Antitumor Activity of Enzastaurin as Radiation Sensitizer in Head and Neck Squamous Cell Carcinoma

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Abstract: Background. The antitumor effect of enzastaurin, a specific protein kinase C beta (PKC-β) inhibitor, was tested on head and neck squamous cell carcinoma (HNSCC) cell lines, as well as HNSCC xenograft model.

Methods. HNSCC cell lines and xenograft were treated with enzastaurin alone or in combination with radiation to evaluate its antitumor effect. Annexin V-FITC staining and Western blot were done to analyze enzastaurin-induced changes in apoptosis and the PIK3/AKT Akt signal transduction pathway.

Results. Enzastaurin showed strong antitumor activity either alone or in combination with radiation in HNSCC cell lines and in xenograft, with a corresponding reduction in the expression of key radioresponsive proteins.

Conclusion. Enzastaurin can inhibit tumor growth and improve the efficacy of radiation for HNSCC both in vitro and in vivo. This suggests that enzastaurin may represent a better strategy for the treatment of HNSCC patients, either as a monotherapy or as a radiosensitizer.

Keywords: PKC-β inhibitor; head and neck squamous cell carcinoma; chemoradiation; radiosensitizer; xenograft

Head and neck squamous cell carcinoma (HNSCC) is a devastating disease, leading to significant morbidity and mortality rates. Concurrent chemoradiation is frequently used as primary treatment for patients with advanced-stage disease, but only a portion of patients have durable responses to cisplatin-based chemoradiation. Concurrent chemotherapy is frequently toxic, with significant short- and long-term side effects.1,2 More effective agents are needed for patients with HNSCC.

Protein kinase C (PKC) is a family of serine/threonine kinases known to play critical roles in cell proliferation, differentiation, and apoptosis.3 Increased level of PKC and/or increased activity have been observed in breast cancer, gastric carcinoma, malignant gliomas, renal cell carcinoma, non-small cell lung carcinoma, prostate cancer, some head and neck cancers, and during early colon carcinogenesis. They are often linked to disease progression.4-6 In addition, tumor-induced angiogenesis requires activation of PKC, particularly PKC-β.7 PKC activation can trigger signaling through the RAS/extracellular...
signal-regulated kinase (ERK) pathway. PKC activity is also linked to the PI3/AKT pathway, the control of cell growth, the apoptotic response, and the modulation of response to radiotherapy. These functions of PKC identify them as potential targets for anticancer therapies.

Enzastaurin is a specific protein kinase C beta (PKC-β) inhibitor that has been demonstrated to be effective for inhibiting tumor cell growth in many other tumor types. Preclinical study of combination enzastaurin with radiation or other antitumor drugs found that enzastaurin enhances radiation-induced tumor cell death, inhibits xenograft tumor growth, and sensitizes tumor cells to common genotoxic drugs. Here, we report the potent antitumor effect of enzastaurin in a variety of HNSCC cell lines, as well as in a murine model either as a single agent or in combination with radiation and demonstrate that enzastaurin has strong antitumor activity in head and neck cancer.

**MATERIAL AND METHODS**

**Reagents.** Enzastaurin was kindly provided by Eli Lilly and Company (Indianapolis, IN). The compounds were stored as powder and stored at room temperature; for use, it is first dissolved in dimethyl sulfoxide (DMSO) then diluted in the appropriate cell culture medium, such that the final DMSO concentration did not exceed 0.05%.

**Cell Culture and Cell Growth Inhibition.** Seven HNSCC cell lines with different sensitivity to radiation and cisplatin, the most commonly used chemotherapy agent for head and neck cancer, were used for this study. UM1, UM11B, UM23, UM38 (kindly provided by Dr. Gregory Wolf, University of Michigan, Ann Arbor, MI) and Cal27 (purchased from American Type Culture Collection [ATCC]) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G and streptomycin, and 1% nonessential amino acids. JHU12 and JHU22 (kindly provided by Dr. David Sidransky, Johns Hopkins University, Baltimore, MD) were grown in RPMI-1640 with 10% fetal bovine serum and 100 U/mL penicillin G and streptomycin. All cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

Logarithmically growing cells were counted and plated at 1000 or 1500 (for slower growing cell lines) cells per well (to have 10% to 15% confluent by the next day), in triplicate, in 96-well plates, and incubated overnight. The next morning, enzastaurin was added to the cell culture medium at increasing concentrations (0, 1.25 μM, 2.5 μM, 5 μM, 7.5 μM, 10 μM, 15 μM, and 20 μM). Cell growth inhibition was examined by MTT assay (Roche Diagnostics, Indianapolis, IN). Briefly, on the fifth day of the culture, 10 μL of MTT labeling reagent was added to each well. After 4 hours of incubation, 100 μL of solubilization buffer was added, and the plates were incubated at 37°C overnight. The absorbance of each well was measured at 595 nm with a Vmax kinetics microplate reader. The concentration of enzastaurin yielding 50% growth inhibition (IC₅₀) were compared with controls for each cell line and expressed as mean values of 4 independent experiments.

Clonogenic assays were used to evaluate the effect of enzastaurin in combination with radiation. Briefly, cells were trypsinized to generate a single cell suspension, and a specific number of cells (to get approximately 100 clones after 12 to 14 days) were seeded into 60-mm plates. After allowing cells to attach, enzastaurin was added at about the IC₂₅ of each cell line (JHU12: 3 μM; UM11B: 4 μM). For enzastaurin in combination with radiation, 16 hours after adding enzastaurin, cells were irradiated with a single dose of 2 Gy, 5 Gy, and 7 Gy from a Cesium137 irradiator. Four hours after radiation, all plates were aspirated and fresh media were added. Twelve days after seeding, colonies were stained with crystal violet, and the number of colonies containing at least 50 cells was counted. The colony survival fraction was calculated for each treatment and data were presented as a log plot. The results shown were mean values of 3 independent experiments with triplicate setting in each experiment. The t tests were used to test the significance of the difference between different treatments.

**Flow Cytometry Analysis of Cell Cycle and Apoptosis.** Cell cycle distribution was measured before and after HNSCC cells were exposed to either 10 μM enzastaurin or 5 Gy radiation for 24 hours. For the combination condition, cells were treated with 10 μM enzastaurin for 16 hours, and then treated with a single fraction of 5 Gy of radiation. Cells were collected 24 hours after exposure to radiation; cells were fixed with 70% ethanol, incubated with propidium iodide (20 μg/mL) and ribonuclease
(200 μg/mL) for 30 minutes at 37°C, and analyzed by flow cytometry (FACS; Becton Dickinson, Franklin Lakes, NJ). A ModFit II software program was used for cell cycle distribution analysis. The experiment was repeated once for consistency.

To test whether cells were undergoing apoptosis, cells were stained with annexin V with Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA). Briefly, cells were plated, treated as above, and collected, 1 × 10⁵ cells were incubated with 5 μg/mL FITC-conjugated annexin V in the presence of 5 μg/mL of PI and then screened by flow cytometry. Annexin V-positive PI-negative cells scored as early apoptotic. Annexin V–positive PI-positive cells corresponded to late apoptotic cells.

**Protein Extraction and Western Blotting.** The drug treatment protocol used was the same as for the cell cycle analysis. The cells were harvested 24 hours after radiation treatment and resuspended in NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 0.5% NP-40) containing 1 tablet of protease inhibitors cocktail (Boehringer Mannheim, Germany) per 10 mL. For in vivo tumor xenograft samples, tumor tissue was minced and homogenized in lysis buffer and spun in a centrifuge to remove debris. The protein extracts were quantitated with a Bio-Rad Bradford protein assay (Bradford Reagent; BioRad, Hercules, CA).

Protein 40 μg was electrophoresed through 12% sodium dodecyl sulfate (SDS) polyacrylamide gels under denaturing conditions and transferred to nitrocellulose membranes. The membranes were blocked in TBS, 0.1% Tween 20, 5% nonfat dry milk, and incubated with the following appropriate primary antibodies at 1:1000 dilution overnight at 4°C: GSK 3β, phosphor-GSK3βser9, AKT, phosphor-AKTser473; all were purchased from Cell Signaling Technologies (Cell Signaling, Beverly, MA). The blots were washed with TBS-T (TBS with 0.1% Tween 20) next day and incubated for 1 hour at room temperature with 1:1000 of HRP-conjugated anti-rabbit IgG antibody (Promaga, Madison, WI). After washing with 1x PBS-T buffer 3 times, the specific antigen-antibody interaction was detected with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and visualized with X-ray film. The blots were then stripped with Western blot strip buffer (62.5 mM Tris-HCL PH 6.8, 2% SDS, 100 mM 2-Mercaptoethanol), re-probed with anti-Tubulin monoclonal antibody (Roche Molecular Biochemicals, Indianapolis, IN) and then HRP-conjugated anti-mouse second antibody (Santa Cruz, CA). This will be used as the internal control for signal expression.

**In Vivo Mouse Tumor Model Studies.** Experiments were conducted in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the rules and regulations of the University of North Carolina Institutional Animal Care and Use Committee (protocol 07-191.0). Female nude mice 7 to 8 weeks old were obtained from Charles River Laboratory (Wilmington, MA) and maintained in ventilated caging.

JHU12 cells were harvested, washed with 1x PBS, suspended in PBS at 3 × 10⁶ cells/100 μL, and then inoculated subcutaneously into the flanks of mice. JHU12 cells were used because it induces large tumor in nude mice within 3 to 4 weeks. After the tumor size reached 2 to 3 mm in diameter (about 6 to 8 days), enzastaurin solution or vehicle was given orally with a mouse feeding tube twice/day, 5 days/week (Monday–Friday) for 4 to 5 weeks.

For the radiation protocol, mice were first anesthetized with 125 mg/kg Avertin by intraperitoneal injection, placed into a cone-shaped lead shield with a hole on the side to expose only the tumor, and then put into the irradiator. A radiation dose of 1.5 Gy was given per fraction and the tumors were radiated 4 times a week (Tuesday, Wednesday, Thursday, and Friday), 6 Gy/w for 4 to 5 weeks. For the combination group, radiation was given 3 to 4 hours after the first dose of enzastaurin. Tumor size and mouse weight were measured weekly (Tuesday and Friday). The experiment was ended when the control mice tumor reached the maximum allowed size (2.0 cm for the longest diameter) or developed ulceration. Enzastaurin was first dissolved in 100 μl DMSO, then 5% dextrose, so the final concentration of DMSO was less than 0.1%.

The following groups were tested: vehicle control, 100 μL 5% dextrose; enzastaurin 25 mg/kg; enzastaurin 50 mg/kg; enzastaurin 75 mg/kg; 1.5 fractioned radiation only; 25 mg/kg enzastaurin plus radiation; 50 mg/kg enzastaurin plus radiation; and 75 mg/kg enzastaurin plus radiation. The 50 mg/kg enzastaurin with and without 1.5 Gy fractioned radiation group was chosen to repeat the experiment.
One mouse in each group was euthanatized with CO₂, and the tumor was excised from the mice about 4 hours after the last treatment at the end of the fourth week of treatment. A portion of the tumor was paraffin embedded and sectioned for hematoxylin and eosin staining and immunohistochemistry study, and a portion was homogenized and protein extracted for immunoblot analysis. The antibodies used and Western blot methods are the same as for in vitro study.

**Immunohistochemical Analysis.** The effect of enzastaurin on tumor angiogenesis is analyzed by immunohistochemistry with anti-CD31 for micro-vessel density with blood vessel staining kit from Millipore. Briefly, the 5 μM paraffin tissue section were deparaffinized/rehydrated, stained and color developed with DAB following the protocol supplied within the kit. After staining, the sections were viewed under a microscope. Blood vessels were quantified by selecting four 400× fields from each corner of the tumor section and counting the number of blood vessels per field. Pictures were taken from representative areas of each group.

**RESULTS**

**Enzastaurin Inhibits HNSCC Cell Growth.** Seven human HNSCC cell lines were chosen for this study because they display differential responses to cisplatin or radiation. Cal27 is sensitive to both cisplatin and radiation, whereas UM11B is relatively resistant to both therapies. In contrast, JHU12 and JHU22 are resistant to cisplatin while relatively sensitive to radiation. These cell lines were initially treated with increasing concentrations of enzastaurin for 4 days, and the effects on cell viability were evaluated by MTT assay. Unlike cisplatin, for which the HNSCC cell lines have a broad range of sensitivities, the sensitivity of HNSCC cell lines to enzastaurin fall within a more restricted range (Figure 1A), with IC₅₀ between 3 and 10 μM. The cells that are sensitive to cisplatin are also relatively sensitive to enzastaurin. This suggests that enzastaurin may be a more broadly active antitumor agent than cisplatin, to which some tumors have proven resistance. Enzastaurin induced growth inhibition in a dose-dependent manner, with a similar biphasic curve for each cell line (Figure 1B).

**Enzastaurin Enhances the Antitumor Growth Effect of Radiation In Vitro.** To determine whether enzastaurin enhances radiation-induced cell death in HNSCC cells, the relative radiation–sensitive cell line JHU12 and relative radiation–insensitive cell line UM11B were exposed to enzastaurin for 16 hours followed by a single dose of radiation (2 Gy, 5 Gy, or 7 Gy). The impact of the single and combination treatments on cell proliferation was then measured by clonogenic assay. As expected, UM11B cells (Figure 2B) were relatively resistant to radiation compared with JHU12 (Figure 2A), a 50% killing dose is about 4 Gy verses about 3 Gy.
for JHU12 cells. When cells were pretreated with enzastaurin before radiation, a strong growth inhibition was observed in both JHU12 and UM11B cells at every dose level tested ($p < .01$ for all radiation doses). This observation suggests that the inhibition of enzastaurin can overcome the radioresistance of the cell lines and enhance the antitumor effect of radiation. Pretreatment of cells with cisplatin before radiation, resulted in an additive effect in both the relative sensitive and resistant lines when analyzed with MTT assay (data not shown).

**Enzastaurin Alone Induces G1 Arrest and Increases Sub-G0 Population When Combined with Radiation.** To determine the effect of enzastaurin on cell cycle progression in HNSCC cells, either alone or in combination with radiation, HNSCC cell lines with either wild-type (JHU12) or mutant p53 (UM11B) were treated with 10 $\mu$M enzastaurin and analyzed by FACS 24 hours after treatment. The G1/G0 population increased by 30% and 20%, respectively, and that of the S+G2 phase decreased accordingly in both cell lines. Treatment with 5 Gy radiation caused increased G2/M accumulation and decreased S population in both cells. When cells were treated with enzastaurin followed by radiation, a significant increase of sub-G0 population, a moderate decrease in S phase population, and a minor increase in the G2/M population was observed (Table 1).

**Enzastaurin Induces Cell Death via Apoptosis.** To confirm that enzastaurin inhibit cell growth and enhance radiation effect is by inducing apoptosis, annexin V stain followed by flow cytometry analysis was performed for JHU12 and UM11B cells treated with enzastaurin or radiation alone or the combination. As shown in Figure 3, the combination of radiation and enzastaurin enhanced apoptosis in both cell lines than did either agent alone.

**Enzastaurin Inhibits Phosphorylation of GSK3β and AKT.** Phosphorylation of GSK3β has been linked to PKC β activity and phosphorylation of GSK3β has also been linked to AKT activity. Therefore we examined the phosphorylation level of GSK3β and AKT after the HNSCC cells were treated with either enzastaurin alone, radiation alone or enzastaurin in combination with radiation. Although enzastaurin has no effect on total level of GSK3β and AKT, there is a clear reduction of phosphorylation level of GSK3β and AKT, and the reduction is more profound when the cells were treated with enzastaurin followed by radiation (Figure 4). These results are consistent with the results that

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**Table 1. Effect of enzastaurin on cell cycle profile of HNSCC cell lines.**

<table>
<thead>
<tr>
<th>Cell lines and treatment</th>
<th>Sub-G0</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>UM11B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>1.2%</td>
<td>41.5%</td>
<td>41.6%</td>
<td>16.6%</td>
</tr>
<tr>
<td>Enzastaurin</td>
<td>3.5%</td>
<td>56.7%</td>
<td>24.3%</td>
<td>15.0%</td>
</tr>
<tr>
<td>Radiation</td>
<td>2.3%</td>
<td>41.3%</td>
<td>32.5%</td>
<td>23.9%</td>
</tr>
<tr>
<td>Enza + XRT</td>
<td>11.3%</td>
<td>37.4%</td>
<td>26.0%</td>
<td>25.3%</td>
</tr>
<tr>
<td>JHU12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>1.0%</td>
<td>40.8%</td>
<td>44.3%</td>
<td>14.7%</td>
</tr>
<tr>
<td>Enzastaurin</td>
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<td>50.2%</td>
<td>36.3%</td>
<td>7.9%</td>
</tr>
<tr>
<td>Radiation</td>
<td>3.4%</td>
<td>41.6%</td>
<td>30.8%</td>
<td>24.4%</td>
</tr>
<tr>
<td>Enza + XRT</td>
<td>13.5%</td>
<td>37.9%</td>
<td>22.3%</td>
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</tr>
</tbody>
</table>
Enzastaurin inhibits HNSCC cell growth. Neither enzastaurin nor radiation alone has an effect on c-raf level, but the combination significantly inhibited the c-Raf expression (Figure 4).

Enzastaurin Enhances Antitumor Growth Effect of Radiation in HNSCC Xenografts. The radiosensitizing effect of enzastaurin was tested in an in vivo HNSCC xenograft model. In this study, we used JHU12 tumor–bearing mice, because UM11B grow slowly in vivo. Radiotherapy regimens currently in clinical practice for the treatment of HNSCC involve fractionated daily radiation often in combination with concurrent chemotherapy, because the biologic basis of chemosensitization may vary with single-dose radiation. Therefore, in our xenograft studies, we applied the same approach and treated mice with fractionated radiation 4 times a week (Tuesday through Friday) for 4 to 5 weeks. Enzastaurin was given twice daily and 5 days/w (Monday–Friday) for a total of 4 to 5 weeks (24–30 Gy total). As shown in Figure 5A, enzastaurin alone exhibited dose-dependent inhibition of HNSCC xenograft growth. A minimum of 50 mg/kg was required to inhibit tumor growth effectively. After more than 3 weeks of treatment, tumors in mice treated with 75 mg/kg enzastaurin stabilized in size. Radiation alone 1.5 Gy only moderately inhibits tumor growth (Figure 5B).

When combined with 50 mg/kg enzastaurin (we chose the dose with moderate effect of each therapy in the combination study to maximize the potential for observing a synergistic effect), the inhibition effect is significantly improved. Tumor size in the enzastaurin only group is about 63% of control group and 49% of the control in the 1.5 Gy radiation only group, whereas, in the combination

FIGURE 3. Enzastaurin induce cells to undergo apoptosis. JHU12 (A) and UM11B (B) cells were treated with 10 μM enzastaurin, or 5 Gy of radiation for 24 hours or with enzastaurin 16 hours before radiation. Cells were harvested 24 hours after radiation. Annexin V was used to stain for apoptotic cells. Annexin V-positive PI-negative cells scored as early apoptotic. Annexin V-positive PI positive cells corresponded to late apoptotic cells. Annexin V staining shows that enzastaurin inhibits tumor cell growth by inducing apoptosis. The percentage of apoptotic cells increased significantly when cells were treated with both enzastaurin and radiation (2.34- to 3.01-fold increase), this also confirms that the combination of enzastaurin with radiation enhanced the radiation effect on HNSCC cells. Data shown were the average of 2 experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
group, the tumor size is only 10.4% of the control after 4 weeks of treatment. We observed the radiation only (2 animals) and enzastaurin only (1 animal) treated mice along with those mice treated with enzastaurin plus radiation for 4 more weeks without any further treatment. The tumors did not regrow, tumor size basically did not change, and the mice were killed at the end of the 4 weeks of observation. This confirms the inhibiting effect of enzastaurin on tumor growth.

Weight loss in the single modality groups was less than 5% relative to controls and 8% to 10% in the combination treatment groups, suggesting that enzastaurin was well tolerated (data not shown). Five weeks after tumor cell inoculation, most tumors in the control group reached the size of 15 mm in diameter, whereas some tumors in single treatment groups also grew to more than 10 mm and developed ulceration, requiring that those mice be killed. In contrast, tumors in the combination treatment were much smaller, and some tumors began to regress. These data demonstrate that enzastaurin, an orally available PKC β inhibitor, has strong antitumor activity in a HNSCC tumor model and sensitizes the tumors to radiation according to afractionated treatment that mimics current clinical regimens.

Enzastaurin Inhibits the PI3K/AKT Pathway and Angiogenesis in HNSCC Xenograft. To determine the effect of enzastaurin on PI3K/AKT signal pathway in vivo, tumors were harvested 28 days after beginning treatment. Western blotting is used to analyze enzastaurin-induced cell signal pathway related protein level changes. Changes in PI3K/AKT expression and phosphorylation were similar to HNSCC cell lines in culture with the following exception. Although enzastaurin inhibits the phosphorylation of GSK 3β and AKT, it has no effect on total GSK3β and AKT in HNSCC cell lines in monolayer culture; in HNSCC xenografts, enzastaurin in combination with radiation inhibits the phosphorylation of GSK 3β and AKT. Although not statistically significant, the combination therapy suggests some inhibition of total GSK3β and AKT level (Figure 6). These results may explain why the inhibition effect of enzastaurin in combination with radiation is better in vivo than in vitro and confirms that enzastaurin’s antitumor effect mechanism is through inhibiting the PKCβ and PI3K/AKT pathways.

The effect of enzastaurin on angiogenesis in tumors was determined. On gross examination, enzastaurin plus radiation–treated tumors had more necrosis than tumors in other groups, although the tumor size was smaller, and this
was verified on microscopic examination (left side of Figure 7, section d). CD31 immunohistochemistry section revealed decreased blood vessel density in enzastaurin-treated and the combination-treated tumor, and the combination treatment group was most effective at inhibiting blood vessel formation. Figure 7A, shows the mean number with standard error of blood vessel in each group ($p < .05$ vs control in enzastaurin only treatment; $p < .01$ vs control in combination treatment group). Figure 7B, shows a representative field of each group.

**DISCUSSION**

The goal of radiotherapy is to kill tumor cells efficiently with as little damage to normal tissue as possible. Unfortunately, radiotherapy alone often fails to eliminate tumors in advanced head and neck cancer, leading to locoregional recurrence and the development of distant metastasis. Concurrent radiation and chemotherapy with cisplatin-based regimens has improved tumor control significantly. However, a subset of patients does not respond well to chemoradiotherapy, and cisplatin-related toxicity is of major concern.

PKC has become a therapeutic target for treating several malignancies. The PKC-β specific inhibitor enzastaurin is a novel bisindolylmaleimide that abrogates signaling cascades in which PKC-β is involved by competing at the enzyme’s ATP-binding site. Enzastaurin has been tested in clinical trials on patients with diffuse large B-cell lymphoma and advanced lung cancer. Here we investigated the antiproliferation activity of enzastaurin against HNSCC alone or in combination with radiation in vitro and in vivo HNSCC xenograft.

Enzastaurin alone can inhibit tumor cell growth effectively in all cell lines tested, with the IC50 falling into a much more narrow range than cisplatin. When combined with radiation, enzastaurin exhibited strong antiproliferative effects.
and synergistically enhanced the antitumor growth effect of radiation both in HNSCC cell lines and in vivo HNSCC xenograft. Annexin V stain followed by flow cytometry analysis confirmed that the antiproliferation effect of enzastaurin is by inducing cells to undergo apoptosis. By studying non-small cell lung cancer, colon, and thyroid cancer cell lines and freshly explanted tumor tissue with human tumor cloning assay (HTCA), Hanauiske et al17 confirmed that enzastaurin has direct antitumor activity not only in established tumor cell lines from different tissue, but also against freshly explanted human tumors. Jasinski18 found that the pro-apoptosis effect of enzastaurin in combination with radiation is through activation of caspase-3 and caspase-9. They also confirmed that enzastaurin can reverse radiation induced activation of PKC α, β II, and ε—all anti-apoptotic PKC isoforms.

GSK 3β is a pharmacodynamic marker for enzastaurin, has been linked to PKC-β directly and also is a downstream target of the AKT pathway. Our results showed that although enzastaurin has no obvious effect on total GSK3 β and AKT in vitro, it inhibited the phosphorylation of GSK 3β and AKT compared with untreated control. Radiation only had very limited effect on the expression of pGSK 3β and pAKT, but when combined with enzastaurin, the expression of pGSK 3β and pAKT was decreased dramatically. In HNSCC xenograft, enzastaurin seems not only to inhibit the phosphorylation of GSK 3β and AKT, it may also have some inhibition effect on total GSK3 β and AKT level when enzastaurin is combined with radiation, although the effect is not significant. This may explain why the in vivo antitumor effect of enzastaurin in combination with radiation is better than in vitro cell lines. Like pAKT, C-raf is also important for tumor cell survival and radioresistance in HNSCC. Our results also show that c-raf expression is reduced in cells treated with enzastaurin and radiation. These indicate that the radiosensitizing effect of enzastaurin on HNSCC cells may be carried out through inhibition of the expression of radioresistance-associated oncoproteins, thereby sensitizing the cells to radiation and resulting in cell death. Consistent with our observation, Spalding et al19 found that enzastaurin in combination with 2 Gy of radiation can significantly inhibit both GSK3β and PKC β phosphorylation very shortly after adding enzastaurin in pancreatic cancer cell lines BxPC3 and Panc1 and xenograft.

A fractionated regimen was used in our in vivo efficacy studies, which showed that enzastaurin at minimal required doses because monotherapy (50 mg/kg) can effectively inhibit tumor growth when combined with fractionated radiation. The therapeutic effect of the combined treatment was much more effective compared with either treatment alone and was more pronounced than in vitro experiment. After 4 weeks of combined treatment, some tumors start to regress, and the tumor did not resume growth 4 weeks after stopping treatment. Spalding’s work with pancreatic cancer xenograft also shows that enzastaurin (100 mg/kg twice daily) with 2 Gy fractioned radiation for 10 days can effectively inhibit tumor growth and increase mouse survival.

Enzastaurin was developed as a specific PKC β inhibitor and was initially evaluated in preclinical tumor models for its antiangiogenic activity. It has been found that enzastaurin can dramatically suppress the growth of new vasculature in the rat corneal micropocket20 and results in decreased microvessel in the Panc1 pancreatic cell line.10 To investigate the antivascular effects of enzastaurin, we stained mice tumor tissue for CD31 to determine its effect on blood vessel density of each treatment group. We found that enzastaurin alone and enzastaurin in combination with radiation both resulted in decreased blood vessel density compared with no-treatment control, and the combination treatment was most effective at inhibiting blood vessel formation and also induced tumor tissue necrosis; whereas radiation alone was not obviously different from control except for more fibrosis.

In summary, our studies have shown that enzastaurin, a specific PKC β inhibitor demonstrated strong antitumor effects in HNSCC cell lines and xenograft models. Enzastaurin has direct antitumor cell growth and antivascular effects, and furthermore, enzastaurin synergizes with radiation, significantly enhancing the efficacy of the latter. All these properties make enzastaurin a promising antitumor agent for the treatment of patients with HNSCC, by promoting the therapeutic activity of radiation.

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