Protein kinase C beta in malignant pleural mesothelioma

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Malignant pleural mesothelioma (MPM) is a disease with few therapeutic options. Protein kinase C beta (PKCβ) is involved in important cellular functions. Enzastaurin (LY317615.HCl) is a novel inhibitor of PKC in clinical development. MPM cell lines (7) and patient tumor tissues (24) were evaluated for expression of PKCβ by immunoblotting and immunohistochemistry, respectively. In-vitro cell growth assays were performed with enzastaurin with or without cisplatin. Cell migration was evaluated with the wound healing assay. Downstream signaling (survival and focal adhesion pathways) was studied by immunoblotting for related molecules in the presence of phorbol ester with or without enzastaurin. Expression for PKCβ1 was seen in all cases, with a mean integrated optical density of 152.5 (standard deviation = 95.47, $n = 24$), whereas PKCβ2 expression was less intense, with a mean integrated optical density of 11.45 (standard deviation = 16.27, $n = 21$). There was a trend toward lower overall survival among patients expressing above-median PKCβ1 ($P = 0.064$), but not PKCβ2. Robust expression of PKCβ1 and low expression of PKCβ2 were observed in MPM cell lines. Treatment of MPM cell lines with enzastaurin revealed an IC50 of 5 μmol/l, and strong synergism was observed when combined with cisplatin. Wound healing assay revealed that treatment of H2461 cells with enzastaurin reduced migration by 59.2%. Enzastaurin treatment led to disruption of F-actin architecture. Downstream signaling showed reduced phosphorylation of AKT, FAK (focal adhesion kinase), p130Cas, S6 ribosomal protein, and paxillin. PKCβ1 was expressed in the majority of MPM samples. Enzastaurin has preclinical activity against MPM, and exhibited synergism with cisplatin. PKCβ inhibition in MPM might be able to reduce the invasiveness of MPM by affecting cytoskeletal function. *Anti-Cancer Drugs* **19:841–848 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Malignant pleural mesothelioma (MPM) is a rare disease, with approximately one in 100 000 people being diagnosed per year in the US. This disease can affect individuals who have been potentially exposed to asbestos, and in some cases, infection with the simian virus 40 (SV40) has been implicated in the pathogenesis of MPM [1,2]. Median survival from the time of diagnosis is approximately 9 months. Treatment options include surgery and/or chemotherapy, and sometimes radiation therapy [3,4]. Only one chemotherapeutic agent (pemetrexed) has been approved by the Food and Drug Administration in recent years for treatment of this disease, and new and more efficacious therapeutic options are needed [5,6].

The protein kinase C (PKC) family of serine–threonine protein kinases has been implicated in several important cellular functions including proliferation, motility, invasion, and apoptosis [1]. Of the various PKC isoforms, PKCβ expression has been demonstrated in several human cancers, most notably B-cell lymphomas [7]. Its overexpression has been shown to be an adverse prognostic factor in diffuse large B-cell lymphomas [7–9]. This was evaluated in a gene expression study, where 6817 genes were evaluated in relation to refractoriness versus curability in diffuse large B-cell lymphomas; patients whose tumors had higher expression of PKCβ2 had a worse 5-year event-free survival (36 vs. 49%, $P = 0.054$) [7]. PKCβ has been implicated in angiogenesis, making it an attractive target for therapeutic inhibition in cancer [10]. Downstream, PKC can target PI3K/AKT pathway and other signal transduction pathways [11,12].

Enzastaurin (LY317615.HCl) is an oral small-molecule acyclic bisindolylmaleimide inhibitor of PKCβ, currently undergoing phase I–III clinical trials, and able to inhibit PKCβ in the low nanomolar range. At higher dosages, it is able to inhibit other PKC isoforms. It is being studied in multiple myeloma [13], breast cancer [14], cutaneous T-cell lymphoma [15], thyroid cancer [16], colon cancer, glioblastoma [11], and non-small cell lung cancer [17].

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In this non-small cell lung cancer phase II clinical trial where enzastaurin was used as second-line or third-line, the overall survival was 9.9 months at a 12-month rate of 46.3%. Thirty-five percent had a stable disease with no objective responses observed. Most drug-related toxicities were mild, with grade 3 toxicities being uncommon (ataxia, fatigue, thrombo-embolism, anemia).

In this study, we evaluated the expression of PKCβ in MPM and its relationship to prognosis. We also determined the effects of inhibition of PKCβ with enzastaurin and in combination with cisplatin in MPM. PKCβ can affect the cytoskeleton. Inhibition of cell motility/migration and its relationship to the focal adhesion proteins was determined in MPM, and these were considerably affected by enzastaurin treatment.

Materials and methods

Cell lines and cell culture

MPM cell lines H513 (epithelioid), H2461 (epithelioid), and H2596 (sarcomatoid) were cultured as previously described [18,19]. H28 (epithelioid), H2052 (sarcomatoid), H2452 (biphasic), MSTO-211H (biphasic), and the nonmalignant mesothelial cell line (MeT-5A) were obtained from the American Type Culture Collection (Rockville, Maryland, USA). MPM cells were cultured as per our established protocols [20].

Reagents and antibodies

Enzastaurin was provided by Eli Lilly (Indianapolis, Indiana, USA). Cisplatin was purchased from Sigma (St Louis, Missouri, USA). Phorbol ester (phorbol-12-myristate-13-acetate, PMA) was obtained from Calbiochem (San Diego, California, USA). Fetal bovine serum (FBS) was obtained from Gemini Bioproducts (Woodland, California, USA). Cell culture media, penicillin, and streptomycin were obtained from Cellgro (Boehringer Ingelheim, Heidelberg, Germany). Antibodies used included PKCβ1 and PKCβ2 (Santa Cruz, Santa Cruz, California, USA); phospho-AKT (Ser473), phospho-p70 ribosomal protein S6 (Ser240/244), phospho-glycogen synthase kinase (GSK)-3β (Ser9), GSK3β, phospho-pCas130 (Tyr165), phospho-FAK (Tyr925) (Cell Signaling Technology, Beverly, Massachusetts, USA); phospho-paxillin (Tyr31) was purchased from Invitrogen (Carlsbad, Californai, USA); β-actin monoclonal antibody and all other chemicals were purchased from Sigma.

Immunohistochemistry and tissue microarrays

Paraffin-embedded, formalin-fixed tumor tissues were processed into a tissue microarray (TMA) with clinical information, under an institutional review board approved protocol. Immunohistochemistry was performed using biotin-free HRP-labeled polymer complex bound to secondary antibody (DAKO Cytomation, Carpinteria, California, USA), and performed according to previously published procedures [19]. Negative controls were performed by substituting the primary antibody step with nonimmune mouse immunoglobulins. Nuclear staining with PKCβ 1 and 2 antibodies was quantified by using the Automated Cellular Imaging System (ACIS) from Clarient (San Juan Capistrano, California, USA). This system consists of a bright field microscope with several objectives, digital camera, an automated slide loading system, and a computer. The measurement of intensity of the staining is based on three related color parameters: the color defined by hue, the 'darkness' defined as luminosity, and density of the color defined as the saturation. ACIS software was programed by experienced user-pathologist (M.T.), by setting the color-specific thresholds, to determine the intensity of brown positivity of cells within the outlined areas of interest. For each TMA core, we selected representative areas of tumor containing comparable numbers of cells, approximately 50–100 cells. The ACIS software calculated the average intensity for each region as a measure of integrated optical density (IOD) in the nuclear compartment. The IOD of each image (region) is given as the average of optical densities of each molecule (pixel) within the region. Computing of IOD is directly proportional to the concentration of molecule recognized by the stain according to the Beer–Lambert law [21]. IOD is a proxy for antigen content and it is calculated as intensity multiplied by brown area (in microns). For comparison purposes we normalized the IOD value to the entire measured area by calculating IOD/10 μm^2.

Immunoblotting

To examine protein expression in mesothelioma and nonmalignant mesothelial cells under basal conditions, subconfluent cells were cultured in medium supplemented with 10% FBS. To detect the activation and further inhibition of cell transduction pathways, cells grown on 10 cm culture dishes for 24 h were washed twice with phosphate-buffered saline, and incubated at 37°C with enzastaurin 2.5 μmol/l [or dimethylsulfoxide (DMSO)] for 3 h, followed by PMA 50 nmol/l (or DMSO) for 30 min. Whole cell lysates were collected and immunoblotting was performed following routine protocols [20]. The same membranes were subsequently stripped and reprobed in a similar fashion with different primary antibodies. β-actin levels were used to control for equal loading amounts. Quantification of bands was performed by utilizing the ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Ratios of the integrated intensity of the band of interest to the corresponding β-actin band were used for comparisons.

Wound healing assay

In-vitro wound healing assay studies were done using previous methods [20]. Briefly, cells were plated in six-well tissue culture plates with complete culture media for 24 h. Using a pipette tip, a wound was created through

the middle portion of the culture plates. Culture medium containing enzastaurin (or DMSO) was used. Plates were photographed at baseline and after 24 h using an Olympus IX71 research microscope. The areas of reepithelization were measured using ImageJ software (National Institutes of Health). Three separate visual fields were measured in each experiment.

Viability assays and synergism studies

Cells were plated in 96-well plates at 5×10^3 per well, in serum-containing media, and grown for 24 h. Drugs (or drug carrier) were added in serum-free media, and cells were incubated for 72 h. Cell growth was estimated utilizing fluorometric readings after the addition of Alamar Blue, a nonradioactive, nontoxic dye that is reduced and fluorescence is proportional to the metabolic activity. A HT Synergy Plus microplate reader (Biotek, Winooski, Vermont, USA) was used to measure fluorescence. Drug synergism was estimated by the median-effect analysis [22], using the Calcusyn 3.0 software package (Biosoft, Cambridge, UK).

Immunofluorescence

5×10^3 cells were plated on glass cover slips on six-well plates, and grown for 24 h with 10% FBS-containing media. Treatment conditions were applied as described in individual experiments, and immunofluorescence performed as previously published [19]. Visualization was achieved with an Olympus IX81 DSU spinning disk confocal microscope (Olympus America, Center Valley, Pennsylvania) with DIC and a back-thinned EMCCD camera. Images were processed with Slidebook 4.0 (Intelligent Imaging Innovations, Denver, Colorado, USA) and ImageJ (National Institutes of Health).

Statistical analysis

For comparisons of frequencies among different categories, Fisher's exact test was used. Kaplan–Meier curves were generated and differences between curves were compared by the log-rank test. The statistical software used was SPSS, version 15.0 (SPSS Inc, Chicago, Illinois, USA).

Results

Protein kinase C beta expression in malignant pleural mesothelioma tumor tissues and relationship to survival

TMAs from 24 patients with MPM were analyzed by immunohistochemistry. Expression for PKCβ1 was seen in all cases, with a mean IOD of 152.5 (standard deviation = 95.47, $n = 24$), whereas for PKCβ2, expression was less intense, with a mean IOD of 11.45 (standard deviation = 16.27, $n = 21$). Histograms depicting the distribution of the IODs observed for PKCβ1 and PKCβ2 are shown in Fig. 1a and b, respectively. A survival analysis was performed, by grouping patients whose tumors expressed values above or below the median IOD. For PKCβ1, patients with IODs above the median tended to have poorer survival (median survival of 894 vs. 296 days, $P = 0.064$, $n = 22$, Fig. 1c), whereas no statistically significant survival differences were observed among patients with PKCβ2 IOD levels above or below the median (523 vs. 482 days, $P = 0.552$, $n = 19$, Fig. 1d). Representative microscopic fields are shown in Fig. 1e and f.

Expression of protein kinase C beta and phospho-protein kinase C beta in malignant pleural mesothelioma cell lines

To evaluate whether available MPM cell lines expressed PKCβ, immunoblots were performed utilizing PKCβ1 and PKCβ2 antibodies. A total of eight cell lines were analyzed (seven MPM cell lines and one benign mesothelial cell line, MeT-5A). Expression was consistently stronger for PKCβ1 as compared with PKCβ2, similar to what was observed in patient tumor samples. Cell lines with strongest reactivity included H28, H2461, H2691, and MeT-5A. The same pattern could be observed for PKCβ2 (Fig. 2).

Decreased cell growth of malignant pleural mesothelioma cells with enzastaurin and synergism with cisplatin

Cell growth studies were conducted to evaluate the dose–effect of enzastaurin. In the two MPM cell lines tested, H28 and H2461, enzastaurin significantly decreased cell growth in the micromolar range, with IC50s at approximately 5 μmol/l after 72 h of incubation with drug (Fig. 3a). Importantly, there was no effect of enzastaurin on MeT-5A cells (data not shown, 0–10 μmol/l). Synergism of enzastaurin with cisplatin for MPM cells was also determined utilizing the median-effect analysis [22]; combination indexes at IC50, IC75, and IC90 were 0.46, 0.46, and 0.70, respectively, demonstrating significant synergism (Fig. 3b).

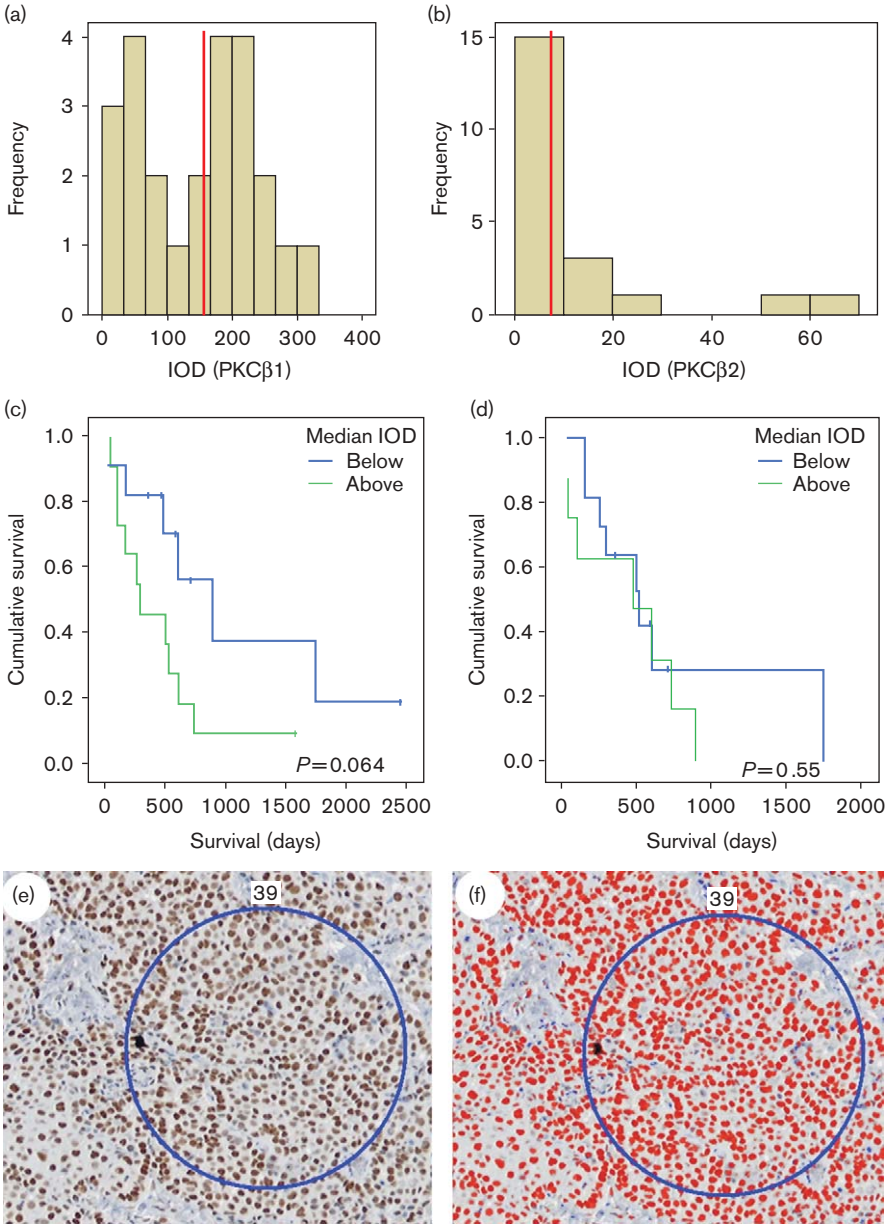
Wound healing assay

To determine the potential biological effects of PKCβ inhibition, cell migration was studied with wound healing assays. The wound areas were quantitated 36 h after the scratch was created and enzastaurin (4 μmol/l) was added. Cells treated with enzastaurin had significantly different areas of reepithelization, revealing that the treated cells had larger wound areas 36 h after treatment, when compared with controls (0.84 and 0.551 mm^2, respectively; $P = 0.002$), a relative reduction of 59.2% in area of reepithelization (Fig. 4a and b).

Inhibition of protein kinase C beta and downstream signaling

We further studied the downstream signaling inhibited by enzastaurin in H2461 cells, with PMA stimulation as positive control. The AKT pathway, an important mechanism for cell survival, was significantly inhibited by enzastaurin, as shown by decreased levels of AKT

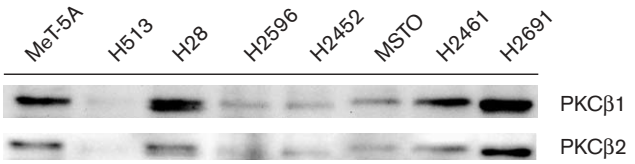
Fig. 1



Immunohistochemical analysis of PKCβ expression and survival analysis in MPM. (a, b) Histogram depicting the distribution of IODs for PKCβ1 (a) and PKCβ2 (b). The vertical red line represents the median values in each group of samples. (c, d) Kaplan–Meier overall survival curves for MPM patients according to PKCβ1 (c) or PKCβ2 expression (d). (e, f) Photomicrographs of MPM tumors, with immunohistochemical staining for PKCβ1 before (e) and after image processing (f) (magnification, ×40). IOD, integrated optical density; MPM, malignant pleural mesothelioma; PKCβ, protein kinase C beta.

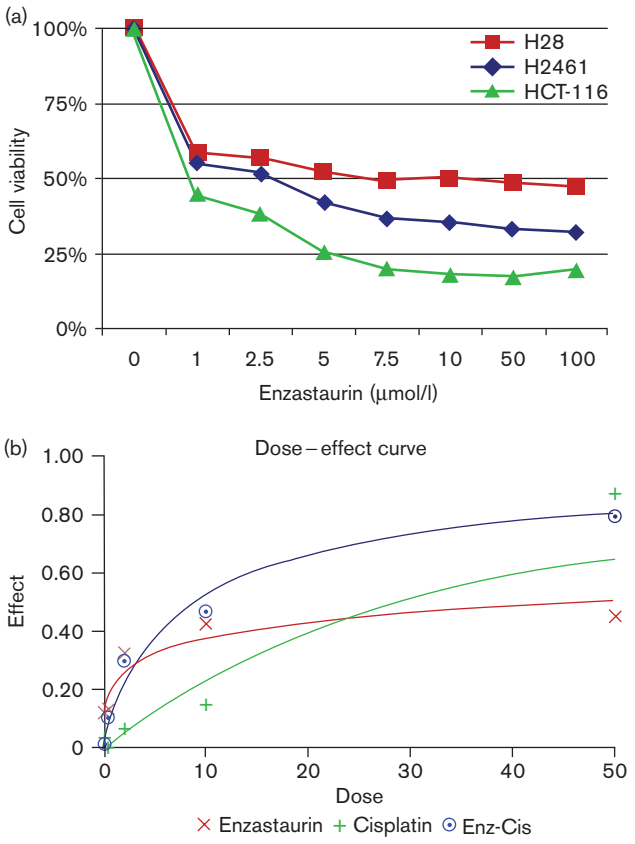
(Ser^{473}), even after treatment with PMA (experiment performed twice, with consistent reproducible results); this effect could be observed in its downstream effectors as well (phospho-GSK3β and phospho-S6). Expression and phosphorylation of focal adhesion molecules, important proteins for cellular migration, were evaluated as well. Enzastaurin decreased phosphorylation of AKT (Ser473) by 30.8%, FAK (Tyr925) by 66.3%, p130Cas (Tyr165) by 67.2%, S6 ribosomal protein (Ser240/244) by 57.8%, and paxillin (Tyr31) by 78.9% (all measurements compared with maximum stimulation with PMA) (Fig. 5a). Immunofluorescence also revealed significant F-actin cytoskeletal changes, such as disorganization of cytoskeletal structure and membranous blebs in more

Fig. 2



Expression of PKCβ in MPM cell lines. Immunoblotting of seven MPM cell lines and one benign mesothelial cell line (MeT-5A), stained with PKCβ1 and PKCβ2 antibodies, revealing variable expression of PKCβ among MPM cell lines. MPM, malignant pleural mesothelioma; PKCβ, protein kinase C beta.

Fig. 3



Enzastaurin's effects on growth of MPM cell lines. (a) Curves of serum-starved MPM cell lines after treatment with enzastaurin at increasing concentrations for 72 h. HCT-116 was used as a positive control. IC50s observed were approximately 5 μmol/l, with no significantly increased effect with increasing concentrations. (b) Dose–effect curves after treatment with enzastaurin, cisplatin, or both for 72 h, at a 1 : 1 ratio. The ordinate axis indicates the dosage of each individual drug. The drug combination displayed significant synergism, with combination indices at IC50, IC75, and IC90 below 1. MPM, malignant pleural mesothelioma.

than 80% of visualized cells, and decreased phosphorylation of p130Cas when cells were exposed to enzastaurin for 24 h (Fig. 5b).

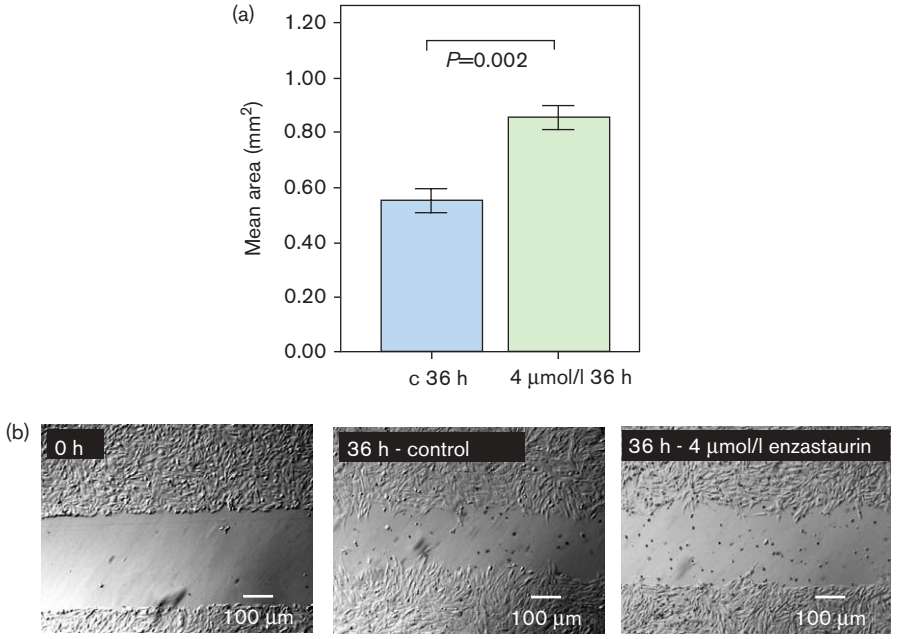
Discussion

MPM is a difficult disease to treat and with an overall poor prognosis. To impact on this disease, new and novel targets have to be identified. In this study, we show that PKCβ was overexpressed in MPM (as compared with normal tissue), and overexpression of PKCβ1 might be associated with an overall poor prognosis. Inhibition of PKCβ with specific small molecule inhibitor enzastaurin led to dose–response growth inhibition of MPM cells and not normal mesothelial MeT-5A cells. Cisplatin is a cytotoxic chemotherapy that is routinely utilized in MPM, and in MPM cells there was synergism of inhibition with cisplatin and enzastaurin. Downstream, enzastaurin led to inhibition of AKT/GSK3b pathways [11], as well as inhibiting the focal adhesion protein phosphorylation. This also led to decreased cell migration with enzastaurin.

Identification of the PKCβ pathway as an important therapeutic target in MPM should ultimately lead to clinical trials in this disease. There have been several novel therapeutic targets that have been evaluated in MPM that have not yielded promising results. The epidermal growth factor receptor (EGFR), a receptor tyrosine kinase overexpressed in 97% of patients with MPM, had been evaluated in a phase II CALGB trial. Treatment with gefitinib, an anti-EGFR small-molecule inhibitor, failed to demonstrate significant activity as a single agent, with responses seen in only 4% of patients, and stable disease observed in another 49%. Median survival was 6.8 months in this trial [23]. Recently, the addition of bevacizumab, an anti-vascular endothelial growth factor antibody, to chemotherapy (gemcitabine and cisplatin) in patients with MPM failed to show improvement in overall survival (15.6 and 14.7 months, nonsignificant) or progression-free survival (6.9 and 6.0 months, nonsignificant) [24]. Our laboratory has shown that c-Met, a receptor tyrosine kinase involved in cellular scattering, invasion, and metastasis, was overexpressed in 82% of MPM tumors, and that targeting c-Met with a small molecule inhibitor is effective *in vitro* [19]. Clinical trials aimed at evaluating this molecule as a target in MPM are currently being planned. It would be interesting, preclinically and ultimately clinically, to determine if there is any synergism between c-Met and PKCβ inhibition in MPM for the future.

PKC expression has been demonstrated in several tumors, including B-cell lymphomas [7], colon [25], and non-small cell lung cancers (71% of samples) [26]; in bladder cancers, PKCβ2 expression has been shown to be dependent upon tumor grade and stage, with tumors with higher grade and stage having lower expression, whereas the opposite was true for PKCζ [27]. In addition, elevated expression of PKCβ2 in colonic epithelium induces cellular hyperproliferation; [28] transgenic mice expressing PKCβ2 in the colonic epithelium displayed

Fig. 4



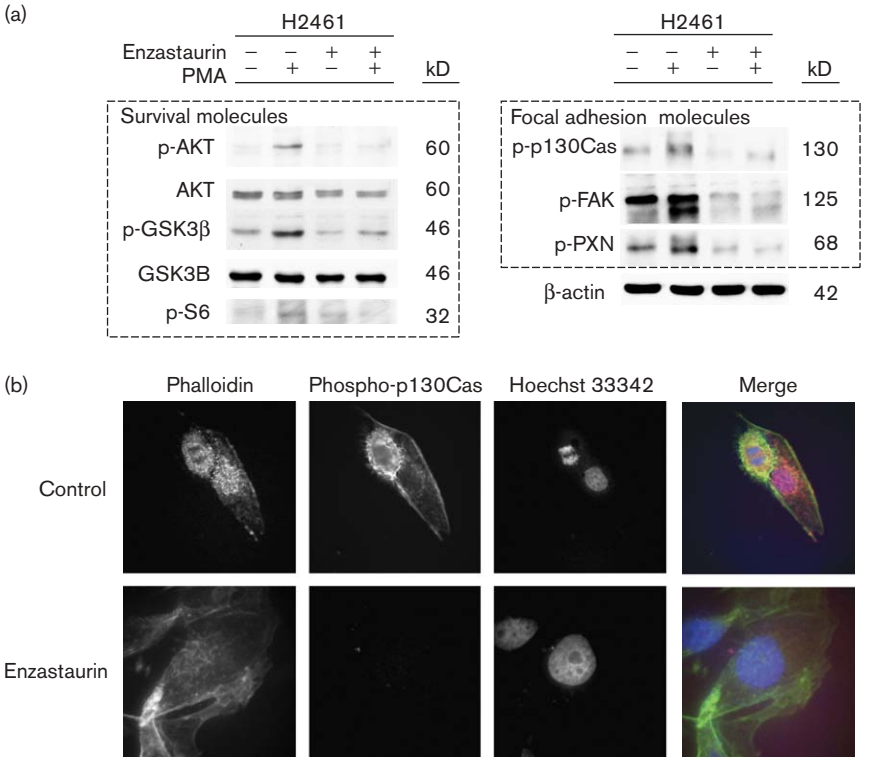
Inhibition of cell motility by enzastaurin, as assessed by wound healing assay. (a) Graphic of the mean areas (mm^2) free of reepithelization in control (c 36 h) and enzastaurin-treated samples (4 µmol/l – 36 h), with statistically different values (error bars represent standard error of the mean of three measured fields per group). (b) Representative photomicrographs of wound healing assay at baseline (0 h) and 36 h after creation of wound and initiation of treatment conditions in control (36 h – control) and enzastaurin-treated samples (36 h – 4 µmol/l enzastaurin) (magnification, × 10).

an increased propensity to development of colon tumor formation [25]. Interestingly, in mesothelial cells, inhibition of PKCα prevented asbestos-induced c-fos and c-jun proto-oncogene expression, therefore proving that inhibition of PKC may have a role in disrupting asbestos-induced oncogenic signaling. Furthermore, PKCβ2 localization to the cellular membrane predicts for worse overall survival in diffuse large B-cell lymphoma (14 vs. 64%, $P = 0.005$), confirmed in multivariate analysis as being the strongest adverse prognostic factor [29]. It would now also be interesting to determine the other isoforms of PKC expression in MPM.

Several strategies to inhibit PKC have been attempted. Agents with a broad spectrum against PKC isoforms (in particular α and β) have been evaluated, such as PKC412 in metastatic melanoma *in vivo* [30] and UCN-01 (7-hydroxystaurosporine) against breast cancer *in vitro* [31]. UCN-01 also was shown to abrogate DNA damage-induced S and G(2) arrest, making p53-mutant cells more susceptible to cytotoxicity [32]. Go6976, an inhibitor against isoforms α and β of PKC, has been shown to have direct effects on checkpoint kinases Chk1 and Chk2, and was able to enhance cytotoxicity of the topoisomerase inhibitor SN-38, a metabolite of irinotecan [33]. An antisense oligodeoxynucleotide against PKCα, ISIS3521 (aprinocarsen, LY9000003), has been clinically evaluated in a phase III study in combination with carboplatin and paclitaxel in patients with advanced non-small cell lung cancer. This study failed to reveal significant improvement in survival (10.0 vs. 9.7 months), time to progression (4.7 vs. 4.5 months), and both arms had equal response rates as well as toxicity rates [34].

Enzastaurin (LY317615.HCl) is the newest specific PKCβ small molecule inhibitor. Enzastaurin also leads to considerable angiogenesis inhibition, through modulating the PKCβ in endothelial cells [35]. In addition, treatment with paclitaxel or carboplatin followed by enzastaurin produced two-fold to three-fold tumor growth delays of small cell lung cancer cells [36]. One important aspect of PKCβ is its involvement in resistance to chemotherapy; in an in-vitro experiment, inhibition of PKCβ in combination with chemotherapy was able to reverse drug resistance; given that the function of P-glycoprotein 170 (Pgp-170), an important mediator of multidrug resistance, is dependent upon PKC phosphorylation, it has been shown that primary resistance to paclitaxel could be reversed by concomitant treatment of ovarian cancer cells with antisense nucleotides against PKCα and PKCβ [37]. This makes enzastaurin a particularly interesting agent to study in combination with established chemotherapy regimens in other tumor types, in particular MPM.

Fig. 5



Effects of enzastaurin on downstream signaling molecules in H2461 MPM cell line. (a) Immunoblotting of samples after exposure to enzastaurin for 3 h, followed by stimulation with the PKC agonist PMA. Appropriate negative controls are shown. Enzastaurin was able to abrogate PMA-induced phosphorylation of key molecules involved in the AKT pathway and focal adhesion complex. Beta-actin is shown as loading control. (b) Immunofluorescence microscopy showing cellular effects of enzastaurin on the cytoskeleton (phalloidin) and phosphorylation of p130Cas. Nuclei are counterstained with Hoechst 33342 ($\times 60$). MPM, malignant pleural mesothelioma; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate.

Focal adhesion proteins play an important role in cancer cell growth and invasion. FAK is a substrate for Src, an oncogene which confers anchorage-independent growth on chicken embryo fibroblasts [38]. These pathways play a major role in cancer cell migration [39,40], invasion [41], and metastasis [42]. Recently, Cheng and colleagues demonstrated that the mechanism by which PKC may regulate the actin cytoskeleton involves the Src suppressed C kinase substrate (SSeCKS) [43]. SSeCKS colocalized with FAK, and by suppressing SSeCKS with small interfering RNAs, the formation of actin stress fibers and focal adhesions was inhibited. Although enzastaurin does not directly inhibit FAK or its downstream targets, it has been shown that stimulation of PKC with phorbol ester increases phosphorylation of FAK, whereas inhibition of PKC suppresses the formation of adherens junctions in epithelial cells [44]. Our results further corroborate that indeed inhibition of PKCβ is sufficient to decrease phosphorylation of key molecules in the focal adhesion, such as FAK, p130Cas, and paxillin, supporting the notion that PKCβ blockage affects multiple cellular functions. Further studies need to be performed to verify *in vivo* whether these changes translate into decreased tumor growth, invasion, and metastasis.

In summary, we have shown that targeting PKCβ is an effective strategy in an in-vitro MPM model. The cytoskeletal changes associated with enzastaurin treatment might indicate that tumor invasion might be minimized by this strategy. Our future goals will be to further these studies to evaluate the mechanisms by which enzastaurin synergizes with cisplatin and other drugs, including small molecule tyrosine kinase inhibitors.

Acknowledgements

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