48 h after treatment (data not shown). Thus, HCMV-mediated induction of NFκB is required for synthesis of both the IE1 and IE2 proteins in HEL cells at early and late times after infection.

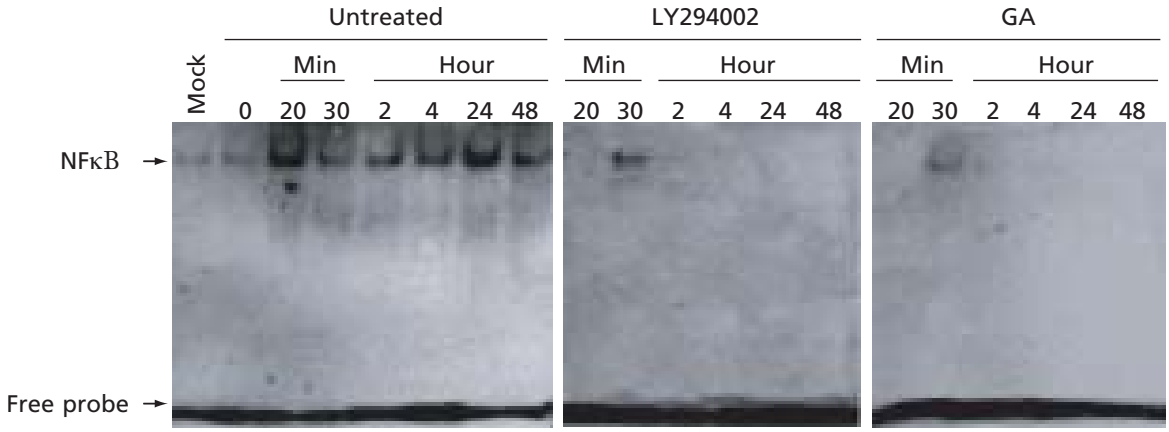
Effect of medium change on synthesis of viral proteins and infectious HCMV

Finally, we evaluated the possibility that, if the medium containing GA is changed frequently, the efficacy of GA to inhibit HCMV replication could be improved. For this purpose, HCMV-infected HEL cells were maintained in the presence of GA for 6 days, with or without medium changes at 48 h intervals, and the syntheses of viral proteins and infectious progeny virus were analysed. The expression level of IE1 protein in untreated and GA-treated cells at 1–6 days pi was basically the same and was not affected by medium changes (Figure 8A and 8B). In untreated cells, synthesis of IE2 protein elevated markedly at 2 days pi and high levels were continuously detectable during the 6-day period of this experiment (Figure 8A). The early and late proteins, first synthesized at 2 days pi, increased in amount with time after infection (Figure 8A), whereas GA delayed the elevation of the IE2, early and late protein synthesis until 4 days pi (Figure 8A). In untreated cells a marked difference in the synthesis of viral proteins was not induced by medium changes, except that the synthesis of the IE2 protein was stimulated (Figure 8A and 8B). On the contrary, if the medium was replaced with fresh medium containing GA at 2 and 4 days pi, the increase induced in the IE2 protein levels at 3–6 days pi was substantially inhibited (Figure 8A and 8B). Moreover, the synthesis of both the early and late viral proteins could be abolished by treatment with fresh GA (Figure 8B). Typical growth kinetics of HCMV in either untreated or GA-treated HEL cells are shown (Figure 8C and 8D). Although the treatment of infected HEL cells with GA blocked HCMV replication at 3–6 days by approximately 10- to 1000-fold, HCMV replication still progressed rapidly and markedly (Figure 8C). In contrast, in cells treated with GA with medium changes at 2-day intervals the commencement of the synthesis of infectious HCMV at 3 days pi and the subsequent rapid increase in virus yield, seen in GA-treated cells maintained without medium change, could be inhibited (Figure 8D).

Discussion

In the present study, we have shown that the replication of HCMV is greatly inhibited by the treatment of virus-infected HEL cells with 40 nM GA, a specific inhibitor of Hsp90. The inhibitory effect of GA was also found in other human diploid KMS-6 fibroblasts (derived from a whole embryo) and in two human cell lines, TPC-1 (derived from epithelial thyroid papillary carcinoma) and U373MG (derived from astrocytoma). Moreover, HCMV replication in these cells could be similarly inhibited by treatment with the same concentration of another Hsp90-specific inhibitor 17AAG (17-allylamino-17-demethoxygeldanamycin) (data not shown). These results indicate that the inhibition of HCMV replication by the inhibitors of Hsp90 is not cell-type-specific and that Hsp90 plays an important role in the HCMV replication cycle. The

Figure 6. LY294002 and GA block HCMV-mediated induction of NFκB in HEL cells



HEL cells were infected with HCMV at a multiplicity of 1 PFU/cell and maintained in medium without or with either 20 μM LY294002 or 40 nM GA. At the indicated times pi nuclear extracts were prepared, and binding activity of the extracts to the oligonucleotide NFκB probe was examined by EMSA. Mock represents the nuclear extracts prepared from mock-infected HEL cells at 48 h pi.

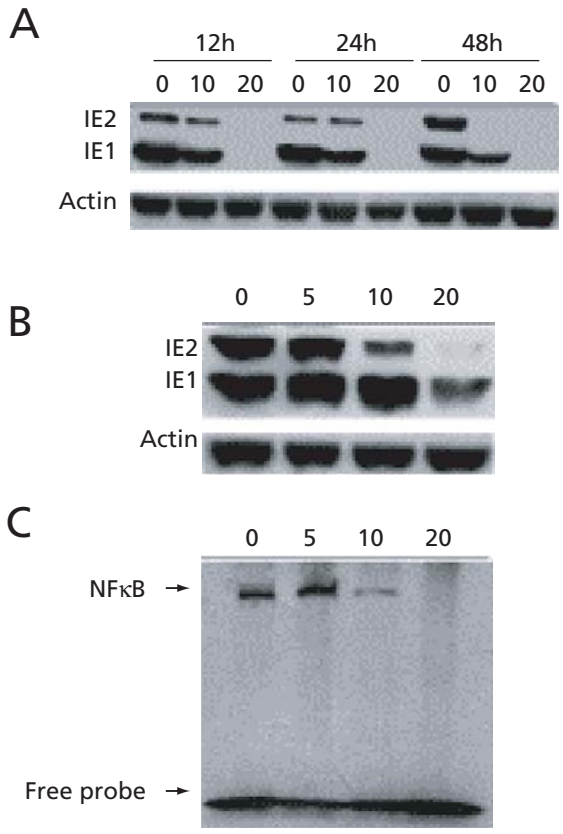
importance of Hsp90 for completing viral replication cycles has been shown by using HIV-1 (O'Keefe *et al.*, 2000), hepatitis C virus (Waxman *et al.*, 2001), vaccinia virus (Hung *et al.*, 2002), and herpes simplex virus type 1 (Li *et al.*, 2004).

One possible mechanism responsible for the marked difference between untreated and GA-treated HEL cells with respect to HCMV production is the cytotoxicity of GA. However, no significant difference was found in the rate of cell survival in either untreated or GA-treated cells (Figure 1C). Moreover, a relatively short (12 h) treatment was enough to cause inhibition of MIE gene expression at 48 h pi (Figure 4A) and HCMV replication at 5 days pi (data not shown). Consequently, the big difference observed in HCMV production in untreated and GA-treated cells does not seem to be due to cytotoxicity of GA.

Kinetic analysis revealed that the MIE proteins were synthesized in a two-tiered manner (Figures 2A and 5A). It is interesting to note that in the second tier of synthesis the increase in the level of the IE2 protein was much more prominent compared to that of the IE1 protein. However, the second tier of MIE, especially IE2, protein synthesis was greatly inhibited in GA-treated cells. Also, the increase in the levels of both the early pUL44 and the late pp65 proteins, induced in untreated cells after 40 h pi, was markedly blocked by GA. There was a good correlation between the level of IE2, but not IE1, protein synthesized and the levels of the early and late proteins synthesized at a given time. The same correlation was also observed in HCMV-infected HEL cells treated with or without GA for prolonged periods (Figures 8A and 8B), indicating that the level of the IE2 protein synthesized in HEL cells is an important determinant for whether or not HCMV can express the early and late genes. Since the pUL44 is required for the initiation of viral DNA replication (Pari & Anders, 1993; Weiland *et al.*, 1994), the failure to synthesize normal levels of this protein in GA-treated cells should lead to a process that fails to synthesize viral DNA, late proteins and consequently progeny HCMV. Therefore, the block to HCMV replication in GA-treated HEL cells is due to the block to IE2 gene expression at the transcriptional level in these cells (Figure 3).

Consistent with previous reports (Johnson *et al.*, 2001; Kowalik *et al.*, 1993; Sambucetti *et al.*, 1989; Yurochko & Huang, 1999; Yurochko *et al.*, 1995, 1997a, 1997b), infection of HEL cells with HCMV induced the activation of Akt and NFκB, and this induction was markedly inhibited in the presence of LY294002, an inhibitor of PI3-K (Figures 5 and 6; King *et al.*, 1997; Wennstrom & Downward, 1999). This inhibitor also blocked both the first and the second tiers of MIE (especially IE2) protein synthesis (Figure 5B). Thus, the activation of Akt and NFκB by HCMV and the expression of the MIE genes in HEL cells are dependent on the PI3-K activity, as shown previously (Johnson *et al.*, 2001). Moreover, GA also blocked both the HCMV-mediated activation of Akt and NFκB and the two-tiered MIE protein synthesis (Figure 5C). The kinetics of Akt and NFκB activation and MIE protein synthesis in GA-treated HEL cells are closely correlated with the respective time courses in LY294002-treated cells (Figures 5 and 6). This correlation may imply that GA disrupts the PI3-K signalling pathway leading to the activa-

Figure 7. Effect of SN50 and TPCK, inhibitors of NFkB, on synthesis of the MIE proteins and activation of NFκB in HEL cells



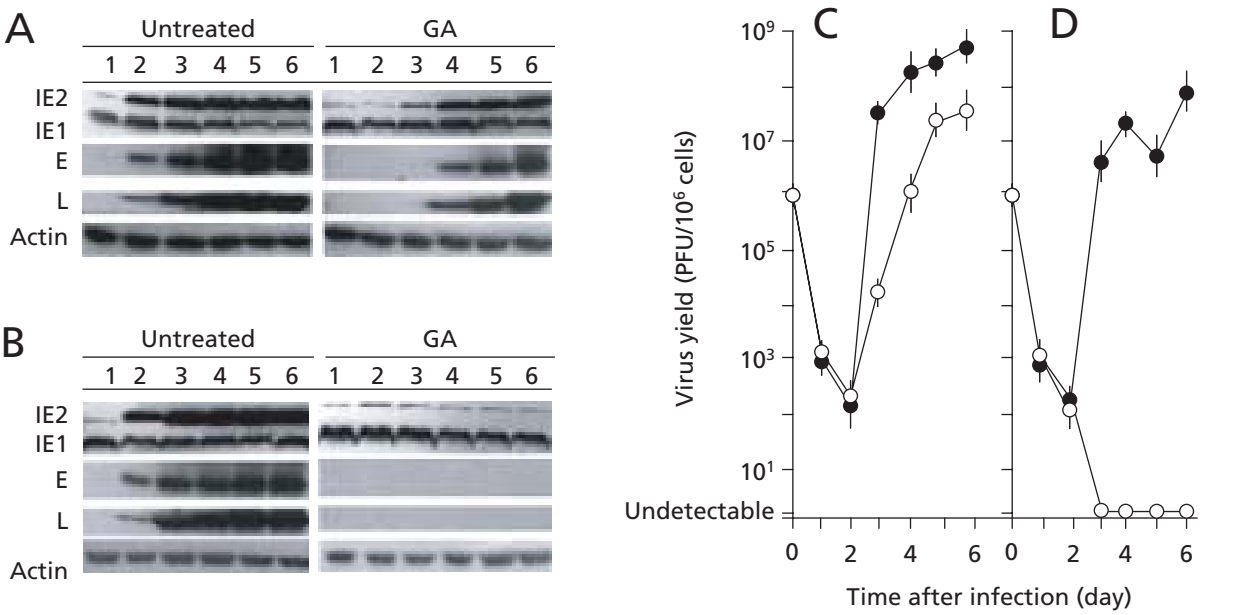
HEL cells were treated with 0–20 μM, either (A) SN50 or (B and C) TPCK 1 h before infection, during the 1 h absorption period, and from 1–12–48 h after infection with HCMV at a multiplicity of 1 PFU/cell. (A) at 12, 24 and 48 h pi and (B) at 48 h pi cells were lysed and examined for expression levels of the MIE proteins. (C) At 24 h pi nuclear extracts were prepared and binding activity of the extracts to the oligonucleotide NFκB probe was examined by EMSA.

tion of the MIE genes. GA binds with a high level of specificity within the ADP/ATP binding pocket of Hsp90 and inhibits the function of this chaperone (Prodromou *et al*., 1997; Sullivan *et al*., 1997). This interaction results in inappropriate functioning and rapid degradation ofHsp90-associated client proteins, such as Akt kinase and 3-phosphoinositide-dependent protein kinase-1 (PDK-1), which phosphorylate Akt (Sato *et al*., 2000; Fujita *et al*., 2002; Basso *et al*., 2002; Blagosklonny, 2002; Richter & Buckner, 2001). Therefore, these results suggest that the inhibitory effect of GA on MIE gene expression is primarily expressed through the ability of GA to disrupt the proper function of some downstream effector molecules of PI3-K, such as Akt and PDK-1 client proteins.

Akt is activated by PDK-1 and once Akt is activated it can phosphorylate a number of cellular factors, including IκB kinases (IKKs), to ensure cell survival (Datta *et al*., 1999). IKKs then phosphorylate the cytoplasmic NFκB inhibitors, IκBs, and tags them for proteasomal degradation. It thus allows NFκB proteins to translocate into the nucleus and activate the transcription of the HCMV MIE genes by binding to the NFκB responsive elements present in their enhancer region. However, since the binding of HCMV glycoproteins located in the virion to host cell receptors results in the activation of a number of cellular factors (Boyle *et al*., 1999; Kowalik, 1993; Yurochko, 1995), it is possible that some cellular proteins induced by this receptor-ligand interaction other than NFκB may be responsible for activation of the MIE genes. Our results indicated that the treatment of HCMV-infected HEL cells with SN50, a cell-permeable recombinant peptide that blocks translocation of NFκB to the nucleus (Lin *et al*., 1995), results in an inhibition of the MIE protein synthesis at 12–48 h pi to an undetectable level (Figure 7A).

Previous works have shown that SN50 has no effect on IκB-degradation (Lin *et al*., 1995) or on the activity of several other transcription factors including AP-1, CREB and OCT (Lin *et al.* 1995; Maggirwar *et al*., 1998). Moreover, TPCK, a protease inhibitor that blocks IκB degradation (Henkel *et al*., 1993) also inhibited MIE protein synthesis (Figure 7B) and NFκB activation in HEL cells (Figure 7C). Therefore, the NFκB induced after HCMV infection is essential for expression of MIE genes in HEL cells at both early and late times after infection. This conclusion is supported by the findings that active NFκB is critical for the transactivation of the HCMV MIE promoter (DeMeritt *et al*., 2004; Lee *et al*., 2004; Prosch *et al*., 1995). Because phosphorylated Akt and NFκB induced in untreated and drug-treated cells within 4 h pi (Figure 6) could also be induced by infection with UV-inactivated HCMV, the proteins found from 20 min to 4 h pi seem to be produced as a result from the binding of HCMV glycoproteins on the virion to the host cell receptor (data not shown). Therefore, the NFκB induced very early after infection by receptor-ligand interaction may be necessary for first-tier MIE gene expression in both untreated and GA-treated HEL cells, and the NFκB induced within 12 h pi is presumably required for the expression of second-tier MIE gene expression in untreated cells. When considered together, our results suggest that the inhibitory effect of GA on HCMV replication is primarily caused by disrupting the proper function of Akt, a downstream effector molecule of PI3-K, and the IE stages in virus replication cycle are most likely to be under control of GA action.

Figure 8. Effect of medium change on synthesis of viral proteins and infectious HCMV



HEL cells were infected with HCMV at a multiplicity of 1 PFU/cell and maintained in medium containing 0 or 40 nM GA for 6 days (A, C) without or (B, D) with medium changes at 2-day intervals. At 1–6 days pi (A, B) the expression levels of the MIE (upper), early (middle) and late (lower) proteins were examined and (C, D) the total amounts of infectious HCMV produced in untreated (black circle) and GA-treated (white circle) cells were determined.

Currently, patients who are suffering from diseases caused by HCMV are treated with chemical compounds, such as ganciclovir, foscarnet or cidofovir, all of which inhibit HCMV replication by blocking viral DNA synthesis. However, the substantial toxicity of these drugs and emergence of drug-resistant strains hinder AIDS patients from prolonged maintenance therapy (Baldanti *et al*., 1995, 1998; Chou *et al*., 1995; Erice *et al*., 1989; Lurain *et al*., 1994) and, therefore, other drugs with different mechanisms of action are expected. Since GA has been shown to possess the ability to eliminate production of infectious HCMV in HEL cells by inhibiting MIE protein synthesis when medium with GA is changed frequently (Figures 8B and D), the inhibitors for Hsp90, such as GA and 17AAG, may prove to be useful antiviral therapeutics.

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References

Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P & Hemmings BA (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO Journal* **15:**6541–6551.

Atlas D & Adler M (1981) Alpha-adrenergic antagonists as possible calcium channel inhibitors. *Proceedings of the National Academy of Sciences, USA* **78:**1237–1241.

Baldanti F, Silini E, Sarasini A, Talarico CL, Stanat SC, Biron KK, Furione M, Bono F, Palu G & Gerna G (1995) A three-nucleotide deletion in the UL97 open reading frame is responsible for the ganciclovir resistance of a human cytomegalovirus clinical isolate. *Journal of Virology* **69:**796–800.

Baldanti F, Underwood MR, Talarico CL, Simoncini L, Sarasini A, Biron KK & Gerna G (1998) The Cys607 Tyr change in the UL97 phosphotransferase confers ganciclovir resistance to two human cytomegalovirus strains recovered from two immunocompromised patients. *Antimicrobial Agents & Chemotherapy* **42:**444–446.

Basso AD, Solit DB, Munster PN & Rosen N (2002) Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. *Oncogene* **21:**1159–1166.

Blagosklonny MV (2002) Are p27 and p21 cytoplasmic oncoproteins? *Cell Cycle* **1:**391–393.

Blagosklonny MV, Schulte TW, Nguyen P, Mimnaugh EG, Trepel J & Neckers L (1995) Taxol induction of p21WAF1 and p53 requires c-raf-1. *Cancer Research* **55:**4623–4626.

Boyle KA, Pietropaolo RL & Compton T (1999) Engagement of the cellular receptor for glycoprotein B of human cytomegalovirus activates the interferon-responsive pathway. *Molecular & Cellular Biology* **19:**3607–3613.

Brugge JS (1986) The p35/p36 substrates of protein-tyrosine kinases as inhibitors of phospholipase A2. *Cell* **46:**149–150.

Chan YJ, Chiou CJ, Huang Q & Hayward GS (1996) Synergistic interactions between overlapping binding sites for the serum response factor and ELK-1 proteins mediate both basal enhancement and phorbol ester responsiveness or primate cytomegalovirus major immediate-early promoters in monocyte and T-lymphocyte cell types. *Journal of Virology* **70:**8590–8605.

Chavany C, Mimnaugh E, Miller P, Bitton R, Nguyen P, Trepel J, Whitesel L, Schnur R, Moyer J & Neckers L (1996) p185erbB2 binds to GRP94 *in vivo*. Dissociation of the p185erbB2/GRP94 heterocomplex by benzoquinone ansamycins precedes depletion of p185erbB2. *Journal of Biological Chemistry* **271:**4974–4977.

Cherrington JM & Mocarski ES (1989) Human cytomegalovirus ie1 transactivates the alpha promoter-enhancer via an 18-base-pair repeat element. *Journal of Virology* **63:**1435–1440.

Chou S, Guentzel S, Michels KR, Miner RC & Drew WL (1995) Frequency of UL97 phosphotransferase mutations related to ganciclovir resistance in clinical cytomegalovirus isolates. *Journal of Infectious Diseases* **172:**239–242.

Datta SR, Brunet A & Greenberg ME (1999) Cellular survival: a play in three Akts. *Genes & Development* **13:**2905–2927.

DeMeritt IB, Milford LE & Yurochko AD (2004) Activation of the NF-κB pathway in human cytomegalovirus-infected cells is necessary for efficient transactivation of the major immediate-early promoter. *Journal of Virology* **78:**4498–4507.

Dimanche-Boitrel MT, Pelletier H, Genne P, Petit JM, Le Grimellec C, Canal P, Ardiet C, Bastian G & Chauffert B (1992) Confluence-dependent resistance in human colon cancer cells: role of reduced drug accumulation and low intrinsic chemosensitivity of resting cells. *International Journal of Cancer* **50:**677–682.

Erice A, Chou S, Biron KK, Stanat SC, Balfour HH Jr & Jordan MC (1989) Progressive disease due to ganciclovir-resistant cytomegalovirus in immunocompromised patients. *New England Journal of Medicine* **320:**289–293.

Fortunato EA & Spector DH (1999) Regulation of human cytomegalovirus gene expression. *Advance in Virus Research* **54:**61–128.

Fujita N, Sato S, Ishida A & Tsuruo T (2002) Involvement of Hsp90 in signaling and stability of 3-phosphoinositide-dependent kinase-1. *Journal of Biological Chemistry* **277:**10346–10353.

Gradin K, McGuire J, Wenger RH, Kvietikova I, Fhitelaw ML, Toftgard R, Tora L, Gassmann M & Poellinger L (1996) Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the arnt transcription factor. *Molecular & Cellular Biololgy* **16:**5221–5231.

Hartson SD & Matts RL (1994) Association of Hsp90 with cellular Src-family kinases in a cell-free system correlates with altered kinase structure and function. *Biochemistry* **33:**8912–8920.

Henkel T, Machleidt T, Alkalay I, Kronke M, Ben-Neriah Y & Baeuerle PA (1993) Rapid proteolysis of IκBα· is necessary for activation of transcription factor NF-αB. *Nature* **365:**182–185.

Hung JJ, Chung CS & Chang W (2002) Molecular chaperone Hsp90 is important for vaccinia virus growth in cells. *Journal of Virology* **76:**1379–1390.

Jaiswal RK, Weissinger E, Kolch W & Landreth GE (1996) Nerve growth factor-mediated activation of the mitogen-activated protein (MAP) kinase cascade involves a signaling complex containing B-Raf and HSP90. *Journal of Biological Chemistry* **271:**23626–23629.

Johnson RA, Wang X, Ma XL, Huong SM & Huang ES (2001) Human cytomegalovirus up-regulates the phosphatidylinositol 3-kinase (PI3-K) pathway: inhibition of PI3-K activity inhibits viral replication and virus-induced signaling. *Journal of Virology* **75:**6022–6032.

Kane LP, Shapiro VS, Stokoe D & Weiss A (1999) Induction of NF-κB by the Akt/PKB kinase. *Current Biology* **9:**601–604.

King PD, Sadra A, Teng JM, Xiao-Rong L, Han A, Selvakumar A, August A & Dupont B (1997) Analysis of CD28 cytoplasmic tail tyrosine residues as regulators and substrates for the protein tyrosine kinases: EMT and LCK. *Journal of Immunology* **158:**580–590.

Kowalik TF, Wing B, Haskill JS, Azizkhan JC, Baldwin AS Jr & Huang ES (1993) Multiple mechanisms are implicated in the regulation of NF-κB activity during human cytomegalovirus infection. *Proceedings of the National Academy of Sciences, USA* **90:**1107–1111.

Lee Y, Sohn WJ, Kim DS & Kwon HJ (2004) NF-κB- and c-Jun-dependent regulation of human cytomegalovirus immediate-early gene enhancer/promoter in response to lipopolysaccharide and bacterial CpG-oligodeoxynucleotides in macrophage cell line RAW 264.7. *European Journal of Biochemistry* **271:**1094–1105.

Li YH, Tao PZ, Liu YZ & Jiang JD (2004) Geldanamycin: a ligand of heat shock protein 90: inhibits the replication of herpes simplex virus type 1 *in vitro*. *Antimicrobial Agents & Chemotherapy* **48:**867–872.

Lin YZ, Yao SY, Veach RA, Torgerson TR & Hawiger J (1995) Inhibition of nuclear translocation of transcription factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *Journal of Biological Chemistry* **270:**14255–14258.

Liu B & Stinski MF (1992) Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 cis-acting elements. *Journal of Virology* **66:**4434–4444.

Lovric J, Bischof O & Moelling K (1994) Cell cycle-dependent association of Gag-Mil and hsp90. *FEBS Letters* **343:**15–21.

Lurain NS, Spafford LE & Thompson KD (1994) Mutation in the UL97 open reading frame of human cytomegalovirus strains resistant to ganciclovir. *Journal of Virology* **68:**4427–4431.

Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS Jr & Mayo MW (2000) Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-κB. *Molecular & Cellular Biology* **20:**1626–1638.

Maggirwar SB, Sarmiere PD, Dewhurst S & Freeman RS (1998) Nerve growth factor-dependent activation of NF-κB contributes to survival of sympathetic neurons. *Journal of Neuroscience* **18:**10356–10365.

Malone CL, Vesole DH & Stinski MF (1990) Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. *Journal of Virology* **64:**1498–1505.

Mazeron MC, Jahn G & Plachter B (1992) Monoclonal antibody E-13 (M-810) to human cytomegalovirus recognizes an epitope encoded by exon 2 of the major immediate early gene. *Journal of General Virology* **73:**2699–2703.

Meier JL & Stinski MF (1996) Regulation of cytomegalovirus immediate early genes. *Intervirology* **39:**331–342.

Mocarski ES, Abenes GB, Manning WC, Sambucetti LC & Cherrington JM (1990) Molecular genetic analysis of cytomegalovirus gene regulation in growth:persistence and latency. *Current Topics in Microbiology & Immunology* **154:**47–74.

Mocarski ES & Courcelle CT (2001) Cytomegalovirus and their replication. In *Fields Virology*; pp. 2629–2673. Edited by D Knipe & P Howley. Philadelphia: Lippincott, Williams & Wilkins.

Morimoto RI & Milarski KL (1991) Expression and function of vertebrate hsp70 genes. In *Stress Proteins in Biology and Medicine*; pp. 323–360. Edited by RI Morimoto, A Tissieres & C Georgopoulos. New York: Cold Spring Harbor Laboratory.

Nair SC, Toran EJ, Rimerman RA, Hjermstad S, Smithgall TE & Smith DF (1996) A pathway of multi-chaperone interactions common to diverse regulatory proteins: estrogen receptor, Fes tyrosine kinase, heat shock transcription factor Hsf1 and the aryl hydrocarbon receptor. *Cell Stress & Chaperones* **1:**237–250.

Ohgitani E, Kobayashi K, Takeshita K & Imanishi J (1999) Biphasic translocation of a 70 kDa heat shock protein in human cytomegalovirus-infected cells. *Journal of General Virology* **80:**63–68.

O'Keefe B, Fong Y, Chen D, Zhou S & Zhou Q (2000) Requirement for a kinase-specific chaperone pathway in the production of a Cdk9/cyclin T1 heterodimer responsible for P-TEFb-mediated tat stimulation of HIV-1 transcription. *Journal of Biological Chemistry* **275:**279–287.

Pari GS & Anders DG (1993) Eleven loci encoding trans-acting factors are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA replication. *Journal of Virology* **67:**6979–6988.

Phillips RJ & Ghosh S (1997) Regulation of IκB, in WEHI 231 mature B cells. *Molecular & Cellular Biology* **17:**4390–4396.

Pratt WB & Toft DO (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocrine Reviews* **18:**306–360.

Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW & Pearl LH (1997) Identification and structural characterization of the ATP/ADP binding site in the Hsp90 molecular chaperone. *Cell* **90:**65–75.

Prosch S, Staak K, Stein J, Liebenthal C, Stamminger T, Volk HD & Kruger DH (1995) Stimulation of the human cytomegalovirus IE enhancer/promoter in HL-60 cells by TNFα is mediated via induction of NF-κB. *Virology* **208:**197–206.

Richter K & Buckner J (2001) Hsp90: chaperoning signal transduction. *Journal of Cellular Physiology* **188:**281–290.

Sambucetti L-C, Cherrington J-M, Wilkinson G-W & Mocarski E-S (1989) NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T-cell stimulation. *EMBO Journal* **18**:281–290.

Santomenna LD & Colberg-Poley AM (1990) Induction of cellular hsp70 expression by human cytomegalovirus. *Journal of Virology* **64:**2033–2040.

Sato S, Fujita N & Tsuruo T (2000) Modulation of Akt kinase activity by binding to Hsp90. *Proceedings of the National Academy of Sciences, USA* **97:**10832–10837.

Schulte TW, Blagosklonny MV & Ingui C (1995) Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. *Journal of Biological Chemistry* **270:**24585–24588.

Shirakata M, Terauchi M, Ablikim M, Imadome K, Hirai K, Aso T & Yamanashi Y (2002) Novel immediate-early protein IE19 of human cytomegalovirus activates the origin recognition complex I promoter in a cooperative manner with IE72. *Journal of Virology* **76:**3158–3167.

Stancato LF, Chow YH, Hutchison KA, Perdew GH, Jove R & Pratt WB (1993) Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. *Journal of Biological Chemistry* **268:**21711–21716.

Stepanova L, Leng X, Parker SB & Harper JW (1996) Mammalian p50Cdc37 is a protein kinase-targeting subunit of Hsp90 that binds and stabilizes Cdk4. *Genes & Development* **10:**1491–1502.

Sullivan W, Stensgard B, Caucutt G, Bartha B, McMahon N, Alnemri ES, Litwack G & Toft D (1997) Nucleotides and two functional states of hsp90. *Journal of Biological Chemistry* **272:**8007–8012.

Wartmann M & Davis RJ (1994) The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. *Journal of Biological Chemistry* **269:**6695–6701.

Waxman L, Whitney M, Pollok BA, Kuo LC & Darke PL (2001) Host cell factor requirement for hepatitis C virus enzyme maturation. *Proceedings of the National Academy of Sciences, USA* **98:**13931–13935.

Weiland KL, Oien NL, Homa F & Wathen MW (1994) Functional analysis of human cytomegalovirus polymerase accessory protein. *Virus Research* **34:**191–206.

Wennstrom S & Downward J (1997) Analysis of CD28 cytoplasmic tail tyrosine residues as regulators and substrates for the protein tyrosine kinases: EMT and LCK. *Journal of Immunology* **158:**580–590.

White EA, Clark CL, Sanchez V & Spector DH (2004) Small internal deletions in the human cytomegalovirus IE2 gene result innonviable recombinant viruses with differential defects in viral gene expression. *Journal of Virology* **78:**1817–1830.

Wu M, Lee H, Bellas RE, Schauer SL, Arsura M, Katz D, FitzGerald MJ, Rothstein TL, Sherr DH & Sonenshein GE (1996) Inhibition of NF-κB/Rel induces apoptosis of murine B cells. *EMBO Journal* **15:**4682–4690.

Yurochko AD, Kowalik TF, Huong SM & Huang ES (1995) HCMV upregulates NF-κB activity by transactivating the NF-κB p105/p50 and p65 promoters. *Journal of Virology* **69:**5391–5400.

Yurochko AD, Hwang ES, Rasmussen L, Keay S, Pereira L & Huang ES (1997a) The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-κB during infection. *Journal of Virology* **71:**5051–5059.

Yurochko AD, Mayo MW, Poma EE, Baldwin AS Jr & Huang ES (1997b) Induction of the transcription factor Sp1 during human cytomegalovirus infection mediates upregulation of the p65 and p105/p50 NF-κB promoters. *Journal of Virology* **71:**4638–4648.

Yurochko AD & Huang ES (1999) Human cytomegalovirus binding to human monocytes induces immunoregulatory gene expression. *Journal of Immunology* **162:**4806–4816.

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