Enzastaurin shows preclinical antitumor activity against human transitional cell carcinoma and enhances the activity of gemcitabine

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Abstract
Enzastaurin, an oral serine/threonine kinase inhibitor, suppresses signaling through protein kinase C (PKC)-β and the phosphatidylinositol 3-kinase/AKT pathways. We preclinically evaluated enzastaurin alone and in combination with gemcitabine for transitional cell cancer (TCC). Immunohistochemistry (IHC) was done on 105 human samples from a microarray to show the expression of PKC-β. The preclinical antitumor activity of enzastaurin and gemcitabine as single agents and in combination against aggressive human -lines (-SUP and 5637) and murine subcutaneous xenografts bearing 5637 cells was determined. Western Blot was done on tumor cells in vitro to detect signaling through PKC-β, GSK-3β, and AKT. The effect on cell migration was determined in vitro. Modulation of proliferation (Ki-67), apoptosis (cleaved caspase-3), and angiogenesis (CD31) in vivo was determined by IHC. IHC done on human TCC samples from a microarray showed the expression of PKC-β in 33% of tumors. Enzastaurin induced significant apoptosis and inhibited proliferation in vitro at low micromolar concentrations. The in vitro inhibitory activity of combination enzastaurin and gemcitabine by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay seemed synergistic. Western Blotting revealed down-regulation of Akt, PKC-β, and GSK-3 β phosphorylation. Enzastaurin inhibited migration at an earlier time point independent of antiproliferative activity. Combination therapy had significantly superior antitumor activity in murine xenografts compared with untreated controls, whereas single agents did not. IHC showed reduced Ki-67 and CD31 and increased cleaved caspase-3 with combination therapy compared with controls. Enzastaurin showed preclinical antitumor activity against human TCC and enhanced the activity of gemcitabine. [Mol Cancer Ther 2009;8(7):1772–8]

Introduction
There were ~65,000 new cases of bladder cancer this year in the United States, of whom ~25% will be muscle-invasive or metastatic (1). Front-line chemotherapy for metastatic transitional cell carcinoma (TCC) of the urothelium with either methotrexate, vinblastine, doxorubicin, and cisplatin or gemcitabine and cisplatin yields a median survival of ~15 months (2). Although the response rate with these regimens is 40% to 70%, subsequent progression is universal and salvage chemotherapy is relatively ineffective (3). A significant proportion of patients is ineligible to receive optimal cisplatin-based combination chemotherapy due to renal dysfunction or poor performance status. Neoadjuvant cisplatin-based combination chemotherapy is accepted as standard for locally advanced muscle-invasive bladder cancer, although the improvement in outcomes is modest (4). Therefore, efficacious and tolerable agents need to be discovered for TCC.

Enzastaurin is an orally administered serine/threonine kinase inhibitor that suppresses the protein kinase C (PKC)-β, phosphatidylinositol 3-kinase/AKT, GSK-3 β, and vascular endothelial growth factor signaling pathways (5, 6). Enzastaurin is feasible as a single agent and in combination with cisplatin plus gemcitabine in early phase I clinical trials, and is active in phase II trials of lymphomas (7–10). PKC-β is expressed in TCC and other PKC-β inhibitors have preclinical antitumor activity (11, 12). The phosphatidylinositol 3-kinase/Akt pathway also seems to be a driver of urothelial TCC (13). Additionally, angiogenesis and vascular endothelial growth factor seem to possess key roles in TCC initiation, progression, and invasion (14–18). Preclinical synergism has been observed between chemotherapy and other antiangiogenic agents in TCC (19–21). Enzastaurin also seems to enhance the preclinical activity of chemotherapy in non-small cell lung cancer and thyroid cancer (22, 23). Therefore, a rationale can be made to assess the preclinical antitumor activity of enzastaurin alone and in combination with gemcitabine for human TCC.

Materials and Methods
Human TCC Tissue Microarray Analysis
Formalin-fixed, paraffin-embedded specimens were available from 105 patients with TCC who underwent...
radical cystectomy between 1995 and 2002 at the Baylor College of Medicine affiliated hospitals. Tumor tissue was cored (one tissue cylinder, 2 mm) and a microarray was constructed using the manual tissue arrayer (Beecher Instruments Microarray Technology). Immunohistochemistry (IHC) for the PKC-β protein was done on the microarray to detect expression.

**Cell Culture and Reagents**

The human TCC cell lines 5637 and TCC-SUP were obtained from American Type Culture Collection. 5637 was cultured as a monolayer in RPMI 1640 (Invitrogen) supplemented with 2.5 g/L-glucose, 2 mmol/L l-glutamine, 10 mmol/L sodium pyruvate, 10 mmol/L HEPES, 10% fetal bovine serum, and 1% penicillin-streptomycin. TCC-SUP was cultured as a monolayer in Eagle’s MEM (Invitrogen) with 10% fetal bovine serum, and 1% penicillin-streptomycin. All cells were grown in a humidified incubator with 5% carbon dioxide at 37°C.

**Cell Viability and Activity Assay**

For cell viability and activity assays, 2.5 × 10^3 cells were plated per well in 96-well plates and treated with DMSO 10 μg/mL (vehicle), gemcitabine (Gemzar; Eli Lilly) dissolved in DMSO 10 mg/mL, or enzastaurin (Eli Lilly) dissolved in DMSO 10 mg/mL, or a combination of gemcitabine and enzastaurin. We did not assess the independent activity of the solvent, DMSO, compared with a non-DMSO exposed group of cells. Cell viability and activity was determined using the formula of a rational ellipse

\[
m = 2 \times 0.5236 \times \frac{2m_1}{m_2}
\]

as described and apoptosis was determined by flow cytometry using an Annexin V-FITC apoptosis detection kit (Sigma). The independent activity of DMSO was not assessed. The results were reported as the average percentage of apoptotic cells for each group.

**Cell Apoptosis Assays**

Cells were exposed to gemcitabine and/or enzastaurin in DMSO vehicle (compared with a control group of DMSO treated group) for 48 h in culture media at 37°C/5% CO₂ as described and apoptosis was determined by flow cytometry using an Annexin V-FITC apoptosis detection kit (Sigma). The independent activity of DMSO was not assessed. The results were reported as the average percentage of apoptotic cells for each group.

**Western Blot Analysis**

Changes in cellular expression and phosphorylation of signaling intermediates resulting from exposure to enzastaurin were determined by Western blot. 5637 or TCC-SUP cells were cultured in media alone or in media containing 1 μmol/L enzastaurin for 24 h before harvesting. Membranes were probed with each of the anti–phospho-antibodies, then stripped and reprobed with the antibody for the corresponding protein and β-actin as follows: total Akt1 (clone C73H10; Cell Signaling Technology), phospho-Akt1 (polyclonal to Ser 473; Cell Signaling Technology), total PKC-βII (clone Ab-642; GenWay Biotech), phospho-PKC-βII (polyclonal to Thr 641; Millipore), total GSK-3β (clone 27C10; Cell Signaling Technology), phospho-GSK-3β (Cell Signaling Technology), and β-Actin. Membranes were imaged using ECL Plus Western blotting detection reagent and Hyperfilm (Amersham Biosciences). Densitometry was done using ImageJ software (Rasband, W.S., NIH, Bethesda, MD).

**Migration Assays**

Cell cultures grown in 100-mm dishes to 90% confluence were scraped with a soft plastic cell scraper to create a cell-free zone with a straight wound edge. The edge of the wound was marked on the underside of the plate with a fine gauge hypodermic needle as a migration reference point. Cells were incubated in normal culture medium or normal medium containing 1 μmol/L enzastaurin. The distance of cell migration into the cell-free zone was evaluated by microscopy at 6, 12, and 24 h. An MTT assay was also done to distinguish antimigration activity independent of antiactivity viability at the 24-h time point.

**Murine Tumor Xenografts**

5637 cells were passed at 80% to 90% confluence every 2 to 3 d a total of thrice before use in the tumor experiments to ensure exponential growth. The cells were trypsinized, washed once in sterile PBS, and the cell pellet resuspended in RPMI 1640 at 10⁵ cells/mL. Immediately before injection, cells were mixed (1:1) with Matrigel (BD Biosciences) and 5 × 10⁶ cells were injected s.c. (100 μL/mouse) into one site of the flanks of 6- to 8-wk-old female athymic nude mice (Harlan Sprague-Dawley, Inc.). Five mice were housed per cage in barrier plastic cages and maintained on a 12-h light/dark cycle with free access to food in a pathogen-free animal facility. Mice developing measurable subcutaneous tumors within 2 to 3 wk were randomized to the treatment and control groups. Tumor growth was followed by measuring the size of the tumors 2 to thrice per week. Tumors were measured in two dimensions with calipers and tumor volume was estimated using the formula of a rational ellipse

\[
V = \frac{1}{3} \pi m_1^2 m_2
\]

where \(m_1\) is the length of the short axis and \(m_2\) is the length of the long axis. At the end of the experiment, tumors were resected and prepared for histologic examination.

**Administration of Agents to Mice**

All experiments involving animals were done under approved protocol granted by the Institutional Animal Care and Use Committee of Baylor College of Medicine. In a pilot study, five cohorts of five mice each bearing subcutaneous 5637 tumors, were randomly assigned to groups with similar average tumor sizes. The 5 cohorts were treated as follows: no treatment (control), gemcitabine 20 or 40 mg/kg/wk i.p. dissolved in distilled water, or enzastaurin at 4 http://rsb.info.nih.gov/ij/
100 mg/kg/d (suspended in distilled water) for 5 d a week by oral gavage in a volume of 0.05 mL and 0.1 mL, respectively; all treatments were administered for 4 wk. In the second pilot study, 5 mice per group were given enzastaurin 100 mg/kg thrice a day or were not treated. In the third and confirmatory study, 4 cohorts of mice with 10 to 18 mice per group were given enzastaurin 100 mg/kg thrice a day, gemcitabine 20 mg/kg/wk, or the combination of enzastaurin 100 mg/kg thrice a day plus gemcitabine 20 mg/kg/wk. All treatments were administered for 4 wk.

**IHC of Murine Tumors**

Xenograft tumors removed from mice were fixed in formaldehyde and embedded in paraffin. Expression of Ki-67, cleaved caspase-3, and CD31 in histologic sections were analyzed by IHC (Dako) in five randomly selected murine xenografts from each group. Appropriate positive and negative controls were also stained. Four high power fields (×400) were examined per tumor, to derive an average per tumor per group. Each image was interpreted for immunoreactivity using a 0 to 3+ scoring system for both the intensity of stain and percentage of positive cells by using the Image-pro Plus software, (Media Cybernetics, Inc.). Cells were considered positive for Ki-67 if they displayed 2+ or 3+ expression, whereas cells with any expression of cc-3 and CD31 were considered positive. Comparisons between indices of proliferation and apoptosis were done using Mann-Whitney nonparametric tests.

**Statistical Analysis**

The significance of differences was determined by Student’s *t* test or one way ANOVA measurement. Comparisons of inhibiting tumor growth between treatment groups or the control were done using one-way ANOVA and Tukey-Kramer HSD test (SAS, JMP 5,1.2). *P* values were considered significant by Student’s *t* test, Tukey-Kramer HSD test, and if *P* value was <0.01 by one way ANOVA measurement.

**Results**

**IHC of Human TCC Tissue Microarrays Showed PKC-β Expression**

PKC-β expression (any percentage of cells) by IHC was found in 34 (33%) of 105 tumors with nuclear and cytoplasmic expression. When examined tumor pathologic stage, expression was found in 23 (40%) of 57 cases of Ta (superficial papillary tumors), 9 (34%) of 26 of T1 (tumors invading the lamina propria), and 2 (16%) of 12 cases of T2 (muscle-invasive tumors). When examining by grade, 11 (37%) of 29 cases of grade 1, 15 (42%) of 35 cases of grade 2, and 7 (18%) of 38 cases of grade 3 TCC expressed PKC-β. The number of tumors in each category of stage and grade were small and formal comparisons to obtain any statistically significant differences in expression were not considered reliable due to insufficient power.

**Enzastaurin Inhibits Viability and Activity of TCC Cells In vitro and Is Synergistic With Gemcitabine**

Gemcitabine inhibited cell viability and activity by MTT assay with an estimated IC50 of 0.3 nmol/L in 5637 cells and 0.8 nmol/L in TCC-SUP cells (Fig. 1A). Enzastaurin reduced cell viability with an estimated IC50 concentration of 1 μmol/L in both cell-lines (Fig. 1B). Enzastaurin combined with gemcitabine showed increased inhibition from 46% to 64% in 5637 cells and from 48% to 69% in TCC-SUP cells (Fig. 1C). The combination of enzastaurin and gemcitabine was synergistic.

**Enzastaurin Induces Apoptosis of TCC In vitro and Enhances the Activity of Gemcitabine**

Enzastaurin induced significant apoptosis at 1 μmol/L in both TCC cell-lines (Fig. 2A and B). The combination of enzastaurin 1 μmol/L and gemcitabine at 0.3 nmol/L in
5637 cells and 0.8 nmol/L in TCC-SUP cells was also evaluated. Enzastaurin induced significant apoptosis, and apoptosis induced by enzastaurin plus gemcitabine was higher than apoptosis induced by both single agents ($P < 0.05$ in TCC-SUP cell line).

**Enzastaurin Inhibits Signaling Via GSK-3β, AKT, and PKC-β**

There was a down-regulation of the level of GSK-3β, P-GSK-3β, AKT, P-AKT, PKC-β II, and P-PKC-β II expression by enzastaurin (1 μmol/L) in the 5637 cell-line (Fig. 3), as determined by Western blot. A similar profile was observed in the TCC-SUP cell-line, except for a lack of down-regulation of GSK-3β.

**Enzastaurin Inhibited TCC Cell Migration Independent of Cell Viability**

The distance of cell migration into the cell-free zone was reduced in both cell-lines compared with untreated controls by administration of enzastaurin 1 μmol/L at 6, 12, and 24 hours ($P < 0.05$; Fig. 4). MTT assay at 24 hours did not show inhibitory activity (data not shown).

**Enzastaurin Combined With Gemcitabine Showed Significant In vivo Antitumor Efficacy Against 5637 Human TCC Xenografts**

Both doses of gemcitabine (20 and 40 mg/kg i.p. weekly) exhibited significant antitumor activity compared with untreated controls ($P < 0.05$; data not shown). In the first pilot experiment, mice treated with enzastaurin 100 mg/kg orally once daily displayed a trend for improved antitu-
Enzastaurin, a potent and selective competitive inhibitor of PKC-β2, significantly inhibited cell viability and induced apoptosis of both the evaluated aggressive human TCC cell lines at low micromolar concentrations in vitro. These concentrations are within the range of plasma concentrations achievable in humans (1-4 μmol/L). Additionally, the combination of gemcitabine and enzastaurin was synergistic for inhibiting cell viability and metabolic activity in vitro. Similar synergism has been recently shown preclinically between enzastaurin and pemetrexed against non–small cell lung cancer (26). In vitro migration of both cell-lines was inhibited at an earlier time point by enzastaurin in independent of antiviability activity. Enzastaurin inhibited signaling in all of the critical target proteins including PKC-β2, Akt, and GSK-3β. In murine xenografts of human TCC, enzastaurin showed antitumor activity and the combination of gemcitabine and enzastaurin was statistically significantly more active than untreated controls, whereas monotherapy was not. IHC of a subset of randomly selected xenografts showed significant antiproliferative, proapoptotic, and antiangiogenic activity for the combination of enzastaurin and gemcitabine compared with untreated controls. Combination therapy also induced apoptosis significantly compared with monotherapy. The difference between combination therapy and monotherapy for proliferation and angiogenesis was not statistically significant, and this could be a function of the small number of tumors evaluated (five tumors/group).

Although we have shown significant preclinical antitumor activity for enzastaurin, the inhibition of prime targets, and the presence of the target in a significant proportion of human TCC tumors, we raise several caveats and limitations of our experiments. Optimally, the control mice would be treated with placebo and an individual blinded to the treatment groups would measure tumor sizes. However, the distance of cell migration (in millimeters) was reduced by enzastaurin 1 μmol/L in a time-dependent fashion (in hours) in 5637 cells (data for TCC-SUP cells not shown).

Figure 4. The distance of cell migration (in millimeters) was reduced by enzastaurin 1 μmol/L in a time-dependent fashion (in hours) in 5637 cells (data for TCC-SUP cells not shown).

Figure 5. Combination enzastaurin 100 mg/kg thrice daily plus gemcitabine 20 mg/kg i.p. weekly exhibited significant growth inhibition compared with untreated controls (P < 0.05 by one-way ANOVA and Tukey-Kramer HSD test). Gemcitabine 20 mg/kg i.p. weekly or enzastaurin 100 mg/kg thrice daily showed a trend for better outcomes compared with untreated controls.

Enzastaurin for Transitional Cell Carcinoma

Enzastaurin, a potent and selective competitive inhibitor of PKC-β2, significantly inhibited cell viability and induced apoptosis of both the evaluated aggressive human TCC cell lines at low micromolar concentrations in vitro. These concentrations are within the range of plasma concentrations achievable in humans (1-4 μmol/L). Additionally, the combination of gemcitabine and enzastaurin was synergistic for inhibiting cell viability and metabolic activity in vitro. Similar synergism has been recently shown preclinically between enzastaurin and pemetrexed against non–small cell lung cancer (26). In vitro migration of both cell-lines was inhibited at an earlier time point by enzastaurin in independent of antiviability activity. Enzastaurin inhibited signaling in all of the critical target proteins including PKC-β2, Akt, and GSK-3β. In murine xenografts of human TCC, enzastaurin showed antitumor activity and the combination of gemcitabine and enzastaurin was statistically significantly more active than untreated controls, whereas monotherapy was not. IHC of a subset of randomly selected xenografts showed significant antiproliferative, proapoptotic, and antiangiogenic activity for the combination of enzastaurin and gemcitabine compared with untreated controls. Combination therapy also induced apoptosis significantly compared with monotherapy. The difference between combination therapy and monotherapy for proliferation and angiogenesis was not statistically significant, and this could be a function of the small number of tumors evaluated (five tumors/group).

Although we have shown significant preclinical antitumor activity for enzastaurin, the inhibition of prime targets, and the presence of the target in a significant proportion of human TCC tumors, we raise several caveats and limitations of our experiments. Optimally, the control mice would be treated with placebo and an individual blinded to the treatment groups would measure tumor sizes. However, given the intense thrice a day schedule of enzastaurin, it was considered unethical to administer placebo thrice daily and the resources to have a blinded person measure tumor size were not available. Activity in a larger panel of cell-lines, more animal models, and autochthonous/orthotopic models may better predict for clinical activity in human subjects with TCC. Synergistic antiproliferative and proapoptotic activity was noted preclinically against non–small cell lung cancer cells when chemotherapy (gemcitabine or pemetrexed) was followed by enzastaurin, whereas concomitant treatments or enzastaurin given before chemotherapy, resulted in antagonistic effects (27). However, a determination of the sequence dependence of antitumor activity was beyond the scope of our project. Notably, the combination of concurrent enzastaurin, gemcitabine, and cisplatin was well-tolerated in a phase I clinical trial with no significant alterations in the pharmacokinetic variables of any drug (10).

It is unclear that inhibition of PKC-β and Akt can alter the natural history of human subjects with TCC. It is also unclear that the presence and perturbation of the signaling pathways involving these molecular targets predicts for the activity of enzastaurin. Additionally, the effect of the activation of these signaling pathways on prognosis is unknown. Long-term outcomes of patients on the tumor microarray are lacking at this time to enable this determination and future studies are planned. Despite significant activity in preclinical models, enzastaurin did not show significant activity in human subjects with glioblastoma multiforme or heavily pretreated advanced breast cancer (5, 28). Although the primary end point of a 20% PFS rate was not achieved with enzastaurin as salvage therapy in patients with advanced non–small cell lung cancer, 13% exhibited a progression-free survival of ≥6 months, which might indicate a significant benefit in an undefined subset of patients (29). Given the activity in lymphomas, enzastaurin is being evaluated in a phase III trial as maintenance therapy for the treatment of non–Hodgkin’s lymphoma in the Preventing Relapse in Lymphoma Using Daily Enzastaurin trial (8, 9). Phase II studies are also evaluating enzastaurin alone or in combination with chemotherapy or biological

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agents for breast, colon, lung, ovarian, renal, and prostate cancers. The preclinical combination of enzastaurin with sorafenib or sunitinib seems encouraging for renal cell carcinoma (30). However, the activity of enzastaurin and the relevance of its molecular targets need further validation. Notably, the dose of enzastaurin chosen for further development (525 mg orally once daily) is based on optimal biological exposure and is not the clinical maximum tolerated dose. Therefore, further study of the optimal dose and predictive factors for response may be warranted.

Despite all of these caveats, further thoughtful development of enzastaurin for TCC may be warranted based on the significant preclinical activity shown with exceptionally low concentrations in our study. A concurrent focus on elucidating the role of PKC-β and Akt signaling in driving the growth of TCC and affecting clinical outcomes as well as sensitivity to enzastaurin is necessary. Given the unmet need in the salvage and cisplatin-ineligible setting of advanced TCC, and the need for a tolerable and convenient option, a rationale can be made to study enzastaurin alone and in combination with chemotherapy in patients with advanced TCC.

Disclosure of Potential Conflicts of Interest

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