Published in final edited form as:

Cancer Lett. 2008 September 8; 268(1): 46–55. doi:10.1016/j.canlet.2008.03.039.

The Heat Shock Protein Antagonist 17-AAG Potentiates the Activity of Enzastaurin Against Malignant Human Glioma Cells

Esther P. Jane and Ian F. Pollack*

Department of Neurosurgery, University of Pittsburgh School of Medicine, University of Pittsburgh Cancer Institute Brain Tumor Center, Pittsburgh, Pennsylvania 15213

Abstract

Recent studies have suggested that the proliferation of malignant gliomas may result from activation of protein kinase C (PKC)-mediated pathways. Enzastaurin (LY317615), an acyclic bisindolylmaleimide, is an oral inhibitor of PKC β as well as other isoforms. The initial objective of this study was to assess the efficacy of enzastaurin in a series of malignant human glioma cell lines with diverse genomic alterations. Although enzastaurin independently produced a dose-dependent inhibition of cellular proliferation and decreased cell viability in each of the glioma cell lines examined, and partially down-regulated Akt and GSK3 β phosphorylation, median effective concentrations were at the upper limits of, or above, the clinically achievable range in all cell lines tested. We therefore examined whether the efficacy of enzastaurin could be enhanced by combination with the HSP90 antagonist, 17-AAG, which inhibits Akt and other signaling intermediates by a distinct mechanism. In comparison to the effect of enzastaurin alone, combination of enzastaurin with 17-AAG led to marked enhancement of antiproliferative and cytotoxic effects. Simultaneous exposure to both agents significantly increased the release of cytochrome c, as well as caspase 3 activation, Bax cleavage, and inhibition of Akt phosphorylation. Cells exposed to enzastaurin and 17-AAG also displayed a significant reduction in cell cycle regulatory proteins, such as CDK4 and CDK6. Taken together, these findings suggest that the efficacy of enzastaurin can be potentiated by the addition of 17-AAG, and indicate that combining molecularly targeted therapies may provide a more effective strategy than single-agent therapy to treat patients with malignant gliomas.

Keywords

Enzastaurin; PKC; 17-AAG; AKT; GSK3 β ; apoptosis; glioma

Introduction

Human glioblastomas are aggressive tumors that commonly harbor a variety of molecular alterations that favor cell growth and survival. Malignant glioma cells have significantly elevated levels of protein kinase C (PKC) activity in comparison to non-neoplastic astrocytes [1], and recent studies from our laboratory [2] and others [3,4] indicate that the aberrant proliferation of these tumors may result in part from excessive activation of various isoforms

*To whom all correspondence should be addressed: Ian F. Pollack, M.D., F.A.C.S., F.A.A.P., Department of Neurosurgery, Children's Hospital of Pittsburgh, 3705 Fifth Avenue, Pittsburgh, PA15213, Phone: 412-692-5881, Fax: 412-692-5921, E-mail: ian.pollack@chp.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of PKC, driven by dysregulated upstream growth factor-mediated signaling cascades [5]. PKC activation has been implicated in modulating several major signaling pathways. For example, PKC activation can trigger signaling through the Ras/extracellular signal-related kinase (ERK) pathway, which may be involved in controlling cell proliferation and apoptosis [6]. In addition, recent work has shown a link between PKC and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which also plays a role in regulating apoptotic responses [7] and is frequently activated in malignant tumors [8]. PKC α , PKC β , and PKC η directly phosphorylate Akt at Ser⁴⁷³, which is essential for Akt activity [9–11]. Moreover, both PKC [12,13] and Akt [14] can phosphorylate glycogen synthase kinase 3 β (GSK3 β) at Ser⁹, suggesting that these signaling intermediates may have overlapping effects on downstream targets. .

Enzastaurin (LY317615), a cyclic bisindolylmaleimide, is an oral inhibitor of several PKC isoforms, and competes with ATP for the enzyme's ATP-binding site [15], leading to inhibition of downstream signaling through AKT. This agent is currently in phase II clinical trials in patients with refractory glioblastoma, diffuse large B-cell lymphoma, lung, head and neck [16] and non-small-cell lung cancer (NSCLC) [17]. In previous studies, enzastaurin has been observed to induce cell growth arrest in the malignant human glioma cell line U87MG in a concentration dependent manner [15].

To evaluate the generalizability of this inhibition, we examined the concentration response profile of enzastaurin in a series of glioma cell lines. Because we observed only partial inhibition of proliferation and downstream growth and survival signaling at clinically achievable concentrations in these lines, we next questioned whether therapeutic efficacy could be enhanced by combination of enzastaurin with another signaling inhibitor that interfered with downstream signaling by a distinct mechanism.

Studies from our laboratory [2,18] and others [19,20] have demonstrated that the heat shock protein (HSP) inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG), interferes with processing of multiple signaling pathway components, and inhibits proliferation of a number of tumor types in vitro, as well as cells transformed with a variety of oncogenes [21]. HSPs are involved in the conformational maturation of a number of signaling proteins critical to the proliferation of malignant glioma cells, including Akt [22], and disruption of HSP90 function by agents such as 17-AAG causes proteasome-dependent degradation of Akt [23,24] as well as several transmembrane tyrosine kinases implicated in glial tumorigenesis [25].

Because enzastaurin and 17-AAG interfere with Akt-mediated signaling by distinct mechanisms, we hypothesized that the HSP90 inhibitor 17-AAG would potentiate enzastaurin-mediated glioma cytotoxicity by decreasing the activation status of PKC as well as down-regulating the levels of other relevant signaling effectors. We therefore, examined the effects of enzastaurin alone and in combination with 17-AAG, on signal transduction and apoptosis in malignant human glioma cell lines.

Our findings show that 17-AAG markedly potentiated the effects of enzastaurin in inhibiting cellular proliferation and Akt phosphorylation, and inducing caspase activation and apoptosis. These data support the notion that combination of molecularly targeted agents may be a promising approach to enhance therapeutic efficacy of these agents against human malignant glioma cells.

Material and Methods

Reagents and Cell Culture

Enzastaurin was obtained from Eli Lilly (Indianapolis, IN). 10mM stock aliquots were stored at -20°C . 17-AAG was purchased from Calbiochem (La Jolla, CA). The established malignant

glioma cell lines U87, T98G, U373 and A172 were obtained from the American Type Culture Collection. LN18, LN-Z308, and LN-Z428 were generously provided by Dr. Nicolas de Tribolet (Lausanne, Switzerland). Human astrocytes (HAs) and human umbilical vein endothelial cells (HUVECs) were obtained from ScienCell Research Laboratories (San Diego, CA). U87, T98G, and U373 were cultured in growth medium composed of minimum essential medium supplemented with sodium pyruvate and non-essential amino acids; A172, LN18, LN-Z428 and LN-Z308 in α -minimal essential medium supplemented with L-glutamine. All growth media contain 10% fetal calf serum, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin (Life Technologies, Inc., Bethesda, MD). HAs were grown in Astrocyte Growth Medium and HUVECs in Endothelial Cell Medium (ScienCell Research Laboratories).

Cell Proliferation and Cytotoxicity Assay

Cells (1×10^3 /well) were plated in 96-well microtiter plates (Costar, Cambridge, MA) in 100 μ l of growth medium. After an overnight attachment period, cells were exposed for 3 days to various concentrations of enzastaurin and, in subsequent experiments to various concentrations of enzastaurin and 17-AAG, alone and in combination. Control cells received vehicle alone (DMSO). All studies were performed in triplicate and repeated at least three times independently. After the 3-day treatment, cells were washed in inhibitor-free medium and the number of viable cells was determined with a colorimetric MTS (3-[4,5-dimethylthiazol-2yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H tetrazolium) cell proliferation assay (CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI) [26].

Western Blotting Analysis

Total cell lysates were prepared and analyzed by Western Blot analysis as described previously [27]. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred onto a nylon membrane (Invitrogen, Carlsbad, CA). Primary antibodies were purchased from Cell Signalling Technology and used according to the manufacturer's recommendations. Secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The proteins were visualized by enhanced chemiluminescence (Cell Signaling Technology). Where indicated, the blots were reprobed with antibodies against β -actin to ensure equal loading and transfer of proteins.

Annexin V Apoptosis Assay

Apoptosis induction in control (DMSO-treated) or inhibitor-treated cells was assessed using an Annexin V assay kit according to the manufacturer's protocol (Molecular Probes, Carlsbad, CA). In brief, 2×10^5 cells were harvested 24 hrs after treatment and washed with ice-cold PBS and resuspended in 200 μ l of binding buffer. Annexin V-FITC and 1 μ g/ml propidium iodide were added to individual samples and incubated for 15 min in a dark environment. The reaction was stopped by adding 300 μ l of 1x binding buffer. Labelling was analyzed by flow cytometry with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) [27].

Immunocytochemistry and Fluorescence Microscopy

Cells were grown on chamber slides (Nalge Nunc, Naperville, IL) in growth medium, and, after an overnight attachment period, were exposed to selected concentrations of inhibitor or vehicle (DMSO) for various durations. Cells were fixed with 3.7% formaldehyde for 15 min, washed in PBS, and permeabilized with 0.1% Triton X-100 in PBS for 15 min. After blocking with 0.3% bovine serum albumin and 1% goat serum for 1 h, cells were incubated with a mouse monoclonal antibody against cytochrome c (BD Biosciences PharMingen, San Diego, CA) at 1:100 and a rabbit antibody against active Caspase 3 (Promega) at 1:250 overnight at 4°C.

After PBS wash, the slides were incubated with secondary antibody (TRITC goat anti-mouse and FITC-goat anti-rabbit; Invitrogen) and Draq-5 (Alexis Biochemicals, San Diego, CA) for 1 h at room temperature. Cells were then washed, mounted, and examined under an Olympus IX81 confocal microscope and imaged using the Olympus Fluoview software (Version 1.5).

RESULTS

Enzastaurin suppresses cell proliferation and induces cytochrome c release in human malignant glioma cell lines

To determine the effect of enzastaurin on a panel of glioma cell lines, cells were cultured with increasing concentrations of enzastaurin and cell proliferation was assessed by MTS assay after 72 h. Control cells were treated with vehicle only (DMSO). Treatment with enzastaurin resulted in a dose-dependent inhibition of cell proliferation (Figure 1A). Over the range of concentrations used, there were no significant effects on normal cells, such as human astrocytes and HUVECs. Examination of the dose-response curves in the malignant glioma cell lines demonstrated that only partial inhibition of cell proliferation was obtained with drug concentrations in the clinically achievable low micromolar concentration range in each of the six lines tested [16].

Because the antiproliferative effects observed were most apparent in T98G, we questioned whether enzastaurin was inducing apoptotic cell death. Since the redistribution of cytochrome c [28] and nuclear translocation of caspase 3 [29] have been reported to be early events in the apoptotic process, we examined these features in the T98G cells. Immunofluorescence microscopy demonstrated that exposure to 5 μ M enzastaurin produced a time-dependent redistribution of cytochrome c and nuclear translocation of caspase 3 (Figure 1B).

This result was confirmed by Western immunoblotting analysis using specific antibodies for caspase 3 and PARP, a caspase substrate. Treatment with 5 μ M enzastaurin significantly increased expression of the cleaved forms of both caspase 3 and PARP, particularly with prolonged exposures (Figure 1C). Taken together, these results indicate that enzastaurin alone was capable of inhibiting glioma cellular proliferation, with substantially greater efficacy against some cell lines than others. In the most sensitive T98G line, treatment with concentrations on the upper border of the clinically achievable range produced mitochondrial injury and apoptosis induction, as evidenced by the release of cytochrome c, activation of caspase 3, and PARP cleavage, although even at these high concentrations did not entirely abrogate cell proliferation.

Enzastaurin decreases AKT and GSK3 β phosphorylation in human malignant glioma cell lines through a non-PKC β -dependent mechanism

Because enzastaurin demonstrated a substantially different response profile in T98G versus the other glioma cell lines tested, we questioned whether this reflected significant differences in the expression of PKC isoforms. Enzastaurin has been demonstrated to inhibit a number of PKC isoforms at clinically achievable concentrations with most dramatic effects on PKC β , γ , δ , ϵ , and θ [15]. We therefore first examined expression of a panel of PKC isoforms in three glioma cell lines (U87, T98G and LNZ308) by Western immunoblot analysis using isoform-specific antibodies. Higher levels of expression of PKC α , PKC γ , PKC δ , PKC ϵ , PKC λ , and PKC μ were seen in all three lines. The expression of PKC β and PKC θ was low in U87, T98G and LNZ308 cell lines (Figure 2A), suggesting that the activity of enzastaurin in glioma cells may well reflect inhibition of isoforms other than PKC β that are effected by submicromolar concentrations of this agent (e.g., α , δ , and ϵ). However, the lack of clear association between expression profiles of the various PKC isoforms and response to this agent indicates that factors other than PKC expression status may strongly influence efficacy.

Because recent reports have described a role for several PKC isoforms in promoting AKT [9–11] and GSK activity [12,13] and have noted that the efficacy of enzastaurin correlates with downstream inhibition of AKT and GSK, we next investigated the effect of enzastaurin on AKT and GSK3 β phosphorylation in the T98G cell line. Time course analysis revealed that treatment of T98G cells with 5 μ M enzastaurin had minimal effects on the expression of PKC α , δ , and ϵ with up to 48 h of exposure (Figure 2B), but a more apparent decrease in AKT and GSK3 β phosphorylation, which was visible by 3 h (Figure 2C). Exposure to enzastaurin for intervals of 12 h or more diminished but did not eliminate their phosphorylation as measured with phosphospecific antibodies, without changes in the levels of total AKT and GSK3 β proteins. (Figure 2C). This suggests that enzastaurin may exert its cytotoxic activity in susceptible glioma cells by downstream inhibition of AKT signaling, rather than having any direct effect on PKC isoform expression, which is consistent with observations in other types of tumor cell lines [30–33].

Combined exposure of glioma cells to enzastaurin and 17-AAG enhances antiproliferative effects and down-regulates expression of cell cycle regulatory proteins

Because enzastaurin only partially inhibited glioma cell proliferation at clinically achievable concentrations, we questioned whether antiproliferative effects could be potentiated by administration of an additional inhibitor of targets involved in PKC-mediated signaling. In that context, administration of heat shock protein antagonists has been associated with down-regulation of AKT as well as a number of other signaling mediators and disruption of AKT-mediated survival signaling in glioma cells [2]. These observations prompted us to examine whether combining enzastaurin and 17-AAG could achieve synergistic effects in human glioma cell lines. To examine the antiproliferative efficacy of administering enzastaurin with 17-AAG in human malignant glioma cells, a combinatorial cell proliferation assay was conducted. Cells were treated with various concentrations of enzastaurin with or without 50 nM 17-AAG, a concentration that produced only modest independent antiproliferative effects in our previous studies [2], and then cell proliferation was measured with an MTS assay. As shown in Figure 3A, the dose-dependent inhibitory effect of enzastaurin was substantially potentiated by adding 17-AAG.

To evaluate potential mechanisms for this potentiation, we also assessed the effects of enzastaurin and 17-AAG, alone or in combination, on the expression level of various cell cycle regulatory proteins in T98G. Results from Western blot analysis showed that the combination of enzastaurin and 17-AAG significantly decreased the levels of cyclin-D1, cyclin-D3, CDK4 and CDK6, suggesting that this combination was synergistically inhibiting cell cycle progression (Figure 3B).

Combined exposure of glioma cells to enzastaurin and 17-AAG induces cytotoxicity by caspase 3 and BAX activation and down-regulates the expression of PKC ϵ and phosphorylation of AKT and GSK3 β

Because heat shock protein interferes with the induction of apoptosis through several pathways by its direct interaction with cytochrome *c*, AKT, and PKC, we also speculated that 17-AAG, might augment the apoptotic effects of enzastaurin in malignant human glioma cell lines. U87 and T98G cells were exposed to 5 μ M enzastaurin or 150 nM 17-AAG or the combination of both, and apoptosis was assessed after 24 h by Annexin V assay, which measures a late stage in the apoptotic process, specifically the membrane externalization of phosphatidylserine. Although only modest effects were seen with both agents alone at these concentrations, the combination of 5 μ M enzastaurin and 17-AAG (150 nM) significantly potentiated glioma cell cytotoxicity (Figure 4A).

Recent reports have suggested that the association of PKC ϵ with BAX neutralizes apoptotic signals propagated through mitochondrial death-signaling pathway [34,35]. We therefore questioned whether combining PKC inhibition with 17-AAG might potentiate early steps in the induction of apoptotic signaling by this mechanism. We compared the levels of the active, cleaved forms of caspase 3 and BAX in cells treated with enzastaurin and 17-AAG alone, and the two combined, using specific antibodies that recognize the respective cleaved fragments by Western immunoblotting [27]. In T98G cells, treatment with 17-AAG alone had no effect on the levels of cleaved BAX and caspase 3. Although enzastaurin alone did induce caspase 3 cleavage, particularly with prolonged exposure, as noted in Figure 1C, levels of cleavage at 24 hours were modest and no Bax cleavage was apparent (Figure 4B). In contrast, combined treatment with both agents resulted in the appearance of substantial immunoreactivity for cleaved forms of BAX (18kDa cleaved fragment) and significant increases in the expression of activated caspase 3 (17kDa cleaved fragment) in a dose- and time-dependent manner (Figure 4B). In addition to the above effects, we observed by immunostaining that translocation of cytochrome *c* from the mitochondria to the nucleus was dramatically enhanced after co-administration of enzastaurin and 17-AAG. The normal distribution of mitochondrial cytochrome *c* was lost within an hour of drug treatment and redistributed around the nucleus with an effect that was substantially more extensive and rapid than after administration of either agent alone (data not shown).

We next examined the effects of these agents alone, or in combination on AKT phosphorylation as well as the expression of PKC ϵ . Results from Western immunoblot analysis showed that the combination of enzastaurin and 17-AAG significantly decreased the levels of AKT phosphorylation compared to that obtained with each agent individually. The combination also led to significant decreases in the expression of PKC ϵ (Figure 4C), one of the PKC isoforms that is a target of enzastaurin, and has been most strongly implicated in the progression of gliomas as well as other tumor types [36].

Discussion

Enzastaurin is an acyclic bisindolylmaleimide derived from staurosporine and acts as an ATP competitor. It interacts with protein kinase C, thereby modulating signal transduction pathways mediating tumor cell survival and proliferation [15]. Although initially developed as an inhibitor of PKC β , it was later noted to inhibit multiple PKC isoforms. It has demonstrated significant *in vitro* and *in vivo* cytotoxicity in B-cell malignancies, as well as in various other cancer cell lines [32], and significant interest has been directed toward the clinical use of this agent against malignant gliomas.

Our detailed dose-response data, however, indicate that this agent exhibits widely different response profiles among a group of glioma cell lines, and only partially blocked the proliferation of many of these lines at therapeutically achievable concentrations, which parallels the partial inhibition of AKT phosphorylation that is achieved at these concentrations. These differences in response did not reflect discernible distinctions between cell lines in their patterns of PKC isoform expression, and may well relate to differences in recruitment of alternative signaling pathways in maintaining proliferation in the setting of PKC inhibition.

We therefore questioned whether combination of enzastaurin with an agent that blocked downstream signaling by an alternate mechanism might potentiate therapeutic efficacy. The combination of enzastaurin with the HSP inhibitor 17-AAG potentiated the inhibition of tumor cell proliferation that was achieved as well as the inhibition of AKT and GSK3 β phosphorylation. The PI3K/AKT pathway is a key regulator of cellular survival and is often constitutively activated in glioblastoma [37]. Convergent inhibition of AKT and GSK signaling

by enzastaurin and 17-AAG may play an important functional role in the synergistic induction of glioma cytotoxicity that was observed with this combination.

This combinatorial effect also potentiated the induction of tumor cell apoptosis. We [2] and others [38] have shown that release of cytochrome *c* from the mitochondria is a critical step for the induction of apoptosis in human malignant glioma cells and that this process precedes nuclear condensation [39], chromatin fragmentation, Annexin V labeling, and cell death [40]. Although we found some degree of translocation of cytochrome *c* from the mitochondria to the nucleus, as well as caspase 3 cleavage, after treating the cells with enzastaurin alone, co-administration of enzastaurin and 17-AAG dramatically enhanced the speed and extent of this process. The combination also markedly potentiated the appearance of later stages of apoptotic progression, as evidenced by a significant enhancement in Annexin V labeling.

In summary, our observations indicate that combining enzastaurin with other strategies for blocking AKT-mediated signaling may significantly potentiate therapeutic efficacy in malignant glioma cells in terms of inhibiting cell proliferation and promoting tumor cell killing. These findings highlight the fact that combining molecularly targeted therapies may provide a more effective strategy than single-agent therapies, which to date have often led to disappointing clinical results, as a way to treat patients with malignant gliomas.

Acknowledgements

We thank Naomi Agostino for maintaining the cell stocks and Maria Branca for assisting with the microscope imaging.

Grant support: This work was supported by NIH grant P01NS40923

REFERENCES

1. Couldwell WT, Uhm JH, Antel JP, Yong VW. Enhanced protein kinase C activity correlates with the growth rate of malignant gliomas in vitro. *Neurosurgery* 1991;29:880–886. [PubMed: 1758601] discussion 886–887
2. Premkumar DR, Arnold B, Jane EP, Pollack IF. Synergistic interaction between 17-AAG and phosphatidylinositol 3-kinase inhibition in human malignant glioma cells. *Mol Carcinog* 2006;45:47–59. [PubMed: 16267832]
3. Baltuch GH, Yong VW. Signal transduction for proliferation of glioma cells in vitro occurs predominantly through a protein kinase C-mediated pathway. *Brain Res* 1996;710:143–149. [PubMed: 8963653]
4. von Deimling A, Louis DN, Wiestler OD. Molecular pathways in the formation of gliomas. *Glia* 1995;15:328–338. [PubMed: 8586467]
5. da Rocha AB, Mans DR, Regner A, Schwartzmann G. Targeting protein kinase C: new therapeutic opportunities against high-grade malignant gliomas? *Oncologist* 2002;7:17–33. [PubMed: 11854544]
6. Goekjian PG, Jirousek MR. Protein kinase C inhibitors as novel anticancer drugs. *Expert Opin Investig Drugs* 2001;10:2117–2140.
7. Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR. Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms. *FEBS Lett* 2000;484:217–223. [PubMed: 11078882]
8. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002;2:489–501. [PubMed: 12094235]
9. Aeder SE, Martin PM, Soh JW, Hussaini IM. PKC-eta mediates glioblastoma cell proliferation through the Akt and mTOR signaling pathways. *Oncogene* 2004;23:9062–9069. [PubMed: 15489897]
10. Kawakami Y, Nishimoto H, Kitaura J, Maeda-Yamamoto M, Kato RM, Littman DR, Leitges M, Rawlings DJ, Kawakami T. Protein kinase C betaII regulates Akt phosphorylation on Ser-473 in a cell type- and stimulus-specific fashion. *J Biol Chem* 2004;279:47720–47725. [PubMed: 15364915]

11. Partovian C, Simons M. Regulation of protein kinase B/Akt activity and Ser473 phosphorylation by protein kinase Calpha in endothelial cells. *Cell Signal* 2004;16:951–957. [PubMed: 15157674]
12. Fang X, Yu S, Tanyi JL, Lu Y, Woodgett JR, Mills GB. Convergence of multiple signaling cascades at glycogen synthase kinase 3: Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. *Mol Cell Biol* 2002;22:2099–2110. [PubMed: 11884598]
13. Goode N, Hughes K, Woodgett JR, Parker PJ. Differential regulation of glycogen synthase kinase-3 beta by protein kinase C isoforms. *J Biol Chem* 1992;267:16878–16882. [PubMed: 1324914]
14. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995;378:785–789. [PubMed: 8524413]
15. Graff JR, McNulty AM, Hanna KR, Konicek BW, Lynch RL, Bailey SN, Banks C, Capen A, Goode R, Lewis JE, Sams L, Huss KL, Campbell RM, Iversen PW, Neubauer BL, Brown TJ, Musib L, Geeganage S, Thornton D. The protein kinase Cbeta-selective inhibitor, Enzastaurin (LY317615.HCl), suppresses signaling through the AKT pathway, induces apoptosis, and suppresses growth of human colon cancer and glioblastoma xenografts. *Cancer Res* 2005;65:7462–7469. [PubMed: 16103100]
16. Carducci MA, Musib L, Kies MS, Pili R, Truong M, Brahmer JR, Cole P, Sullivan R, Riddle J, Schmidt J, Enas N, Sinha V, Thornton DE, Herbst RS. Phase I dose escalation and pharmacokinetic study of enzastaurin, an oral protein kinase C beta inhibitor, in patients with advanced cancer. *J Clin Oncol* 2006;24:4092–4099. [PubMed: 16943527]
17. Herbst RS. Targeted therapy using novel agents in the treatment of non-small-cell lung cancer. *Clin Lung Cancer* 2002;3:S30–S38. [PubMed: 14720353]
18. Premkumar DR, Arnold B, Pollack IF. Cooperative inhibitory effect of ZD1839 (Iressa) in combination with 17-AAG on glioma cell growth. *Mol Carcinog* 2006;45:288–301. [PubMed: 16550610]
19. Clarke PA, Hostein I, Banerji U, Stefano FD, Maloney A, Walton M, Judson I, Workman P. Gene expression profiling of human colon cancer cells following inhibition of signal transduction by 17-allylamino-17-demethoxygeldanamycin, an inhibitor of the hsp90 molecular chaperone. *Oncogene* 2000;19:4125–4133. [PubMed: 10962573]
20. Hostein I, Robertson D, DiStefano F, Workman P, Clarke PA. Inhibition of signal transduction by the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin results in cytostasis and apoptosis. *Cancer Res* 2001;61:4003–4009. [PubMed: 11358818]
21. Munster PN, Marchion DC, Basso AD, Rosen N. Degradation of HER2 by ansamycins induces growth arrest and apoptosis in cells with HER2 overexpression via a HER3, phosphatidylinositol 3'-kinase-AKT-dependent pathway. *Cancer Res* 2002;62:3132–3137. [PubMed: 12036925]
22. Fujita N, Sato S, Ishida A, Tsuruo T. Involvement of Hsp90 in signaling and stability of 3-phosphoinositide-dependent kinase-1. *J Biol Chem* 2002;277:10346–10353. [PubMed: 11779851]
23. Sato S, Fujita N, Tsuruo T. Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci U S A* 2000;97:10832–10837. [PubMed: 10995457]
24. Basso AD, Solit DB, Munster PN, Rosen N. Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. *Oncogene* 2002;21:1159–1166. [PubMed: 11850835]
25. Xu W, Mimnaugh E, Rosser MF, Nicchitta C, Marcu M, Yarden Y, Neckers L. Sensitivity of mature ErbB2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. *J Biol Chem* 2001;276:3702–3708. [PubMed: 11071886]
26. Riss TL, Moravec RA. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay Drug Dev Technol* 2004;2:51–62. [PubMed: 15090210]
27. Jane EP, Premkumar DR, Pollack IF. AG490 influences UCN-01-induced cytotoxicity in glioma cells in a p53-dependent fashion, correlating with effects on BAX cleavage and BAD phosphorylation. *Cancer Lett* 2007;257:36–46. [PubMed: 17900801]
28. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996;86:147–157. [PubMed: 8689682]

29. Kamada S, Kikkawa U, Tsujimoto Y, Hunter T. Nuclear translocation of caspase-3 is dependent on its proteolytic activation and recognition of a substrate-like protein(s). *J Biol Chem* 2005;280:857–860. [PubMed: 15569692]
30. Moreau AS, Jia X, Ngo HT, Leleu X, O'Sullivan G, Alsayed Y, Leontovich A, Podar K, Kutok J, Daley J, Lazo-Kallanian S, Hatjiharissi E, Raab MS, Xu L, Treon SP, Hideshima T, Anderson KC, Ghobrial IM. Protein kinase C inhibitor enzastaurin induces in vitro and in vivo antitumor activity in Waldenstrom macroglobulinemia. *Blood* 2007;109:4964–4972. [PubMed: 17284528]
31. Podar K, Raab MS, Zhang J, McMillin D, Breitzkreutz I, Tai YT, Lin BK, Munshi N, Hideshima T, Chauhan D, Anderson KC. Targeting PKC in multiple myeloma: in vitro and in vivo effects of the novel, orally available small-molecule inhibitor enzastaurin (LY317615.HCl). *Blood* 2007;109:1669–1677. [PubMed: 17023575]
32. Querfeld C, Rizvi MA, Kuzel TM, Guitart J, Rademaker A, Sabharwal SS, Krett NL, Rosen ST. The selective protein kinase C beta inhibitor enzastaurin induces apoptosis in cutaneous T-cell lymphoma cell lines through the AKT pathway. *J Invest Dermatol* 2006;126:1641–1647. [PubMed: 16645590]
33. Tabatabai G, Frank B, Wick A, Lemke D, von Kurthy G, Obermuller U, Heckl S, Christ G, Weller M, Wick W. Synergistic antiglioma activity of radiotherapy and enzastaurin. *Ann Neurol* 2007;61:153–161. [PubMed: 17212356]
34. Hurbini A, Coll JL, Dubrez-Daloz L, Mari B, Auburger P, Brambilla C, Favrot MC. Cooperation of amphiregulin and insulin-like growth factor-1 inhibits Bax-and Bad-mediated apoptosis via a protein kinase C-dependent pathway in non-small cell lung cancer cells. *J Biol Chem* 2005;280:19757–19767. [PubMed: 15767261]
35. McJilton MA, Van Sikes C, Wescott GG, Wu D, Foreman TL, Gregory CW, Weidner DA, Harris Ford O, Morgan Lasater A, Mohler JL, Terrian DM. Protein kinase Cepsilon interacts with Bax and promotes survival of human prostate cancer cells. *Oncogene* 2003;22:7958–7968. [PubMed: 12970744]
36. Okhrimenko H, Lu W, Xiang C, Hamburger N, Kazimirsky G, Brodie C. Protein kinase C-epsilon regulates the apoptosis and survival of glioma cells. *Cancer Res* 2005;65:7301–7309. [PubMed: 16103081]
37. Sansal I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. *J Clin Oncol* 2004;22:2954–2963. [PubMed: 15254063]
38. Joseph B, Marchetti P, Formstecher P, Kroemer G, Lewensohn R, Zhivotovsky B. Mitochondrial dysfunction is an essential step for killing of non-small cell lung carcinomas resistant to conventional treatment. *Oncogene* 2002;21:65–77. [PubMed: 11791177]
39. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999;397:441–446. [PubMed: 9989411]
40. Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, Li CY, Sasaki T, Elia AJ, Cheng HY, Ravagnan L, Ferri KF, Zamzami N, Wakeham A, Hakem R, Yoshida H, Kong YY, Mak TW, Zuniga-Pflucker JC, Kroemer G, Penninger JM. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* 2001;410:549–554. [PubMed: 11279485]

Fig. 1. Effects of Enzastaurin on cellular proliferation and cytotoxicity

A. logarithmically growing glioma cells were incubated with various concentrations of enzastaurin for 3 days. The relationship between enzastaurin and cell numbers was assessed semiquantitatively by spectrophotometric measurement of MTS bioreduction in six established human malignant glioma cell lines and two types of non-neoplastic cells (HA and HUVECs). Points represent the mean of four measurements $\pm$ standard deviation. There was a dose-dependent reduction in cell growth in the tumor cell lines that far exceeded the effects in non-neoplastic cells. Control cells were treated with equivalent concentrations of vehicle (DMSO). **B.** logarithmically growing T98G cells were incubated with 5 μ M of enzastaurin for 3h and 6h. Cells were fixed, permeabilized, and stained with Draq5 (blue) to visualize nuclear morphology. Anti-ACTIVE Caspase 3 antibody (green) was used to stain cells undergoing apoptosis. T98G cells treated with enzastaurin for three hours showed nuclear staining of active-caspase 3, indicated by white arrows. Under normal conditions, mitochondria appeared as filamentous structures as shown by the staining of cytochrome *c* (red) in the untreated cells. After treatment with enzastaurin, mitochondrial filamentous structure was lost and cytochrome *c* staining became more condensed around the nucleus, which represents an early event in the apoptotic process associated with mitochondrial damage. **C.** The effect of enzastaurin on the cleavage of caspase 3 and PARP at different time points. T98G cells incubated with enzastaurin (5 μ M) showed the activation of caspase 3 by 24 h, along with cleavage of PARP. The uncleaved form of PARP is seen at 116 kDa, whereas the cleaved form of PARP is detected at 85 kDa.

Fig. 2. Enzastaurin inhibits PKC isoforms and decreases the phosphorylation of AKT and GSK3 β

A. Expression profile of specific PKC isoforms in three glioma cell lines (U87, T98G and LNZ308), as assessed by Western immunoblot analysis using specific antibodies. Higher levels of expression of PKC α , PKC δ , PKC ϵ , PKC γ , PKC λ , and PKC μ were seen in all three lines.

The expression of PKC β , PKC θ was low. **B.** T98G cells were treated with enzastaurin (5 μ M) for different time points. With prolonged expression (48 hr), expression of PKC α , δ , and ϵ isoforms were slightly reduced. **C.** The effect of enzastaurin on AKT and GSK3 β phosphorylation was assessed by immunoblotting. Exposure of T98G cells to 5 μ M enzastaurin resulted in a discernible, although incomplete, diminution in AKT and pGSK3 β phosphorylation as early as 3 h but there were no marked changes in the expression of AKT and GSK3 β . β -actin was added as a control for equal protein loading

Fig. 3. Combination of enzastaurin and 17-AAG decreases cell proliferation and inhibits expression of cell cycle regulatory proteins

Combination of enzastaurin and 17-AAG preferentially inhibits the growth of glioma cells.

A. Logarithmically growing glioma cell lines were incubated with various concentrations of enzastaurin with or without 50 nM of 17-AAG for 3 d. The relationship between enzastaurin concentration and cell numbers was assessed semiquantitatively by spectrophotometric measurement of MTS bioreduction in U87, T98G, U373, and LN-Z428 established malignant human glioma cell lines. Points represent the mean of three measurements $\pm$ standard deviation. There was a dose-dependent reduction in cell growth, and addition of 50 nM 17-AAG potentiated the enzastaurin-induced toxicity. No significant inhibition was detected in control cells treated with equivalent concentrations of vehicle (DMSO). **B.** Combination of enzastaurin and 17-AAG cooperatively down-regulates expression of cell cycle regulatory molecules. Exponentially growing T98G cells were exposed to various concentrations of enzastaurin with or without 150 nM 17-AAG for 24 h. Results from Western blot analysis showed that the combination of enzastaurin and 17-AAG significantly decreased the levels of cyclin-D1, cyclin-D3, CDK4 and CDK6.

A **B**

Fig. 4. Cotreatment of glioma cells with 17-AAG and enzastaurin enhances cytotoxicity by inducing apoptosis, inhibits PKC ϵ and decreases the phosphorylation of AKT and GSK3 β

A. Logarithmically growing U87 and T98G cells were incubated with 5 μ M enzastaurin or 150nM 17-AAG or the combination of both for 24 h. Apoptosis was determined by flow cytometry using Annexin V-Alexa Flour 488. Points represent the mean of three experiments $\pm$ standard error. Although only modest effects were seen with both agents alone at these concentrations, the combination of 5 μ M enzastaurin and 17-AAG (150 nM) significantly potentiated glioma cell cytotoxicity. **P values <0.05 vs both reagents were considered statistically significant. **B.** Logarithmically growing T98G cells were incubated for 24 h with various concentrations of enzastaurin with or without 150 nM 17-AAG. The cells were lysed, and proteins were separated by SDS-PAGE and probed with anti-BAX antibody, which recognizes both the full length (p21) and cleaved (p18) products of BAX or anti-Caspase 3 antibody, which recognizes the cleaved fragment (arrow). Treatment with enzastaurin or 17-AAG alone had little effect on the levels of cleaved BAX and only modest effects on caspase 3. Combined treatment with both agents resulted in a significant increase in the cleaved forms of BAX and caspase 3 in T98G cells. **C.** To determine the effect of enzastaurin on PKC ϵ , pGSK3 β and pAKT, logarithmically growing T98G cells were incubated for 24 h in the presence of various concentrations of enzastaurin with or without 150 nM 17-AAG. Control cells received DMSO. The cells were lysed and equal amounts of proteins were separated by SDS-PAGE and probed with specific antibodies against PKC ϵ , pGSK3 β and pAKT. Blots were subsequently stripped and reprobbed with β -actin. The combination of enzastaurin and 17-AAG significantly decreased the levels of AKT phosphorylation compared to that obtained with each agent individually whereas the total protein levels of AKT and GSK3 β remain unchanged.