ORIGINAL ARTICLE

Inhibition of Glycogen Synthase Kinase-3 Increases the Cytotoxicity of Enzastaurin

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Cutaneous T-cell lymphomas (CTCL) represent a spectrum of several distinct non-Hodgkin's lymphomas that are characterized by an invasion of the skin by malignant, clonal lymphocytes. Our laboratory has previously demonstrated that the protein kinase C (PKC) β inhibitor Enzastaurin increases apoptosis in malignant lymphocytes of CTCL. These results directly led to a clinical trial for Enzastaurin in CTCL in which it was well tolerated and showed modest activity. To ascertain a means of improving the efficacy of Enzastaurin, we investigated complementary signaling pathways and identified glycogen synthase kinase-3 (GSK3) as important in survival signaling in CTCL. Enzastaurin combined with GSK3 inhibitors demonstrated an enhancement of cytotoxicity. Treatment with a combination of Enzastaurin and the GSK3 inhibitor AR-A014418 resulted in upregulation of β-catenin total protein and β-catenin-mediated transcription. Inhibition of β-catenin-mediated transcription or small hairpin RNA (shRNA) knockdown of β-catenin decreased the cytotoxic effects of Enzastaurin plus AR-A014418. In addition, treatment with Enzastaurin and AR-A014418 decreased the mRNA levels and surface expression of CD44. shRNA knockdown of β-catenin also restored CD44 surface expression. Our observations provide a rationale for the combined targeting of PKC and GSK3 signaling pathways in CTCL to enhance the therapeutic outcome.

INTRODUCTION

Cutaneous T-cell lymphomas (CTCL) represent a spectrum of several distinct extranodal non-Hodgkin's lymphomas. These lymphomas are characterized by an invasion of the skin by malignant, clonal CD4+ lymphocytes (Jakob *et al.*, 1996; Rook *et al.*, 1997). Although treatment may achieve remission, early chemotherapeutic treatment of CTCL has not demonstrated survival benefits and no treatments have proven to be curative. Therefore, new therapeutic approaches to this disease are needed.

Our laboratory has previously demonstrated that treatment with the protein kinase C (PKC) β inhibitor Enzastaurin, which is, to our knowledge, previously unreported, increases apoptosis in CTCL (Querfeld *et al.*, 2006). These results directly led to a clinical trial for Enzastaurin in CTCL in which it was well tolerated and demonstrated modest biological activity, suggesting that it may serve as a good platform for combination drug therapies (Querfeld *et al.*, in press). To improve the efficacy of Enzastaurin, we surveyed complementary signaling pathways to identify potential synergistic activity with Enzastaurin. We have identified glycogen synthase kinase-3 (GSK3) as important in survival signaling in CTCL. GSK3 is a constitutively active serine/threonine kinase involved in a wide variety of cellular processes including glycogen metabolism, transcription, translation, cell cycle, and apoptosis (Cohen and Goedert, 2004).

One critical downstream target of both PKC and GSK3 is β-catenin. β-Catenin is a multifunctional protein that has been implicated in a number of biological processes including cell–cell adhesion, embryonic development, and the Wnt signaling pathway. β-Catenin is phosphorylated on conserved serine and threonine residues, which leads to its ubiquitination and proteasomal degradation. Inhibition of β-catenin phosphorylation results in accumulation of cytoplasmic and nuclear β-catenin and subsequent binding to transcriptional cofactors such as the TCF/LEF family of proteins (Gumbiner, 1995; He *et al.*, 1998; Mann *et al.*, 1999).

A number of genes involved in apoptosis and survival signaling have been shown to be regulated by β-catenin, including CD44 (Wielenga *et al.*, 1999; Voutilainen *et al.*, 2006). CD44 is a transmembrane glycoprotein that has been shown to deliver survival signals although a number of intracellular components, including PI3K/Akt, Erk, Ras, and Lyn (Bates *et al.*, 2001; Lin and Yang-Yen, 2001; Bourguignon *et al.*, 2005, 2006; Kothapalli *et al.*, 2008).

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Abbreviations: CTCL, cutaneous T-cell lymphoma; DTT, dithiothreitol; GSK3, glycogen synthase kinase-3; NK, natural killer; PKC, protein kinase C; shRNA, small hairpin RNA

In addition, CD44 increases resistance to cellular stress induced by cytotoxic agents and is able to block p53-dependent cytostatic and apoptotic signals (Godar *et al.*, 2008; Xu *et al.*, 2010).

Our data demonstrate the observation that co-treatment with Enzastaurin and GSK3 inhibitors results in an increase in apoptosis in part through upregulation of β-catenin levels and modulation of the transcriptional activity of β-catenin. To our knowledge, this is previously unreported. This increase in β-catenin represses expression of the pro-survival factor CD44 at the mRNA and protein level. Knockdown of β-catenin restored cell survival as well as expression of CD44. Combination therapy, which targets both PKC and GSK3, may offer better clinical options for CTCL.

RESULTS

Previous data from our laboratory indicate that treatment with Enzastaurin results in an increase in apoptosis in HH and HuT-78 CTCL cell lines (Querfeld *et al.*, 2006). Because of the modest efficacy of Enzastaurin in phase II clinical trial in CTCL, we sought to identify pathways that could enhance the cytotoxicity of Enzastaurin. Recent *in vitro* and *in vivo* studies have suggested that the GSK3 signaling pathway is important for survival of malignant cells (Ougolkov *et al.*, 2005, 2007). To test whether GSK3 is an important survival factor in CTCL, we treated HuT-78 cells with three structurally distinct GSK3 inhibitors; LiCl, AR-A014418, and SB216763. None of the GSK3 inhibitors alone significantly affected the rate of apoptosis. However, when combined individually with Enzastaurin all three achieved a statistically significant increase in the rate of apoptosis compared with Enzastaurin alone (Figure 1a, see Supplementary Figure S1 online). AR-A014418 was chosen as a GSK3 inhibitor for remaining experiments due to its high specificity for GSK3 and lack of off-target effects compared with the other two inhibitors (Cohen and Goedert, 2004).

The combination of Enzastaurin and AR-A014418 was also examined in other CTCL-derived cell lines to determine whether GSK3 inhibition increased the cytotoxicity of Enzastaurin in a similar manner. Enzastaurin combined with AR-A014418 increased apoptosis compared with DMSO control or either drug alone in the cell lines HH, H9, and MJ (Figure 1b). These results demonstrate that the increase in apoptosis induced by this combination of drugs is not restricted to a single cell line.

To determine the therapeutic potential of inhibiting PKC and GSK3, we extended our studies beyond cell lines to include *ex vivo* samples isolated from CTCL patients. Malignant cells from several CTCL patients were collected, incubated with the inhibitors, and assessed for percentage of cells undergoing apoptosis. The program Calcusyn was used to determine whether the combination of Enzastaurin and AR-A014418 exhibited synergy (http://www.biosoft.com/w/calcusyn.htm). Cells were treated with the inhibitors and the combination index was calculated. A combination index of less than one is interpreted as synergy between the two compounds, whereas a combination index equal to one suggests additivity. Treatment with the combination of
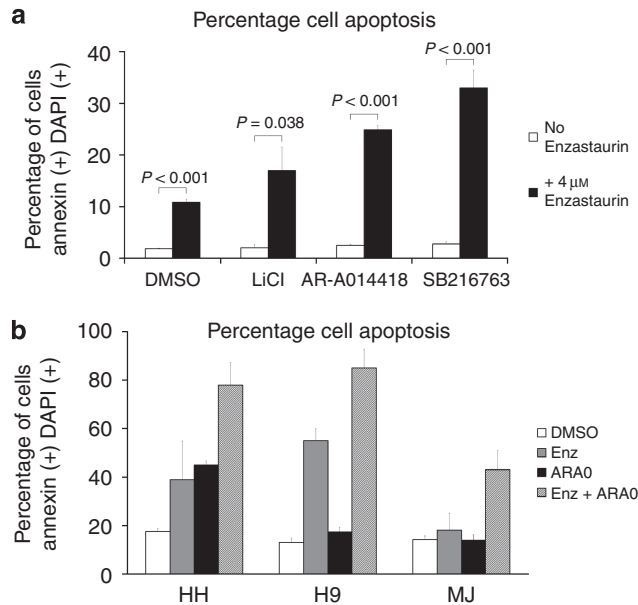
Figure 1. **Glycogen synthase kinase-3 inhibitors combined with Enzastaurin increase apoptosis.** (**a**) HuT-78 cells were cultured in 4 μM Enzastaurin, 5 mM LiCl, 5 μM AR-A014418, 5 μM SB216763, or DMSO (vehicle control) and collected after 5 days. (**b**) HH, H9, and MJ cells were cultured in 4 μM Enzastaurin (H9), 8 μM Enzastaurin (HH, MJ), 5 μM AR-A014418 (H9), 10 μM AR-A014418 (MJ), 20 μM AR-A014418 (HH), or DMSO (vehicle control) and collected after 5 days. Detection of Annexin V and 4′-6-diamidino-2-phenylindole (DAPI) staining for all cells was performed as described previously. Data from three separate experiments were averaged to quantify apoptotic cells (double positive cells) as a percentage of total cells. Error bars represent standard deviation.

Table 1. CI values at different concentrations of Enzastaurin and AR-A014418 in *ex vivo* patient samples

Patient	Enzastaurin (μM)	ARA014418 (μM)	CI
P1	2	5	0.805
	4	5	0.784
P2	2	5	0.737
	4	5	0.825
P3	2	5	0.872
	4	5	0.936
P4	2	5	0.563
	4	5	0.954
P5	2	5	0.568
	4	5	0.801

Abbreviation: CI, combination index.

Enzastaurin and AR-A014418 increased apoptosis in a synergistic or additive manner in all patient samples, suggesting that this drug combination holds potential in treating CTCL (Table 1).

Both PKC and GSK3 can phosphorylate β-catenin and target it for subsequent proteasomal degradation (Chen *et al.*, 2000; Gwak *et al.*, 2006; Raab *et al.*, 2009). Inhibition of
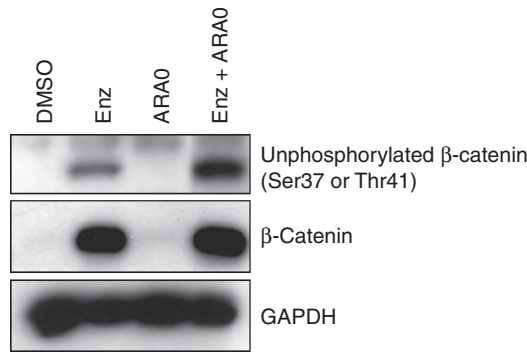
Figure 2. Enzastaurin combined with AR-A014418 increases unphosphorylated (active) β-catenin levels. HuT-78 cells were cultured in standard drug conditions of DMSO, 4 μM Enzastaurin, 5 μM AR-A014418, or the combination of Enzastaurin and AR-A014418 and lysates were prepared at 24 hours. Immunoblots were performed as described previously. Blot is representative of three separate experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

β-catenin phosphorylation can lead to increased transcriptional activity of β-catenin and modulation of gene expression, including a number of genes involved in apoptosis and survival signaling (Clevers and van de Wetering, 1997; He et al., 1998; Mann et al., 1999). To determine whether PKC and GSK3 regulate β-catenin levels in CTCL, we cultured cells in the presence of Enzastaurin and AR-A014418. Treatment with Enzastaurin alone or Enzastaurin and AR-A014418 resulted in an upregulation of β-catenin protein levels (Figure 2). We also used an antibody that detects unphosphorylated β-catenin to determine whether the phosphorylation state of β-catenin was affected by the inhibitors. Treatment with the combination of Enzastaurin and AR-A014418 decreased phosphorylation of β catenin compared with Enzastaurin alone.

Unphosphorylated β-catenin is targeted to the nucleus to form a nuclear complex with TCF/LEF transcription factors (Huber et al., 1996; Eastman and Grosschedl, 1999). To determine whether the combination of inhibitors increased the nuclear localization and transcriptional activity of β-catenin, cells were incubated with the two inhibitors and cells were fractionated to separate nuclear and cytoplasmic fractions. α-Tubulin was used as a cytoplasmic marker and hnRNP A0 was used as a nuclear marker. Treatment with Enzastaurin and AR-A014418 resulted in an increase of nuclear β-catenin compared with DMSO control or either inhibitor alone (Figure 3a). To examine whether inhibitor treatment affected β-catenin-mediated transcription, we used a TCF/β-catenin luciferase reporter. Cells were transfected with the reporter and treated with the inhibitors Enzastaurin and AR-A014418. Neither Enzastaurin nor AR-A014418 alone significantly increased luciferase activity (Figure 3b). However, the combination of both inhibitors resulted in an increase in luciferase activity. Cells were also transiently transfected with ICAT, a protein which represses TCF/β-catenin-mediated transactivation by inhibition of the interaction between β-catenin and TCF (Tago et al., 2000). ICAT expression decreased luciferase activity compared with vector control in cells treated with Enzastaurin and
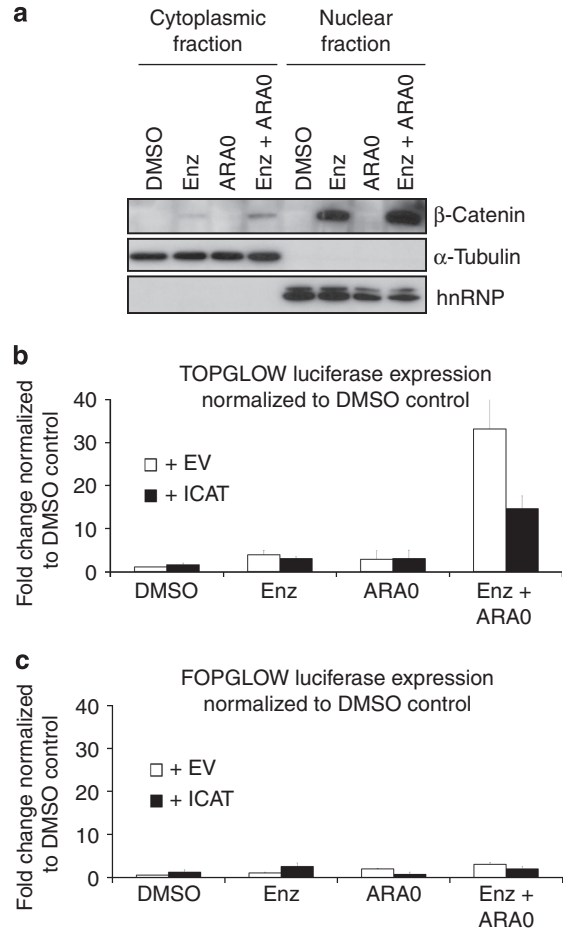
Figure 3. Enzastaurin combined with AR-A014418 increases nuclear localization and transcriptional activity of β-catenin. (**a**) HuT-78 cells were cultured in the standard drug conditions of DMSO, Enzastaurin, or AR-A014418 and collected at 24 hours. Cells were fractionated and resultant lysates were immunoblotted with indicated antibodies. (**b**) HuT-78 cells were transfected with 3 μg TOPGLOW luciferase reporter (Millipore, Billerica, MA) and either PC3DNA or ICAT vector, treated with inhibitors, and collected after 24 hours. (**c**) HuT-78 cells were transfected with 3 μg FOPGLOW luciferase reporter (Millipore) and either PC3DNA or ICAT vector, treated with inhibitors, and collected after 24 hours. Luciferase reporter assays were carried out using manufacturer's instructions (Promega).

AR-A014418. In a separate experiment, cells were transiently transfected with a control luciferase reporter, FOPGLOW (mutant TCF binding sites) and no significant difference in activity was observed between cells treated with DMSO control or any of the inhibitors (Figure 3c).

To determine whether β-catenin-mediated gene regulation is important in modulating cell survival, we transiently transfected HuT-78 cells with either the ICAT vector or an empty vector control, treated with the inhibitors and examined the percentage of cells undergoing apoptosis. In cells co-treated with Enzastaurin and AR-A014418, ICAT expression decreased apoptosis compared with vector control (Figure 4a). This suggests that β-catenin-mediated gene regulation is in part responsible for the activation of apoptosis in cells treated with Enzastaurin and AR-A014418. To
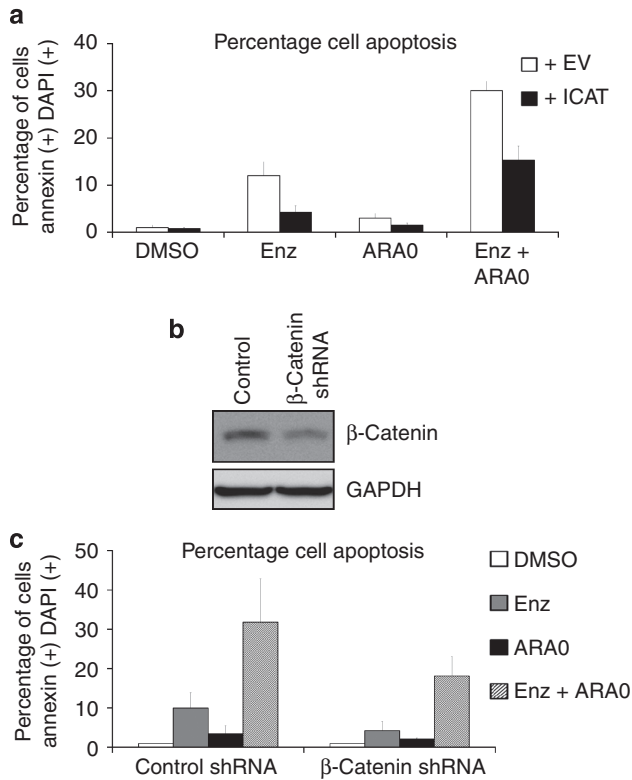
Figure 4. **β-Catenin-mediated transcription increases apoptosis in cutaneous T-cell lymphomas.** (**a**) HuT-78 cells were transfected with 3 µg green fluorescent protein expressing vector and either PC3DNA or ICAT vector. Detection of Annexin V and 4′-6-diamidino-2-phenylindole (DAPI) staining was performed as described previously. Data from three separate experiments were used to quantify double positive cells as a percentage of total cells. Error bars represent standard deviation. (**b**) β-Catenin expression was knocked down in HuT-78 cells using a small hairpin RNA (shRNA)-expressing lentivirus. Cells were then treated with inhibitors and β-catenin protein was examined by immunoblot after 24 hours. (**c**) Detection of Annexin V and DAPI staining was performed on HuT-78 cells transduced with lentivirus as described previously. Data from three separate experiments were used to quantify double positive cells as a percentage of total cells. Error bars represent standard deviation. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

confirm these results, we knocked down β-catenin using a small hairpin RNA (shRNA)-expressing lentivirus. Cells were transduced with either control lentivirus or β-catenin shRNA lentivirus and selected in puromycin. Cells were then treated with Enzastaurin and AR-A014418 and β-catenin protein was examined by immunoblot. Total β-catenin levels were decreased in cells transduced with the β-catenin shRNA lentivirus compared with cells transduced with control lentivirus (Figure 4b). Apoptosis was also measured in the β-catenin knockdown and control cells. A decrease in the percentage of cells undergoing apoptosis when treated with Enzastaurin and AR-A014418 was observed in the β-catenin knockdown cells compared with control cells (Figure 4c, see Supplementary Figures S2 and S3 online). Similarly, β-catenin levels were also knocked down in the CTCL cell lines HH, H9, and MJ. Knockdown of β-catenin levels in all three cell lines resulted in a decrease in the percentage of

cells undergoing apoptosis when treated with Enzastaurin and AR-A014418 compared with control cells (Supplementary Figures S2 and S3 online).

β-Catenin can modulate the transcription of several genes involved in survival signaling, including CD44. We examined mRNA levels of CD44 in cells treated with Enzastaurin and AR-A014418 to determine whether the increase in β-catenin levels resulted in a decrease in gene expression. Treatment with Enzastaurin or the combination of Enzastaurin and AR-A014418 resulted in a decrease in CD44 mRNA levels (Figure 5a). To determine whether treatment with the inhibitors nonspecifically decreases all transcriptional targets of β-catenin, we examined the effect of Enzastaurin and AR-A014418 on c-Myc and cyclin D1. Treatment with the two inhibitors did not significantly affect expression of c-Myc or cyclin D1 (data not shown), suggesting that co-treatment with Enzastaurin and AR-A014418 modulates only a subset of β-catenin responsive genes.

To determine whether this decrease in CD44 mRNA resulted in lower surface expression of CD44, cells were treated with the two inhibitors and examined by flow cytometry. Surface expression of CD44 decreased in cells treated with the two inhibitors compared with cells treated with either inhibitor alone or DMSO control (Figure 5b). To confirm that the observed decrease in CD44 surface expression was mediated by β-catenin, β-catenin was knocked down and cells were treated with the two inhibitors. Knockdown of β-catenin resulted in a restoration of surface CD44 levels, suggesting that β-catenin was indeed responsible for the drug-induced decrease in CD44 (Figure 5c).

DISCUSSION
The goal of cancer therapeutics is to eliminate cancer cells with minimal damage to nonmalignant cells. The combination of Enzastaurin and AR-A014418 seeks to achieve this goal by inhibiting complementary survival pathways important for tumor cell survival and allows for lower doses of the individual drugs that may be less toxic to nonmalignant cells. Treatment with Enzastaurin and AR-A014418 results in enhanced apoptosis in CTCL cell lines as well as in patient samples, suggesting that GSK3 and PKC signaling pathways have important roles in CTCL. Treatment with Enzastaurin and AR-A014418 resulted in a decrease in β-catenin phosphorylation, an accumulation of cytoplasmic and nuclear β-catenin and an increase in transcriptional activity. This increase in transcriptionally active β-catenin resulted in an increased rate of apoptosis and transcriptional changes in CD44. Together, this points to the combination of PKC and GSK3 inhibition as a potential treatment for CTCL.

Although Enzastaurin primarily affects PKC, Enzastaurin has been shown to inhibit other AGC (cAMP-dependent protein kinase/protein kinase G/protein kinase C) family kinases involved in survival signaling, including Akt and p90[RSK] (Graff *et al.*, 2005). We have not ruled out the possibility that the Enzastaurin-mediated increase in apoptosis observed in CTCL may be due in part to additional factors outside of the inhibition of PKC. Additional studies will be needed to better define the critical targets of Enzastaurin.
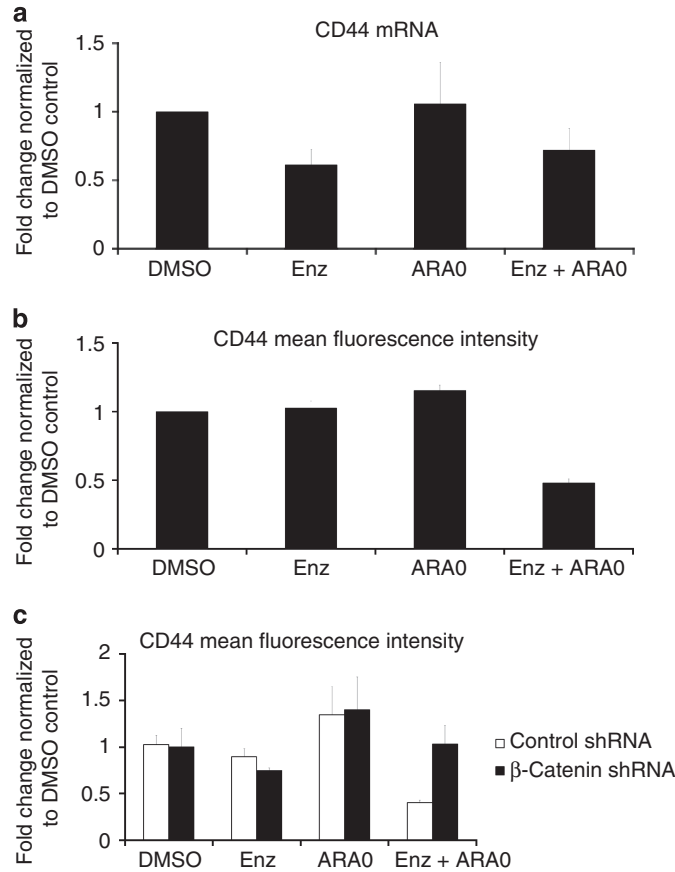
Figure 5. **Enzastaurin combined with AR-A014418 modulates expression of CD44.** (**a**) RNA was isolated from HuT-78 cells treated with inhibitors and data from three separate experiments were used to calculate the change in gene expression as a percentage of DMSO control. Error bars represent standard deviation. (**b**) HuT-78 cells were stained with antibodies that detect CD44 and examined by flow cytometry. Cells in late apoptosis bind antibodies nonspecifically and were therefore gated out during analysis. Error bars represent standard deviation from three separate experiments. (**c**) HuT-78 cells transduced with lentiviruses were stained with antibodies against CD44 and examined by flow cytometry. Error bars represent standard deviation from three separate experiments.

β-Catenin has been shown to be overexpressed in some CTCL patients (Bellei *et al.*, 2004, 2006). β-Catenin accumulation was seen in 31% of the mycosis fungoides samples and 70% of the Sézary syndrome samples in one study and 46% of mycosis fungoides cases in another study (Bellei *et al.*, 2004, 2006). However, the β-catenin observed in these tumor samples was not found in the nucleus and there was no detectable expression of β-catenin/TCF target genes (Bellei *et al.*, 2004). This suggests that the mere presence of β-catenin will not necessarily trigger apoptosis in CTCL. Translocation to the nucleus and transcriptional activity appear to be important in activating apoptosis. It is also likely that the specific cofactors that bind to β-catenin also have a role. In addition to members of the TCF/LEF family of transcription factors, β-catenin can associate with a number of other cofactors to modulate transcription, including p300, CBP, histone deacetylases, and FOXO family proteins (Billin *et al.*, 2000; Hecht *et al.*, 2000; Takemaru and Moon, 2000; Hoogeboom *et al.*, 2008). The expression of ICAT may disrupt the interaction of β-catenin with other proteins besides TCF/LEF family members, such as p300 (Daniels and Weis, 2002).

β-Catenin has been shown to not only upregulate expression of TCF/β-catenin target genes but also repress gene expression (Deng *et al.*, 2002, 2004). For example, β-catenin has been shown previously to inhibit transcription factors involved in survival signaling, such as NF-κB (Deng *et al.*, 2002, 2004). Although some studies have suggested that β-catenin may positively regulate the expression of CD44, β-catenin appears to repress the expression of CD44 in the context of Enzastaurin and AR-A014418 treatment in CTCL cells.

Although Enzastaurin alone was able to decrease mRNA levels of CD44, only the combination of Enzastaurin and AR-A014418 was able to decrease surface expression of CD44. The control of CD44 expression is very complex and likely influenced by a number of factors, including splicing and posttranslational regulation. Expression of specific isoforms of CD44 can be regulated by PI3K and PKC (Fichter *et al.*, 1997). β-Catenin can not only affect CD44 mRNA expression, but has also been shown to modulate splicing of CD44 isoforms (Goncalves *et al.*, 2008). We used primer probe sets and flow cytometry antibodies that could detect all splice variants and isoforms of CD44, respectively. Treatment with Enzastaurin and AR-A014418 may only be inhibiting the surface expression of a subset of all CD44 isoforms. Further experiments will need to be conducted to determine the

specific isoforms of CD44 affected by the combination of Enzastaurin and AR-A014418.

All different subtypes and stages of CTCL have been shown to express CD44 (Orteu *et al.*, 1997). In addition, CD44 functions in PKC-regulated migration of T cells (Fanning *et al.*, 2005). Inhibition of PKC by Enzastaurin as well as the induced decrease of CD44 may be a particularly effective therapeutic against the skin-homing malignant lymphocytes of CTCL.

The concentrations of both Enzastaurin and AR-A014418 used in these experiments have been previously shown to be at or below achievable levels *in vivo*. Phase I clinical trials showed that steady-state plasma concentrations of up to 8 μM Enzastaurin are achievable in patients (Graff *et al.*, 2005). Although no pharmacokinetic data for AR-A014418 exists for humans, blood plasma concentrations of 3.75 μM AR-A014418 have been measured with oral dosing of 1 μmol kg^{-1} in Sprague–Dawley rats and much higher dosages (10-fold) been used with no reported dose-limiting toxicities (Martins *et al.*, 2011; Medina and Avila, 2010; Gould *et al.*, 2004).

The combination of Enzastaurin and AR-A014418 has the potential to affect a number of important signaling pathways. *In vivo* experiments in mice will be needed to address if the combination of Enzastaurin with a GSK3 inhibitor activates synergistic toxicities or if it has other off-target effects. For example, Enzastaurin is able to inhibit both natural and antibody-dependent cellular cytotoxicity of natural killer (NK) cells against tumor targets, and therefore may suppress NK cell-mediated activity against tumor cells (Ogbomo *et al.*, 2011). Interestingly, treatment of NK cells with a GSK3 inhibitor reversed Enzastaurin-mediated inhibition of NK cell cytotoxicity. *In vivo* experiments will be needed to address these and other potential positive and negative off-target effects.

Understanding the role of PKC and GSK3 in survival signaling in CTCL will provide insight into the biology of CTCL as well as define rational targets for therapy. Our observations point to the importance of targeting these two pathways in CTCL and the possibility of an improvement in therapeutic outcome.

MATERIALS AND METHODS

Cell lines and cell culture

HH, H9, HuT-78, and MJ cells were grown in RPMI 1640 (Invitrogen, Baltimore, MD) supplemented with 10% fetal bovine serum (MJ in 20%), 2 mM glutamine, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, and 2.5 mg ml^{-1} amphotericin B. For primary samples, Northwestern University IRB approval was obtained for this study, and each patient provided written, informed consent. The study complied with the Declaration of Helsinki protocols. Blood samples were collected from CTCL patients and peripheral blood mononuclear cells (PBMCs) were isolated using BD Vacutainer CPT tubes (BD Biosciences, San Jose, CA). CD4 positive cells were then isolated using manufacturer's instructions for the EasySep immuno-magnetic cell separation kit (StemCell Technologies, Vancouver, BC, Canada).

Antibodies and reagents

ICAT vector was a gift from the Gottardi Laboratory. Antibodies against β-catenin and active β-catenin were purchased from Millipore (Temecula, CA). Antibodies against α-tubulin and hnRNP A0 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). CD44 antibodies were purchased from BD Biosciences. All other antibodies were purchased from Cell Signaling. Enzastaurin was a gift from Eli Lilly (Indianapolis, IN). Annexin 4′-6-diamidino-2-phenylindole staining was performed according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). Flow cytometry data were recorded by using a DakoCytomation CyAn machine, and samples analyzed by using FCS Express.

Cell viability assay

Cell viability assays were carried out using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reagent from Promega according to the manufacturer's instructions (Madison, WI). The data are expressed as percentage of DMSO control cells.

Transfection

HH and HuT-78 cells were transiently transfected using an Amaxa Nucleofector I device (Lonza, Cologne, Germany). Five million HuT-78 cells were electroporated in 0.1 ml of Solution V using the T-01 program with 3 μg of the appropriate vector.

Luciferase assay

Cells were transiently transfected with 3 μg of plasmid vector and incubated at 37 °C for 24 hours. Lysates were prepared 24 hours post-transfection using the Promega Luciferase Assay System according to the manufacturer's instructions (Promega). Luminescence was measured for 15 seconds using a BioTek Synergy HT plate reader (BioTek, Winooski, VT). Luminescence was normalized to protein concentration of sample.

Cell fractionation

Cells were washed twice in phosphate-buffered saline and resuspended in 1 ml buffer A (10 mM HEPES, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM MgCl$_2$, 5% v/v glycerol, 0.1 mM Na$_3$VO$_4$) for 10 min on ice. A volume of 25 μl buffer B (10 mM HEPES, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM MgCl$_2$, 5% v/v glycerol, 0.1 mM Na$_3$VO$_4$, 10% v/v Nonidet P-40) was added and lysates were spun down at 4,000 r.p.m. for 10 minutes at 4 °C. Supernatant was collected (cytoplasmic fraction) and stored. Remaining pellet was washed twice with 1 ml buffer C (10 mM HEPES, pH 7.5, 10 mM KCl, 1 mM DTT, 1 mM MgCl$_2$) and resuspended in 500 μl of buffer D (20 mM HEPES, pH 7.5, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM MgCl$_2$, 25% v/v glycerol). Lysates were incubated for 15 minutes on ice with intermittent vortexing and then spun down at 15,000 g for 5 min at 4 °C. Supernatants were collected (nuclear fraction) and stored.

Reverse transcription PCR

RNA lysates were prepared using RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcriptase step was run on Eppendorf Master Cycle Thermocycler (Eppendorf, Hauppauge, NY). Real-time PCR was performed using the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA) and run on 7500 Fast Real Time PCR Machine (Applied Biosystems). Samples were run in triplicate. Reverse transcription PCR was performed a minimum of three independent times for each gene. RNA expression

was normalized to three independent control genes using geNorm (http://medgen.ugent.be/~jvdesomp/genorm/).

Immunoblotting and immunoprecipitation

Lysates were prepared and resuspended in SDS loading buffer, heated to 95 °C for 5 min, separated by 10% SDS-PAGE and transferred to Immobilon membranes. Membranes were blocked in BLOK Casein in TBS (G Biosciences, St Louis, MO) for 1 hour at room temperature, incubated overnight in BLOK containing the appropriate primary antibodies at 4 °C, washed three times in Tris-buffered saline with Tween, and incubated with secondary antibodies in BLOK for 1 hour at room temperature. For detection, membranes were washed three times in tris buffered saline with tween and detected with ECL Plus (Pierce, Rockford, IL).

Lentivirus production and transduction

Lentiviruses were produced using the Lentiphos HT kit (Clontech, Mountain View, CA) and GIPZ Lentiviral shRNAmir constructs according to the manufacturer's instructions (Open Biosystems, Huntsville, AL). To transduce the cells, one million cells were plated per well in a 6-well plate with 5 ml of viral supernatant and 5 µl of polybrene. Cells were spun for 2 hours at 3,200 r.p.m. at room temperature.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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