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The novel HSP90 inhibitor, PU-H71, suppresses glial cell activation but weakly affects clinical signs of EAE

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Abstract

Ansamycins are very effective HSP90 inhibitors that showed significant beneficial effects in the treatment of EAE. However, their toxicity and poor stability in solution limit their clinical use. In the present study we have characterized the anti-inflammatory properties of a novel HSP90 inhibitor, PU-H71, and tested its effects in EAE. Our findings show that PU-H71 reduced lipopolysaccharide astrocyte activation but failed to reduce the inflammatory cytokine activation. In contrast to ansamycins, PU-H71 weakly affects EAE clinical course. In conclusion, although PU-H71 displayed some anti-inflammatory properties, it appeared in vivo less effective than the more toxic HSP90 inhibitors.

Keywords

PU-H71; HSP90 inhibitors; Glial activation; EAE; Astrocytes; Microglia

1. Introduction

Geldanamycin (GA) and radicicol are natural compounds that inhibit the N-terminal ATPase activity of heat shock protein 90 (HSP90). HSP90 is a molecular chaperone that modulates the stability and the transport of several intracellular proteins and prevents the nonspecific aggregation of misfolded or unfolded proteins (Barginear et al., 2008). Interestingly, the main role of this chaperone is the regulation of proteins, called ‘client proteins’, at the late stage of folding (near native-state) and mainly involved in signal transduction (Pearl et al., 2008). When the ATPase activity of HSP90 is blocked by the above mentioned inhibitors, client proteins dissociate from the chaperone; some of them become activated (such as the transcription factor, HSF-1) (Whitesell et al., 2003), others lose their function or are targeted for proteasomal degradation. In particular the release of HSF-1 allows its translocation into the nucleus, followed by induction of the heat shock response (HSR). We have demonstrated that the pharmacological induction of the HSR by the administration of HSP90 inhibitor

drugs can suppress glial pro-inflammatory activation and ameliorate the clinical course of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS) (Murphy et al., 2002; Dello Russo et al., 2006), consistent with our previous data using hyperthermia (Heneka et al., 2001). The anti-inflammatory effects of the HSR appear to be mediated by reduced activation of the transcription factor NFkB (Heneka et al., 2000).

However, HSP90 inhibitors can exert their biologic effects either by induction of HSR, via activation of HSF-1 or by inactivation or degradation of HSP90 client proteins. An increasing number of proteins has been identified as being HSP90 client proteins (Zhang and Burrows, 2004; Zhao and Houry, 2007; Makhnevych and Houry, 2012); and some of them including the inducible nitric oxide synthase (NOS2), steroid hormone receptors (SHRs), peroxisome proliferator activated receptors (PPARs), the transcription factor NFkB are directly involved in the regulation of inflammatory responses. Particularly, it has been demonstrated that HSP90 binds to the two constitutive forms of NOS (NOS1 and NOS3) (Osawa et al., 2003) as well as the inducible NOS2 (Yoshida and Xia, 2003), regulating their activity. We have shown that geldanamycin potently inhibits astrocyte NOS2 while increasing HSP70 levels (Murphy et al., 2002). Consistent with these findings, a geldanamycin derivative, 17-allylamino-17-demethoxy-geldanamycin (17-AAG), strongly reduced astrocyte inflammatory responses, while displaying minor inhibitory effects on microglial activation (Dello Russo et al., 2006). However although ansamycins are very effective HSP90 inhibitors, their toxicity as well as limited stability limits their clinical use (Chiosis et al., 2004).

To address these limitations, novel HSP90 inhibitors have been developed with improved pharmacokinetic properties. In particular, Chiosis and colleagues have designed and synthesized small molecular weight inhibitors using purine as a scaffold that binds to HSP90 and blocks its activity (Chiosis et al., 2003; Chiosis, 2006). Within the PU-class, PU-H71 has been characterized as a potent HSP90 inhibitor (IC₅₀=50 nM) (He et al., 2006). Interestingly, GA and PU datasets show a very high degree of correlation with respect to HSP90 inhibition, suggesting that the molecular mechanism of action of these drugs is similar. Hence, we tested the hypothesis that the novel HSP90 inhibitor PU-H71, currently in phase I clinical trials (Kim et al., 2009), can exert beneficial effects in neuroinflammatory diseases. In the present study we first characterized the effects of PU-H71 on glial activation, and then tested its effects in the EAE model. Our findings show that PU-H71 reduced astrocyte activation when this was stimulated by the bacterial endotoxin lipopolysaccharide (LPS) but failed to reduce the activation elicited by inflammatory cytokines. Similar to 17-AAG, PU-H71 displayed milder anti-inflammatory effects on microglia activation. In contrast to ansamycins, PU-H71 slightly influenced the course of EAE disease, although it reduced peripheral T cell activation and IFN γ production. These findings demonstrate that even though PU-H71 displays some anti-inflammatory properties similar to the ansamycins, it is less effective when tested in vivo in an animal model of MS.

2. Materials and methods

2.1. Materials

Cell culture reagents [Dulbecco's modified Eagle's medium (DMEM), DMEM-F12 and fetal calf serum (FCS)] were from Invitrogen Corporation (Paisley, Scotland). Antibiotics were from Biochrom AG (Berlin, Germany). Bacterial endotoxin LPS (*Salmonella* Typhimurium) was from Sigma-Aldrich (St. Louis, MO, USA). Recombinant pro-inflammatory cytokines, namely human tumor necrosis factor- α (TNF α), human interleukin 1 β IL-1 β , and rat interferon- γ (IFN γ), were purchased from Endogen (Pierce Biotechnology, Rockford, IL, USA). PU-H71 was a gift of Dr. G. Chiosis.

2.2. Cell cultures

Primary enriched cultures of rat microglia were prepared from mixed cultures of cortical glial cells (at in vitro day 14), as described previously (Dello Russo et al., 2009). Briefly, microglial cells were detached from the astrocyte monolayer by gentle shaking. The cells were plated in 96-well plates at a density of 3×10^5 cells/cm² using 100 μ L/well DMEM-F12 containing 10% FCS and antibiotics. Under these conditions, the cultures were 95–98% CD11b positive. Experiments were carried out in the same medium used for cell plating to reduce microglial death, which normally occurs after splitting from astrocytes. Microglial activation was induced by incubating cells with pro-inflammatory cytokines (10 UI/mL IFN γ , 10 ng/mL TNF α and 10 ng/mL IL-1 β) or 1 ng/mL LPS for different times as indicated in the figure legend. At the end of each experiment, the incubation medium was collected and used for the measurement of nitrite production. Primary cultures of cortical rat astrocytes were obtained as previously described (Lisi et al., 2011). Briefly, after dissecting and digesting the cortices, the cells were plated in 75-cm² flasks (1 brain/flask). The culture medium was changed within 24 h, and then twice a week until the astrocytes formed a monolayer. At that time the culture medium was replaced with PBS without Ca²⁺ and Mg²⁺ (Sigma-Aldrich) and the flasks were vigorously shaken to remove non-adherent cells, oligodendrocytes and microglia. Subsequently, the astrocytes were detached from the flask by a 5-min 0.05% trypsin–EDTA treatment (Biochrom Ltd., UK). Astrocytes obtained with this procedure were then passaged twice for the first time in 75-cm² flasks and for the second time directly in multi-well plates used for the experimental procedures, carried out in 1% FCS DMEM. In these experimental conditions, astrocyte cultures are >95% positive for the cell specific marker GFAP (glial fibrillary acidic protein) (Vairano et al., 2002). Astrocyte activation was accomplished as described for microglial cells, except that for LI treatment, the amount of LPS was increased to 1 μ g/mL.

Stable transfected rat C6 glioma cells (see below) were grown in DMEM containing 10% FCS and antibiotics, including G418. The cells were passed once a week and used for the experiments after 3–4 days, at which time they had reached almost 100% confluence.

2.3. Nitrite assay

NOS2 activity was assessed indirectly by measuring nitrite accumulation in the incubation media. Briefly, an aliquot of the cell culture media (80 μ L) was mixed with 40 μ L Griess Reagent (Sigma-Aldrich) and the absorbance measured at 550 nm in a spectrophotometric microplate reader (PerkinElmer Inc., MA, USA). A standard curve was generated during each assay in the range of concentrations 0–100 μ M using NaNO₂ (Sigma-Aldrich) as standard. In this range, standard detection resulted linear and the minimum detectable concentration of NaNO₂ was 6.25 μ M.

In the absence of stimuli, basal levels of nitrites were below the detection limit of the assay after 24 h and 48 h incubations.

2.4. IKB α promoter or NFkB activation luciferase assay

C6 cells that are stably transfected with a 1.0 kB fragment of the rat IKB α promoter (Gavrilyuk et al., 2002) or 4 copies of a canonical NFkB element driving luciferase expression were used to monitor effects of PU-H71 on activation of IKB α promoter and NFkB activation. These cells have a low level of basal luciferase activity, which can be induced between 4- and 10-fold upon incubation with LPS plus IFN γ or with a cytokine mixture, including TNF α , IL-1 β and IFN γ (referred as TII). C6 cells were incubated with the indicated NOS2 inducers in DMEM containing 1% FCS and the indicated concentrations of PU-H71. After desired incubation times, the media were removed, and the cells were washed once with cold phosphate-buffered saline. To prepare lysates, 50 μ L of CHAPS

buffer (10 mM CHAPS, 10 mM Tris, pH 7.4) was added. Aliquots of cell lysates (40 μ L) were placed into wells of an opaque, white 96-well microplate. A volume of luciferase substrate (20 μ L) (Steady Glo reagent, Promega) was added to all samples, and the luminescence was measured in a microplate reader (VICTOR X4, PerkinElmer). The data are presented as the percentage of luciferase activity measured in the presence of inducers (cells incubated in media with LI or TII) with or without PU-H71, relative to the activity of control cells (which was set as 1).

2.5. NOS2 mRNA analysis

Total cytoplasmic RNA was extracted from astrocytes using TRIZOL reagent (Invitrogen). RNA concentrations were measured using the Quant-iTTM RiboGreen[®] RNA Assay Kit (Invitrogen). In each assay, a standard curve in the range of 0–100 ng RNA was run using 16S and 23S ribosomal RNA (rRNA) from *Escherichia coli* as standard. Aliquots (1 μ g) of RNA were converted to cDNA using random hexamer primers and the ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA). Changes in mRNA levels were estimated by quantitative real-time PCR (Q-PCR) using the following cycling conditions: 35 cycles of denaturation at 95 °C for 20 s; annealing at 59 °C for 30 s; and extension at 72 °C for 30 s; Brilliant SYBR Green QPCR Master Mix 2 $\times$ (Stratagene, La Jolla, CA, USA) was used. PCR reactions were carried out in a 20 μ L reaction volume in a MX3000P real time PCR machine (Stratagene). The primers used for NOS2 detection were the following: 1704F (50-CTG CAT GGA ACA GTA TAA GGC AAA C-30) and 1933R (50-CAG ACA GTT TCT GGT CGA TGT CAT GA-30), which yield a 230 base pair (bp) product. The primers used for α -tubulin were the following: F984 (50-CCC TCG CCA TGG TAA ATA CAT-30) and 1093R (50-ACT GGA TGG TAC GCT TGG TCT-30), which yield a 110 bp product. Relative mRNA concentrations were calculated from the take-off point of reactions (threshold cycle, Ct) using the comparative quantization method and the software included in the unit. For this analysis, controls were used as calibrators and the Ct values for α -tubulin expression as normalizers. Thus, using the $-\Delta\Delta C_t$ method Livak and Schmittgen, 2001, we calculated the differences (fold changes) in the expression of NOS2 target gene after a specific treatment vs its respective control. Moreover, in each run we calculated the PCR efficiency using serial dilution of one experimental sample; efficiency values were found between 94 and 98% for each primer set (Dello Russo et al., 2009).

2.6. T-cell isolation and cytokine measurements

Splenic T cells were prepared from spleens isolated from EAE mice as described (Sharp et al., 2008). In brief, after red blood cell lysis, the splenocytes were plated into 24-well plates at a density of 2×10^5 cells/well in 500 μ L RPMI media, and activated with antibodies to T cell receptor CD3 and to co-stimulatory receptor CD28. After 1 day, aliquots of the media were assayed for levels of IFN γ by specific ELISA following the recommended procedures (R&D Systems, Minneapolis, MN). Each sample was assayed in triplicate, and calculation of pg/mL cytokine determined from standard curves generated using the IFN γ standard provided. Samples were diluted to insure measurement within the linear range of the assay. The minimum detectable concentration of IFN γ was 15 pg/mL.

2.7. EAE induction and clinical assessment

Female C57BL/6 mice, aged 6–8 weeks were maintained in a controlled 12 h:12 h light/dark environment and provided with food and water. EAE was actively induced in mice using the rat/mouse synthetic myelin oligodendrocyte glycoprotein peptide 35–55, (MOG35–55) (Sigma-Aldrich, St. Louis, MO), as previously described (Feinstein et al., 2002). Briefly the mice were injected subcutaneously (s.c., 200 μ L injections in one hind limb) with an emulsion of 300 μ g MOG35–55 dissolved in 100 μ L phosphate buffered saline (PBS), mixed with 100 μ L complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO), containing

500 µg of *Mycobacterium tuberculosis* (Difco, Detroit, MI). Immediately after MOG35-55 injection, the animals received an intraperitoneal (i.p.) injection of pertussis toxin (PT, 200 ng in 200 µL PBS) (Sigma-Aldrich, St. Louis, MO). Two days later, the mice received a second PT injection, and 1 week later, they received a booster injection of MOG35-55. Clinical signs were scored on a five-point scale: grade 0, no clinical signs; 1, limp tail; 2, impaired righting; 3, paresis of one hind limb; 4, paresis of two hind limbs; 5, moribund. The animals were observed every other day after the MOG35-55 booster injection, and severity of disease was evaluated by calculating an average clinical score. A cumulative clinical score was calculated for each mouse by adding the daily scores from the day of onset until the end of the treatment.

2.8. PU-H71 administration

The mice were randomized into different treatment groups, 6 mice for each treatment. Each experiment was repeated twice with similar results. In both cases clinical observations were continued for one month. PU-H71 was dissolved in phosphate buffer containing DMSO. The HSP90 inhibitor was administered at the peak of disease (19 days after the MOG35-55 booster injection), at a dose of 75 mg/kg in a volume of 200 µL by intra-peritoneal injections (i.p.), given 3 times a week. The vehicle solution was administered to the control group according to the same schedule and via the same administration route used for PU-H71. The mice were monitored for clinical signs every other day.

2.9. Data analysis

All in vitro experiments were done using 5–6 replicates per each experimental group, and repeated at least 3 times. For the RNA analyses, the samples were assayed in triplicate, and the experiments were repeated at least twice. Data were analyzed by one- or two-way ANOVA followed by Bonferroni's post hoc tests or by unpaired *t*-test. *P* values <0.05 were considered significant.

3. Results

3.1. PU-H71 exerts anti-inflammatory effects on glial cells

Primary rat astrocytes were activated using 1 µg/mL bacterial endotoxin LPS and 5 ng/mL IFN γ (LI) or a mixture of proinflammatory cytokines (TII, containing 10 ng/mL IL1 β , 10 ng/mL TNF α , and 5 ng/mL IFN γ). Cells were incubated for 24 or 48 h with the stimuli in the presence of the HSP-90 inhibitor PU-H71 at indicated doses. Both stimuli, LI and TII, significantly increased the amount of nitrites, a stable end product of NO, measured in the incubation medium after 24 h or 48 h respectively. PU-H71 significantly and dose-dependently reduced NOS2 activity (Fig. 1A) and expression (Fig. 1C) in LI-stimulated astrocytes, whereas it displayed only modest effects on TII-stimulated astrocytes (Fig. 1B). In LI-stimulated cells, nitrite production was significantly reduced by 20 nM PU-H71, while NOS2 mRNA levels were significantly inhibited at 10 nM (Fig. 1C). When tested on TII activated astrocytes within the same range of concentrations, PU-H71 tended to reduce nitrite production, but this effect only reached statistical significance at the maximum dose used (30 nM) (Fig. 1B). Moreover, while 30 nM PU-H71 induced a 60% reduction of nitrite production elicited by LI, it provoked only a 20% reduction in TII treated astrocytes. Consistently, PU-H71 failed to reduce NOS2 mRNA levels when in the TII treated astrocytes (Fig. 1D).

Further characterization of the regulation of inflammatory gene expression was carried out using the rat C6 glioma cell line, which shares many properties with primary astrocyte cultures (Lisi et al., 2011). In these cells, PU-H71 also significantly reduced nitrite production stimulated by LI at doses of 30 nM or greater (Fig. 2A), whereas only 50 nM

drug induced significant reduction of nitrite production elicited by TII (Fig. 2B). The similarity of PU-H71 effects in primary astrocytes and in C6 cells prompted us to test the effects of this drug on the activation of NFkB, using C6 cells transfected with 4 copies of a canonical NFkB element driving luciferase expression. In C6-kB cells, PU-H71 reduced LI-stimulated NFkB element activation at 30 nM (Fig. 3A), while the same dose of PU-H71 did not reduce TII-stimulated NFkB activation (Fig. 3B). Surprisingly, PU-H71 did not show any significant effect on activation of the Ikb α promoter, either elicited by LI or by TII (Fig. 3C–D). The activity of Ikb α promoter is often increased in rapid response following NFkB activation.

NOS2 can be also induced in primary cultures of rat cortical microglia by LPS or cytokines (Dello Russo et al., 2009). These cells are more reactive than astrocytes; therefore, a lower dose of LPS is used in the absence of any IFN γ to induce comparable levels of NOS2 expression and activity. This is likely due to the higher constitutive expression of the toll-like receptor 4 (TLR4) in microglia as compared to astrocytes, which mediates LPS signaling. Although higher microglial expression of CD14, the protein binding target of LPS which in turn activates TLR4, could also account for their greater reactivity, the presence of soluble CD14 in the incubation medium is sufficient to trigger NOS2 expression in astrocytes in response to LPS (Galea et al., 1996). PU-H71 displayed similar effects on microglial cells as on astrocytes, with 50 nM PU-H71 needed to significantly reduce the LPS dependent nitrite release (Fig. 4A), and with no significant affect on the TII stimulated nitrite production (Fig. 4B). However, the inhibitory effects of PU-H71 on microglial cells were less in comparison to its suppression of astrocyte activation.

3.2. PU-H71 effects in EAE

Based on our previous findings that HSP90 inhibitors potently reduced clinical disease in EAE (Murphy et al., 2002; Dello Russo et al., 2009), and in view of similar anti-inflammatory effects on glial cells in vitro, we tested effects of PU-H71 in MOG EAE. For this, female C57BL/6 mice were immunized with MOG 35–55 peptide to develop a chronic disease, and near the peak of disease (19 days after MOG booster), the mice were randomized for treatment into two groups (6 animals for each group) that had similar patterns of disease development. One group received vehicle and the other received PU-H71 every other day. In contrast to rapid attenuation of clinical scores due to 17AAG or geldanamycin, PU-H71 displayed limited therapeutic effects on average clinical score (Fig. 5A) and cumulative scores (Fig. 5B) for up to 3 weeks of treatment. After 3 weeks treatment, clinical scores in the vehicle treated group showed a trend towards increasing, while scores in the PU-H71 treated group remained stable; however, this effect was not statistically significant. Despite the lack of effect of PU-H71 on clinical scores, in vitro testing showed that 10 nM PU-H71 significantly reduced IFN γ release elicited by CD3, or CD3 plus CD28, both in splenic as well as in lymph node derived T cells (Fig. 6).

4. Discussion

Despite the findings that GA and its derivative 17-AAG show potent anti-inflammatory effects in vitro and in the EAE model of MS, their toxicity limits possible clinical use. In the present study we characterized the effects of a novel HSP-90 inhibitor PU-H71 on glial activation and tested its effects in EAE. Our findings show that, similar to the ansamycins, PU-H71 reduces astrocyte activation when stimulated by LI but had only modest effects on astrocyte activation elicited by pro-inflammatory cytokines. Moreover, PU-H71 displayed milder inhibitory effects on microglia activation than on astrocytes. Our findings also show that PU-H71 had limited effects on the clinical course of EAE, although in vitro it potently reduced IFN γ production from splenic and lymph node T cells. These results point to important differences in the mechanisms of action of ansamycins versus PU-H71 on glial

cell activation, which could account for the reduced efficacy of PUH-71 in EAE. Furthermore, these findings suggest that, at least at later stages of EAE disease, the lack of effect on parenchymal glial cells is of greater importance than effects on the peripheral immune responses. It is also possible that differences in the pharmacokinetics of PUH-71 versus 17-AAG lead to significant differences in brain accumulation. Although the tissue distribution of PUH-71 has been reported (Cerchietti et al., 2009), its levels in the brain were not determined.

Both 17-AAG and PU-H71 bind to the N-terminal ATP/ADP pocket of HSP-90, inhibiting its ATPase activity. However, the structural formula of PU-H17 is completely distinct from 17-AAG. PU-H71 does not have a complex formulation and the benzoquinone moiety is not present (Breinig et al., 2009). The lack of a benzoquinonoid structure is the first important difference of PU-H71 to 17-AAG that could give diverging pharmacological effects. In fact, the DT-diaphorase status is a major source of variability in response to Hsp90 inhibition with 17-AAG and constitutes a potential resistance mechanism. Correspondingly, the poor performance of 17-AAG in Hep3B cells is well explained by the inactivating hypermethylation of the NAD(P)H dehydrogenase quinone 1 (NQO1) gene. No such restrictions were observed for PU-H71; consequently, the antitumorigenic response towards novel Hsp90 inhibitors is independent of the NQO1 status (Breinig et al., 2009). These distinctive features of the two drugs may explain the different pharmacological properties that we observed in vivo. The limited in vivo efficacy of PU-H71 may be due to differences in cell selectivity. PUH (at 10 nM) was very effective at reducing IFN γ release in vitro when tested on peripheral T cells activated with CD3-CD28, whereas 17-AAG displayed only a minor inhibitory effect on IL-2 and did not reduce IFN γ release (Dello Russo et al., 2006). Conversely, both 30 nM PUH-71 and 30 nM 17-AAG reduced of approximately 60% nitrite production induced by LPS in cortical astrocytes. However, in the present study LPS was used in association with IFN γ , which increased the amount of nitrites accumulated in the incubation medium up to 30 μ M, whereas LPS alone increased such production up to 7 μ M (Dello Russo et al., 2006). This would suggest that PUH-71 is more effective than 17-AAG at inhibiting astrocyte pro-inflammatory activation. On the other hand, in microglial cells 100 nM 17 AAG reduced NO production induced by LPS of approximately 65% (Dello Russo et al., 2006), while 50 nM PUH only induced only 25% inhibition. It should be considered that in the chronic EAE model, the glial component, particularly microglia, is more relevant than T cell activation in sustaining brain inflammation, especially in the later stages of diseases when pharmacological treatments were administered (Constantinescu et al., 2011). Therefore, the inhibitory effects of 17-AAG observed in microglial cells may become more relevant in vivo. Another important difference between the two drugs that may explain the diverging effects observed in vivo is related to the different ability to cross the blood brain barrier (BBB). In fact PU-H71 shows, in normal conditions, very limited capacity to enter the CNS (Caldas-Lopes et al., 2009). However, it should be noted that during the EAE clinical course the properties of the BBB are modified, and a breakdown of the barrier often occurs. Therefore, the BBB becomes more permeable, allowing the passage of cells from the periphery as well as several substances, including drugs (Fletcher et al., 2010).

Finally, the limited effects observed in MOG-induced EAE after PU-H71 treatment may be explained by the mild inhibitory effects that the drug showed in vitro when glial cells were activated with a mixture of cytokines. In fact, TNF α , IFN γ and IL1 β are cytokines more related to neuroinflammatory pathologies like MS and consequently in EAE (Sosa and Forsthuber, 2011). Therefore, a mixture of these cytokines is a more relevant physio-pathological stimulus than the bacterial endotoxin to mimic the environment in which glial cells are exposed in vivo in neurodegenerative pathologies, like MS. In our experience, drugs that exert significant anti-inflammatory properties in vitro and reduce NOS2 activity

induced by TII ameliorate the clinical signs of EAE in vivo (Lisi et al., 2012). The two stimuli (LPS and TII) induced a different profile of activation in glial cells, both in astrocytes and in microglia (Dello Russo et al., 2009; Lisi et al., 2011). LPS exerts its actions by binding the TLR4 (Arroyo et al., 2007), while the cytokines bind to and activate different receptors, using different intracellular signaling (Rivest, 2003; Sanchez et al., 2003). Nevertheless, the anti-inflammatory effects of PU-H71 observed in glial cells are likely mediated by blocking NFkB activation, as suggested by the in vitro data obtained using stably transfected C6 glioma cells. This can account for the effects on nitrite reduction elicited both by LI or TII. In fact, 30 nM for LI-stimulated C6 cells and 50 nM for TII-stimulated C6 cells in parallel with the reduction on NO production are able to reduce the NFkB promoter activity. These observations are in line with our previous findings obtained using hyperthermia to induce a HSR (Heneka et al., 2000).

In conclusion, data presented in this paper show that although the novel PU-H71 HSP90 inhibitor retains the anti-inflammatory effects of ansamycins, these properties are not sufficient to improve the clinical course of EAE. However, in order to completely understand the mechanisms at the basis of these peculiar effects further studies are needed, especially comparisons between other HSP-90 inhibitors belonging to the small molecular weight compounds.

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Fig. 1.

Effects of the HSP90 inhibitor on NO production in activated astrocytes. Astrocytes were activated for 24 h with LI [panel A] or 48 h with TII [panel B]. The HSP90 inhibitor, PU-H71, was added to the cells in nM concentrations at the beginning of the experiments. NO production was assessed indirectly by measurement of nitrite accumulation in the incubation medium (Griess method). Data are expressed as means $\pm$ SEM (n=6). Representative of 3 different experiments. Total cytosolic RNA was prepared from Control, or astrocytes treated with LI [panel C] or with TII [panel D] and PU-H71 for 24 h, and used for Q-PCR analysis of iNOS expression. Data are expressed as fold change vs. each respective Control, taken as calibrator for comparative quantitation analysis of mRNA levels. Each sample was measured in triplicate, and the experiment was repeated 2 times with similar results. Results were analyzed by one-way ANOVA followed by Bonferroni's post-test. ***P<0.001 vs. Controls, oooP<0.001 vs. LI or TII.

Fig. 2.

Effects of the HSP90 inhibitor on NO production in activated C6 cells. C6 glioma cells were activated for 24 h with LI [panel A] or TII [panel B]. The HSP90 inhibitor, PU-H71, was added to the cells in nM concentrations at the beginning of the experiments. NO production was assessed indirectly by measurement of nitrite accumulation in the incubation medium (Griess method). Data are expressed as means $\pm$ SEM (n=6). Representative of 3 different experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's post-test. ***P<0.001 vs. Controls, oooP<0.001 vs. LI or TII.

Fig. 3.

PU-H71 reduces the NFκB promoter in transfected C6 cells. (A–B) To monitor NFκB activation, C6 cells stably transfected with 4 copies of a canonical NFκB element driving luciferase expression were activated either with LPS/IFN γ or with TII in association with different concentrations of PU-H71 (as indicated in the figures). The luciferase activity was measured after 8 h of incubation. (C–D) C6 cells that are stably transfected with a 1.0 kB fragment of the rat IκBα promoter were activated either with LPS/IFN γ or with TII in association with different concentrations of PU-H71 (as indicated in the figures). The luciferase activity was measured after 6 h of incubation. Data are presented as the percentage of luciferase activity measured in the control group (taken as 1). Data are means $\pm$ SEM of n=8 separate cell cultures; results were analyzed by unpaired *t*-test. ****P*<0.01 vs Controls, ^o*P*<0.05; ^{ooo}*P*<0.001 vs. LI or TII.

Fig. 4.

Effects of the HSP90 inhibitor on NO production in activated microglia. Microglia cells were activated for 24 h with LPS [panel A] or TII [panel B]. The HSP90 inhibitor, PU-H71, was added to the cells in nM concentrations at the beginning of the experiments. NO production was assessed indirectly by measurement of nitrite accumulation in the incubation medium (Griess method). Data are expressed as means $\pm$ SEM (n=6). Representative of 3 different experiments. Results were analyzed by one-way ANOVA followed by Bonferroni's post-test. ***P<0.001 vs. Controls, ^{ooo}P<0.001 vs. LPS or TII.

Fig. 5.

Effects of PU-H71 in chronic EAE mice model. Mice were immunized with MOG35-55 peptide. 19 days after the MOG booster injection, animals were treated with PU-H71 (75 mg/kg; i.p.), administered every other day. The data shown is mean $\pm$ SEM and is representative of 2 separate studies with similar results. A) Average daily clinical scores. In both groups, scores were significantly different from baseline values by day 6 post-MOG booster injection. No differences were observed in the average day of clinical onset. B) The average cumulative score for each mouse was shown.

Fig. 6.

PU-H71 treatment affects in vitro responses to CD3 and CD28 in splenocytes and lymph-node from EAE mice. Splenocytes and lymph-node isolated from EAE mice were incubated in vitro with or without CD3 or CD28 for 24 h, and the release of IFN-γ was measured. Values are mean±SEM (n=8). Results were analyzed by one-way ANOVA followed by Bonferroni's post-test. ***P<0.001 vs Controls.