Preclinical Development

Efficacy of the Multi-Kinase Inhibitor Enzastaurin Is Dependent on Cellular Signaling Context

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Abstract

The number of targeted small molecules being developed in oncology is increasing rapidly. Many of these are designed to inhibit multiple kinases, and thus the mechanisms of responsiveness and predictive biomarkers can be difficult to discern. In fact, with few exceptions, multi-kinase inhibitors are developed with limited mechanism-based patient selection. Enzastaurin is a multi-kinase inhibitor being studied in several malignancies that we hypothesized would be active in squamous cell carcinoma of the head and neck, because it inhibits classic and novel protein kinase C isoforms. Indeed, enzastaurin reduced the growth of SQ-20B and CAL27 tumor xenografts, decreased proliferation in these cell lines, inhibited putative target phosphorylation, and induced cell cycle arrest. Gene expression arrays confirmed that expression of cell cycle genes, including cyclins D and E, were significantly altered by exposure to enzastaurin. However, testing a panel of squamous cell carcinoma of the head and neck cell lines revealed variable sensitivity to enzastaurin, which correlated significantly with baseline cyclin D1 protein expression. Moreover, sensitivity and resistance could be reversed, respectively, by expression or depletion of cyclin D1. Furthermore, analysis of sensitive and resistant cell lines revealed distinct differences in cyclin D1 regulation. Enzastaurin modulated cyclin D1 synthesis through an Akt-regulated pathway in the former, whereas high-level CCND1 gene amplification was present in the latter. These results underscore the critical relevance of cellular signaling context in developing cancer therapies in general and suggest that enzastaurin in particular would be most effective in tumors where baseline cyclin D1 expression is low to moderate and physiologically regulated. Mol Cancer Ther; 9(10); 2814–24. ©2010 AACR.

Introduction

Molecularly targeted therapy has changed the landscape of oncology drug development and, in some cancers, has made a tremendous effect on survival (1). However, a continuing challenge, especially with agents that target several kinases simultaneously, is gaining an appreciation of the mechanisms underlying sensitivity, which, in turn, should lead to improved patient selection for specific drugs.

Our previous work in squamous cell carcinoma of the head and neck (SCCHN) cell lines has shown that specific protein kinase C (PKC) isoforms are critical mediators of SCCHN growth and valid therapeutic targets (2–4), but until recently, there has been a paucity of agents that effectively target PKC. Enzastaurin is an acyclic bisindolylmaleimide that displays potent selective inhibition of classic and novel PKC isoforms and other AGC kinases, as well as inducing apoptosis, suppressing proliferation, and inhibiting tumor xenograft growth in other tumor models (5–10).

Therefore, we undertook studies exploring the activity of enzastaurin in SCCHN in vitro and in vivo and discovered variability in responsiveness to the agent, which was inversely associated with baseline cyclin D1 expression. In addition, enzastaurin suppressed cyclin D1 synthesis, at least partially, by inhibiting Akt but only in sensitive cells. These findings underscore the critical relevance and complex nature of deciphering mechanisms of sensitivity and resistance in cancer models during drug development. Furthermore, our results suggest that tumor cyclin D1 expression should be explored as a predictive biomarker of enzastaurin efficacy in clinical trials.

Materials and Methods

Cell lines and reagents

CAL27 and HEK293T cells were purchased from the American Tissue Culture Collection. SQ-20B, SCC25, SCC35, and SCC61 cells were provided by Dr. Ralph
Weichselbaum (University of Chicago, Chicago, IL). HN5 cells were provided by the Ludwig Institute for Cancer Research (London, United Kingdom). HN31 and MSK921 cells were provided by Dr. David Raben (University of Colorado Cancer Center, Denver, CO). OSCC3 cells were provided by Dr. Mark Lingen (University of Chicago, Chicago, IL). The general characteristics of all cell lines used were monitored by checking cell morphology microscopically, measuring the doubling time of each cell line, and confirming no microorganism contamination. No authentication was done by the authors.

Phospho-Akt (Ser473), phospho-p70S6 kinase (p70S6K; Thr389), phospho-glycogen synthase kinase 3β (GSK3β; Ser9), phospho-myristoylated alanine-rich PKC substrate (MARCKS; Ser152/156), Akt1, Akt2, and Akt3 antibodies were purchased from Cell Signaling Technology, Inc. Antibodies against α-tubulin (sc-8035) and cyclin D2 were purchased from Santa Cruz Biotechnology. Cyclin D1 (NB 600-584) was purchased from Novus Biologicals, Inc. IRDye 800–conjugated, affinity-purified anti-mouse IgM μ-chain–specific antibody was purchased from Rockland Immunochemicals for Research. Other IRDye secondary antibodies were purchased from Li-Cor Biosciences. Rapamycin and LY290024 were purchased from Santa Cruz Biotechnology. Cyclin G2 antibodies against MARCKS; Ser152/156), Akt1, Akt2, and Akt3 antibodies were purchased from Cell Signaling Technology, Inc. Enzastaurin was provided by Eli Lilly and Company.

**Cyclin D1 expression vector, pCDH-CMV-D1-MCS-EF1-puro, and shRNA-D1 lentiviral vectors**

Full-length human cyclin D1 cDNA clone in pOTB7 vector was purchased from Open Biosystems. The vector was digested with EcoRI and BglII, and the cyclin D1 fragment was purified and inserted in between EcoRI and BamHI in pCDH-CMV-D1-MCS-pEF1-puro, a lentiviral vector. A set of pLKO.1 lentiviral vectors containing shRNA targeting D1 was purchased from Open Biosystems. The RHS3979-9604728 clone was used for the D1 knockdown experiments. A nontarget shRNA control vector for shRNA D1 vector was purchased from Sigma. Lentivirus production and viral infection were done as previously described (4).

**Small interfering RNA transfection**

Small interfering RNA (siRNA) duplexes targeting Akt1 (ID no. s649) and Akt2 (ID no. s1215) were purchased from Ambion. Negative Control #1 siRNA was used as control. siRNAs (30 μmol/L per 100 μL of transfection solution) were electroporated using Lonza’s Nucleofector and the Cell Line Nucleofector Kit V. Three million cells were transfected with the respective siRNA, and 24 hours after transfection, cells were replated into 60-mm dishes, serum-starved for 2 days, and then treated with serum for the indicated times.

**Expression of constitutively active Akt1 in SCCHN cell lines**

 Constitutively active Akt1, 1036-pcDNA3-MyrHA-Akt1, was purchased from Addgene, Inc. Bacterial host containing pcDNA3-MyrHA-Akt1 was amplified and purified, and cells were transfected with 5 μg of plasmid DNA or an equal amount of control empty vector with Lonza’s Nucleofector system.

**Cell cycle analysis**

Propidium iodide (PI) staining was done after the cells were harvested and fixed in 70% ethanol. Fixed cells were washed in PBS and centrifuged at 2,200 rpm for 10 minutes. Cells were resuspended in 4.5 mL of PBS, and 0.5 mL of RNase A (1 mg/mL) was added to the suspension. After 30 minutes of incubation at 37°C, cells were washed with PBS. Cells were counted and resuspended in PI (10 mg/100 mL in PBS) for 30 minutes on ice. The final cell concentration was approximately 1 million cells/mL. PI-stained cells were analyzed for DNA content with a Becton Dickinson FACScan flow cytometer. Cell sorting results were analyzed with FlowJo software using a cell cycle platform and Watson’s Pragmatic Model to calculate the distribution of cells in G0, G1, and G2 phases.

**BrdUrd proliferation assay**

BrdUrd proliferation assay kit (EMD Biosciences) was used for all experiments according to the manufacturer's instructions with specific modifications as previously described (3, 4).

**Cell viability assay**

Cells were seeded into 96-well plates at 1,000 to 2,000 per well. Cells were treated or not treated with enzastaurin (2.5 μmol/L) for 24 to 48 hours. CellTiter-Blue reagent (Promega) was added to the medium, and the reaction stopped after 1 to 5 hours of incubation. Fluorescence (530 Ex/590 Em) was measured and recorded using a BioTek plate reader.

**Protein immunoblot analysis**

Cell lysis, protein concentration, separation, and transfer were determined as previously described (3). Membranes were blocked using blocking buffer (Li-Cor Biosciences). Secondary antibodies were IR labeled. The Odyssey IR imaging system (Li-Cor Biosciences) was used for protein detection and quantification.

**Gene expression microarray**

CAL27 cells grown on 100-mm dishes were washed with PBS and cultured in DMEM without serum for 48 hours to synchronize the cell cycle. Enzastaurin was added to a final concentration of 2.5 μmol/L with 10% fetal bovine serum. An equivalent volume of DMSO was added to control cultures. Twenty-four hours after enzastaurin treatment; total RNA was purified with TRIzol (Invitrogen) following the manufacturer’s instructions. Total RNA was further purified using RNeasy Mini kit (Qiagen). The quality of purified RNA was checked with the Agilent 2100 Bioanalyzer, then labeled with fluorescent dye and hybridized to Agilent-14850 Whole Human Genome Microarrays 4 × 44K G4112F.
Microarray was done in the Functional Genomic Facility, University of Chicago. Triplicate samples from each treatment were hybridized to triplicate chips. Data were analyzed using the dCHIP (11) and Gene Microarray Pathway Profiler (GenMAPP; ref. 12) programs.

**Growth of human tumor xenografts**

SQ-20B (5 × 10⁶) or CAL27 (1 × 10⁷) cells were injected s.c. into the right hind limb of female athymic nude mice (Frederick Cancer Research Institute, Frederick, MD) as previously described (4). Xenografts (n = 8 in each group) were grown for 2 to 3 weeks, and tumor volume was determined by direct measurement with calipers and calculated using the equation $V = \frac{1}{2} \times W^2 \times L$. Animals were sorted into treatment groups, such that the mean tumor volume was 300 mm³ (SQ-20B) or 100 to 150 mm³ (CAL27). Mice were treated with D5W (control) or enzastaurin at 100 mg/kg twice daily by oral gavage without interruption and were sacrificed at the end of the experiment when any animal lost >20% body weight or seemed to be suffering or when tumor size exceeded 2,000 mm³.

**Quantitative real-time PCR**

RNA was isolated as previously described (4). Genomic DNA was purified with Qiagene Genomic tip 100/G columns (#10243) following the manufacturer’s protocols. Cyclin D1 gene copy differences among cell lines were determined by quantitative reverse transcription-PCR (RT-PCR) done on a StepOnePlus machine (Applied Biosystems) using Power SYBR Green PCR Master mix (P/N 4367659). Cyclin D1 copy number was normalized to LINE-1. Conditions for the amplification were 1 cycle of 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. Threshold cycle numbers were determined automatically with the Applied Biosystems software initially and then checked manually. Cyclin D1 gene copy number determined from all cell lines was normalized to CAL27. The cyclin D1 gene primers were 5'-ACGAGCTCGTGTGCTGCGAGGTG (forward) and 5'-CAGGCCAGACCTCCTTCTGCACA (reverse).

**Protein stability assay**

Cells were cultured to approximately 50% confluence, serum-starved for 24 hours, and treated with cycloheximide (50 μg/mL) in serum-containing medium as indicated.

In MG132 experiments, cells were cultured on 60-mm dishes to approximately 40% confluence and transferred to serum-free medium. After 24 hours, the cells were treated with complete medium containing enzastaurin (2.5 μmol/L) or DMSO. After 18 hours, 50 μmol/L MG132 was added; 3 and 7 hours later, cells were harvested.

**Results**

**The growth and proliferation of SCCHN cell lines are inhibited by enzastaurin**

To determine whether enzastaurin is effective in vivo, we chose SQ-20B and CAL27 cell lines based on our previous work with Gö6976, a PKC inhibitor, or rapamycin, which shows tumor growth inhibition (4). SQ-20B and CAL27 cells were grown as hind-limb xenografts and tumors were treated with vehicle control or enzastaurin alone (Fig. 1A and B). Enzastaurin significantly reduced tumor growth in both SCCHN models. Because other PKC inhibitors exhibit an antiproliferative effect in SCCHN cell lines, we investigated whether enzastaurin would inhibit proliferation in SQ-20B and CAL27 cells using the BrdUrd incorporation assay. Exposure for 24 hours to increasing concentrations of enzastaurin (Fig. 1C) caused inhibition of proliferation at concentrations similar to that of tumor models (13–15). Furthermore, PI staining and cytometry to determine relative DNA content in CAL27 cells revealed a higher fraction of enzastaurin-treated cells in S phase compared with untreated controls (46% versus 36%; Fig. 1D) at 24 hours. Interestingly, in SCC35 cells, which are resistant to enzastaurin (see below), no shift in cell cycle phase was observed. Therefore, in SQ-20B and CAL27 tumors, enzastaurin induced delay in tumor growth, decrease in proliferation, and, in CAL27 cells, cell cycle arrest.

**Specific changes in gene expression after enzastaurin treatment**

The chemical structure of enzastaurin is shown in Fig. 2A. To identify the effects of enzastaurin on gene expression, CAL27 cells were incubated with enzastaurin (2.5 μmol/L) or DMSO for 24 hours, and total RNA was extracted, purified, labeled, and hybridized to Agilent Whole Human Genome Microarrays. Expression data were analyzed with the dCHIP computer program (11) by comparing control and enzastaurin groups with a relative difference threshold of ≥1.5-fold, absolute intensity difference between enzastaurin and control samples of >50, and permutation P value < 0.05. Approximately 4,900 genes were identified from this screen, and among these, 75% were upregulated and 25% downregulated. These genes were further analyzed with the GenMAPP computer program (12). The number of genes used in GenMAPP analysis was 2,675. There were 2,013 genes linked to a pathway and 1,662 genes meeting the criteria of fold change ≥1.5 or ≤–1.5 and permuted P value < 0.05 in finding a MAPP.

The investigation of “Cell Cycle G1 to S Control” gene set (Supplementary Fig. S1; Supplementary Table S1) as one with significantly altered expression levels in CAL27 on exposure to enzastaurin was consistent with our earlier work with the classic PKC inhibitor Gö6976 (4). Among the identified enzastaurin downregulated genes in the pathway were CCND1, CCND2, CCNE1, and E2F2 (Supplementary Table S2). A heat map representing the microarray results focusing on cell cycle genes (Fig. 2B) shows that a number of key cell cycle genes

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4 Submitted for publication.
seem to be regulated by enzastaurin, many of which were confirmed by both RT-PCR and protein immunoblotting, including cyclins D1 and D2 (Fig. 2C). Harvested CAL27 xenografted tumors were analyzed for expression of cyclin D1 mRNA and protein by RT-PCR and immunohistochemistry, but a decrease in cyclin D1 expression was not appreciated (data not shown) despite a reduction in phosphorylated GSK3β, a putative target of enzastaurin. Given the inability to synchronize cells in vivo and the additional signals that can influence cyclin D1 expression including stroma-tumor interactions, this was not surprising.

The differential sensitivity of SCCHN cell lines to enzastaurin is related to cyclin D1 expression

To determine whether the antineoplastic properties of enzastaurin could be generalized to multiple SCCHN cell lines, we incubated nine lines with increasing concentrations of enzastaurin and assessed cell growth using a resazurin-based assay. The readings at each concentration were normalized to that of untreated cells and expressed as a percentage of viable cells compared with untreated control. An approximately 4-fold range in sensitivity was observed between the most sensitive and the most resistant cell lines (Supplementary Table S3), showing that enzastaurin reduces cell growth in most, but not all, SCCHN cell lines.

CCND1 is amplified in 30% to 50% of SCCHN, with a range of protein expression in tumors (16). Because the predominant phenotype we observed during enzastaurin treatment was inhibition of proliferation and cyclin D1 was a downstream target, we hypothesized that cyclin D1 levels would be predictive of sensitivity to the agent (Fig. 3A). Indeed, when cell-based in vitro inhibition was plotted against quantified cyclin D1 protein expression (Fig. 3B), a statistically significant correlation was observed ($r = 0.6476$, $P = 0.03$). Interestingly, in the resistant and high cyclin D1–expressing SCC35 cell line, enzastaurin...
did not modulate cyclin D1 protein levels or cell cycle progression (Figs. 1D and 5). Therefore, only in the sensitive CAL27 cell line is CCND1 expression altered by enzastaurin, whereas baseline cyclin D1 expression is predictive of sensitivity.

To delineate further whether cyclin D1 is mechanistically related to enzastaurin sensitivity, we transfected the sensitive CAL27 cell line with cyclin D1 using a lentiviral expression vector. Protein immunoblotting confirmed increased expression of the protein (Fig. 3C) compared with control, and notably, cyclin D1 overexpression was able to increase resistance in CAL27 cells (Fig. 3D), effectively increasing the number of viable cells approximately 3-fold. Conversely, we depleted cyclin D1 expression in the resistant SCC35 cell line and assayed cell viability. SCC35 cells were transfected with cyclin D1 or control shRNA, and protein immunoblotting confirmed knockdown of cyclin D1 expression (Fig. 3C). The cell viability assay showed that depletion of cyclin D1 partially reversed the resistance phenotype exhibited by control SCC35 cells (Fig. 3D). Taken together, these results indicate that induction of cyclin D1 increases resistance, whereas depletion of cyclin D1 induces sensitivity in SCCHN cell lines. Therefore, cyclin D1 expression is associated with enzastaurin sensitivity and mechanistically linked to its activity.

**Akt regulates cyclin D1 synthesis only in enzastaurin-sensitive cell lines**

To further investigate potential mechanisms underlying the effect of enzastaurin on cyclin D1, we incubated CAL27 cells with or without enzastaurin (2.5 μmol/L) and analyzed cell lysates for phosphorylation of the...
PKC substrate MARCKS, Akt, p70S6K, GSK3β, and eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1) over time. Enzastaurin abrogated phosphorylation of all proteins examined (Fig. 4A); however, inhibition of p70S6K, 4E-BP1, and Akt phosphorylation was most consistent temporally with a reduction in cyclin D1 protein. In support for a role of Akt in cyclin D1 regulation, we observed a reduction of
cyclin D1 protein at 8 hours in CAL27 cells treated with the phosphotyrosinol-3 kinase inhibitor LY294002 (Fig. 4B). The delay between inhibition of Akt phosphorylation and reduction in cyclin D1 protein suggests a transcriptional or translational mechanism, and in fact, Akt, mammalian target of rapamycin (mTOR), p70S6K, and 4E-BP1 are part of a signaling pathway that has been implicated in the translation of cyclin D1 in multiple cell systems (17). To determine whether mTOR was involved in cyclin D1 regulation, we incubated CAL27 cells with rapamycin but did not observe a change in cyclin D1 despite a rapid and sustained reduction in p70S6K phosphorylation (Fig. 4C). Therefore, it seems that Akt regulates cyclin D1 in the enzastaurin-sensitive CAL27 cells via a mechanism that is mTOR independent.

To clarify the role of Akt in the control of cyclin D1 and determine if this regulation affected the efficacy of enzastaurin, we measured the expression of Akt isoforms in SCCHN cell lines (Supplementary Fig. S2) and depleted Akt1 and Akt2 using siRNA. Compared with control or single isoform siRNA, Akt1 plus Akt2 siRNA reduced cyclin D1 (Fig. 4D, left), but interestingly, only in sensitive CAL27 cells and not in SCC35 cells. Moreover, transfecting CAL27 or SCC35 cells with constitutively active myristoylated Akt increased cyclin D1 expression only in the enzastaurin-sensitive cell line CAL27 (Fig. 4D, right). Taken together, it seems that a key determinant of enzastaurin efficacy in SCCHN cells is baseline expression of cyclin D1 and intact regulation of cyclin D1 by Akt.

**Enzastaurin primarily affects cyclin D1 protein synthesis, not degradation**

As shown above, enzastaurin inhibits multiple targets in SCCHN cells, many of which have been implicated in cyclin D1 regulation. However, inhibition of GSK3β, a regulator of cyclin D1 degradation (18–20), would be expected to increase cyclin D1 levels. Therefore, we hypothesized that if cyclin D1 reduction was critical to the efficacy of enzastaurin, then any inhibition of cyclin D1 degradation would be observed early and transiently after exposure. As expected, phospho-GSK3β was rapidly inhibited by enzastaurin in both the sensitive CAL27 and the resistant SCC35 cell lines with a concurrent increase in cyclin D1 expression in CAL27 cells (Fig. 5A). However, when we incubated the cells with cycloheximide to isolate cyclin D1 degradation, we observed that, indeed, this increase in cyclin D1 protein was short-lived (15–45 minutes) and relatively minor in CAL27 cells and did not occur in SCC35 cells (Fig. 5B). Therefore, the multi-kinase inhibitory properties of enzastaurin result in complex effects on cyclin D1 especially in CAL27-sensitive cells, with a transient increase in expression 30 to 40 minutes after exposure, but with a net reduction after several hours.

We then examined what effect enzastaurin had on cyclin D1 synthesis, considering that a reduction in protein level was observed after several hours only in enzastaurin-sensitive cell lines. CAL27 and SCC35 cells were treated with the proteasome inhibitor MG132, and cyclin D1 protein expression was quantified with or without enzastaurin (Fig. 5C). The addition of MG132 dramatically increased cyclin D1 levels in both cell lines, underscoring the importance of protein synthesis (DMSO versus DMSO + MG132 lanes). As predicted, however, only in the sensitive CAL27 cells did addition of enzastaurin reduce cyclin D1 expression (DMSO + MG132 versus enzastaurin + MG132), whereas no change was evident in the resistant SCC35 cells. These data, therefore, support the necessity of an inhibitory effect of enzastaurin on cyclin D1 production for the agent to be effective. When physiologic cyclin D1 regulation is disrupted, enzastaurin is inactive, as exemplified by SCC35 cells. Interestingly, quantitative PCR confirms that SCC35 cells have highly amplified CCND1 gene (Supplementary Fig. S3), suggesting a profound protein dysregulation that is not influenced by kinase inhibition.

**Discussion**

The introduction of molecularly targeted therapy in oncology ushered in an era of personalized medicine based primarily on host and tumor biology rather than anatomic location and pathologic features. The challenge, in most circumstances, has become predicting which patients will benefit from a specific agent, a mandate made more difficult with the development of multi-kinase inhibitors that can have several putative targets. Enzastaurin is one such agent being studied in different cancers, which we hypothesized would be effective in SCCHN because of its inhibitory properties against PKC (3, 4). Indeed, the agent was active against SCCHN cell lines and tumor xenografts, but not universally. We delineate a mechanism of sensitivity to enzastaurin that depends on the physiologic expression and regulation of cyclin D1 by Akt, a putative target of the drug. Conversely, when this regulation is disrupted, as observed in a resistant cell line, enzastaurin is ineffective.

Cyclin D1 plays a critical role in cell cycle progression and thus, not surprisingly, is dysregulated in multiple cancers including SCCHN. In fact, increased cyclin D1 expression in SCCHN, often through amplification of the 11q13 locus (21), has been associated with an increased transformation rate of premalignant lesions to cancer (22), lower survival (23–26), and resistance to therapy including cisplatin (27) or epidermal growth factor receptor inhibitors (28). Moreover, inhibition of cyclin D1 reduces the growth of SCCHN cell lines in vitro and tumor formation in vivo and increases sensitivity to cisplatin (29, 30). That enzastaurin treatment decreases cyclin D1 protein expression in a sustained manner in some SCCHN cell lines, therefore, highlights its potential utility, given that it is well tolerated and can be combined with cytotoxic chemotherapy (31).

The observation that enzastaurin does not universally inhibit in vitro growth in all cell lines examined is not surprising. Arguably, this is a closer reflection of what
Akt is among the putative targets of enzastaurin and contributes to cyclin D1 expression in CAL27 cells. A, CAL27 cells untreated (−) or treated (+) with 2.5 μmol/L enzastaurin for the times indicated. Total cell lysates were analyzed by protein immunoblotting and probed with anti-α-tubulin antibody and total and phospho-specific MARCKS, GSK3β, p70S6K, or Akt antibodies. Fold change represents quantification of the ratio of the signals to that of untreated (−) cells. Right, protein immunoblot done simultaneously probing for relevant total protein. Results are representative of at least three independent experiments. B, CAL27 cells were untreated (−) or treated (+) with LY294001 at a concentration of 25 μmol/L for the duration indicated. Total cell lysates were analyzed by protein immunoblotting and probed with α-tubulin, cyclin D1, and phospho-specific Akt antibodies. Results are representative of at least three independent experiments. C, CAL27 cells were untreated (−) or treated (+) with 50 nmol/L rapamycin (Rapa) for the duration indicated, and total cell lysates were analyzed by protein immunoblotting with cyclin D1, phospho-p70S6K, and α-tubulin antibodies. Results are representative of three independent experiments. D, CAL27 cells were transfected with siRNAs directed against Akt1 and Akt2, scrambled control siRNA, or no siRNA (left), or transfected with a control vector or Myr-Akt1, a constitutively active Akt1 expression vector (right). Total cell lysates were analyzed by protein immunoblotting with cyclin D1, Akt1, Akt2, phospho-specific Akt Ser473, and α-tubulin antibodies. FBS, fetal bovine serum. Results are representative of three independent experiments.

Figure 4.
is likely to occur in patients. In fact, the presence of naturally occurring resistant cell lines offers an opportunity to explore predictive biomarkers of efficacy that could be translated to human research. The finding that cyclin D1 levels are negatively associated with sensitivity to enzastaurin is one that should be further validated.

The effect of enzastaurin on cyclin D1 and the observation that cyclin D1 shRNA seems to abrogate the resistance phenotype underscore a mechanistic association. Arguably, in those cell lines with high baseline cyclin D1 expression, cyclin D1 levels cannot be reduced enough to render the cells sensitive. However, by depleting cyclin D1 expression using shRNA, we can render these cell lines sensitive to enzastaurin. Because the majority of oncology therapies are based on empirical approaches in unselected patient populations, predictive biomarkers that are simple to measure, such as cyclin D1 immunohistochemistry, would allow individualized treatment and thus increase the likelihood of benefit.

Inhibition of Akt by enzastaurin can involve direct binding to the ATP pocket, preventing activation; however, our results would suggest that inhibition of phosphorylation at T308 or S473 occurs several hours after exposure to enzastaurin (Fig. 4 and data not shown), indicating an indirect effect. Conversely, Akt regulation of cyclin D1 by mechanisms other than mTOR has been described in other models, but not in SCCHN (32). Akt stabilizes cyclin D1 protein levels by inhibiting GSK3β function through direct Ser9 phosphorylation. However, we could not show this in the SCCHN cell lines we...
examined (data not shown), and enzastaurin had minimal effect on cyclin D1 degradation. Akt has also been implicated in the transcriptional control of CCND1 through regulation of FoxO family members (33, 34) or cyclic AMP response element–binding protein 1 (35). It is possible that the effects we observed on cyclin D1 are mediated through Akt-regulated CCND1 transcription, and when this is disrupted by other mechanisms driving cyclin D1 expression (e.g., CCND1 amplification), cells become resistant to enzastaurin.

The present study illustrates that signaling context is critical to the understanding and development of novel compounds, especially those with multiple targets, and allows construction of a model specific to enzastaurin efficacy in SCCHN (Fig. 6). Our data support the utility of enzastaurin in SCCHN treatment, but only in tumors with moderate to low baseline cyclin D1 expression that is physiologically regulated. Nonetheless, results from preclinical experiments must be interpreted with caution, as they are de facto artificially engineered to replicate human cancer biology. For example, human head and neck cancer and hind-limb xenografts have clear differences in location, stromal cell species origin, and immunocompetence of the host. Thus, although our results provide a potential predictive biomarker to select patients for enzastaurin therapy and a general paradigm for multi-kinase inhibitor development, further exploration and validation in human studies is warranted.

Disclosure of Potential Conflicts of Interest

E.E.W. Cohen has served as a consultant for Eli Lilly Co. No other conflicts of interest were disclosed.

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