Biochemical Pharmacology 81 (2011) 251–258

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The anti-tumoral drug enzastaurin inhibits natural killer cell cytotoxicity via activation of glycogen synthase kinase-3β

Henry Ogbomo [1], Tsigereda Biru [1], Martin Michaelis, Nadine Loeschmann, Hans Wilhelm Doerr, Jindrich Cinatl Jr.*

Institut für Medizinische Virologie, Klinikum der Johann Wolfgang Goethe-Universität, Paul-Ehrlich-Str. 40, 60596 Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 9 August 2010
Accepted 28 September 2010

Keywords:
Antibody-dependent cellular cytotoxicity
Natural cytotoxicity
NKG2D
Protein kinase Cβ
Glycogen synthase kinase-3β

ABSTRACT

Enzastaurin is a selective protein kinase Cβ inhibitor which is shown to have direct antitumor effect as well as suppress glycogen synthase kinase-3β (GSK-3β) phosphorylation (resulting in its activation) in both tumor tissues and peripheral blood mononuclear cells (PBMC). It is currently used in phase II trials for the treatment of colon cancer, refractory glioblastoma and diffuse large B cell lymphoma. In this study, the direct effect of enzastaurin on effector function of human natural killer (NK) cells was investigated. The results obtained showed that enzastaurin suppressed both natural and antibody-dependent cellular cytotoxicity (ADCC) of NK cells against different tumor targets. This inhibition was associated with a specific down-regulation of surface expression of NK cell activating receptor NKG2D and CD16 involved in natural cytotoxicity and ADCC respectively, as well as the inhibition of perforin release. Analysis of signal transduction revealed that enzastaurin activated GSK-3β by inhibition of GSK-3β phosphorylation. Treatment of NK cells with GSK-3β-specific inhibitor TDZD-8 prevented enzastaurin-induced inhibition of NK cell cytotoxicity. Apart from the known antitumor and antiangiogenic effects, these results demonstrate that enzastaurin suppresses NK cell activity and may therefore interfere with NK cell-mediated tumor control in enzastaurin-treated cancer patients.

1. Introduction

Enzastaurin (LY317615.HCl), an acyclic bisindolylmaleimide, is a selective orally administered protein kinase Cβ (PKCβ) inhibitor. Although initially developed to selectively inhibit PKCβ at nanomolar concentrations (IC_{50} of 6 nmol/l), enzastaurin was demonstrated to also inhibit other PKC isoforms including PKCγ, PKCδ, PKCθ, PKCε, PKCξ, PKCη, PKCζ and PKCα by varying degrees (up to 90% inhibition) and at higher micromolar concentrations [1]. In preclinical studies, enzastaurin suppressed signaling through the AKT pathway, induced apoptosis, and suppressed growth of human colon cancer and glioblastoma xenografts [1]. In addition, enzastaurin inhibited glycogen synthase kinase 3 (GSK3) phosphorylation both in tumor tissues and peripheral blood mononuclear cells (PBMC) from treated mice [1]. It is currently used in phase II trials for the treatment of colon cancer, refractory glioblastoma and diffuse large B cell lymphoma [2,3].

The activation of PKC has been demonstrated to be required for natural killer (NK) cell [4,5] and interleukin 2 (IL-2) activated lymphocytes [6] killing of target cells. Also, PKC activation triggers signaling through the phosphoinositide 3-kinase (PI3K)/AKT and GSK3 pathway [7]. Recently, GSK3 was identified as a crucial regulator of innate inflammatory process, regulating components of the interferon (IFN-γ) signaling pathway in macrophages [8]. In addition, GSK3 was shown to be a potential regulator of NK cell cytotoxicity and IFN-γ secretion [9].

NK cells are vital components of the innate immune response that are specialized in attacking abnormal cells such as virus-infected cells and transformed tumor cells. NK cells are armed with an array of activating receptors that trigger cytolytic granules and cytokine production. Besides expression of several receptors that mediate natural cytotoxicity, NK cells also express CD16 which mediates antibody-dependent cellular cytotoxicity (ADCC) against IgG-coated target cells [10–13]. While NK cell activating receptors like NKp30, NKp44, NKp46, and CD16 associate with the transmembrane adaptors CD3ζ, FcRγ, or DAP12 that contain a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) [14], NKG2D associates with the adaptor protein DAP10 which contains a cytoplasmic YINM motif [15–19]. The ligation or antibody dependent cross linking of the ITAM-dependent receptors initiates NK cell signal transduction cascade by phosphorylation of tyrosine residues located within conserved ITAM sequences

* Corresponding author. Tel.: +49 69 6301 6409; fax: +49 69 6301 4302.
E-mail addresses: hogbomo@ucalgary.ca (H. Ogbomo), tsigereda76@yahoo.com (T. Biru), michaelis@em.uni-frankfurt.de (M. Michaelis), n.loeschmann@kinderkrebsstiftung-frankfurt.de (N. Loeschmann), h.w.doerr@em.uni-frankfurt.de (H.W. Doerr), cinatl@em.uni-frankfurt.de (J. Cinatl Jr.).
[1] Both authors contributed equally.

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.09.026

through Src protein tyrosine kinase (PTK) family [20]. These in turn allows the consequent recruitment and activation of Syk and/or ZAP70 PTK and the transmembrane adaptor proteins, linker for activation of T cells, LAT and LAT2 [20,21], further leading to the recruitment and activation of downstream effector molecules such as PI3K [5,22,23], the phospholipases PLCγ1 and PLCγ2 [24–27], the guanosine triphosphate-guanosine diphosphate exchange factors Vav2 and Vav3, [28], the extracellular signal-regulated kinase (ERK) [29], and finally triggers both cytotoxicity and cytokine production [20]. On the other hand, ligation of DAP10 dependent receptor (NKG2D) directly recruits PI3K and Grb2-Vav1 complex [15–19], triggering cytotoxicity but not cytokine production [30,31].

Since enzastaurin effectively inhibits phosphorylation of PKC, AKT and GSK3 [1], all pathways necessary for NK cell activation, we therefore investigated the direct effect of enzastaurin in NK-mediated cytotoxicity (natural and antibody-dependent) and cytokine secretion as well as the influence of enzastaurin on signaling pathways involved in NK cell activity. We observed that enzastaurin 1) inhibits NK cell cytotoxicity (both natural and antibody-dependent), 2) specifically suppresses the expression of NKG2D and CD16 in primary NK cells, 3) impairs perforin release without affecting levels of intracellular perforin expression in NK cells and 4) activates GSK-3β in IL-2-stimulated NK cells by inhibition of GSK-3β phosphorylation.

2. Materials and methods

2.1. Reagents and monoclonal antibodies

Enzastaurin was purchased from Biozol Diagnostica Vertrieb GmbH (Eching, Germany), TDZD-8 was obtained from Enzo Life Sciences GmbH (Loerrach, Germany), recombinant human IL-2 was from Cell Concepts (Umkirch, Germany). The following phycoerythrin-conjugated anti-human monoclonal antibodies (mAbs) were used: CD16, NKp30, NKp44, NKp46, NKG2D, DNAM-1, KIR- KIR2DL2/DL3 and KIR3DL1, all from Miltenyi Biotec (Bergisch Gladbach, Germany), perforin and granzyme B were from Abcam (Cambridge, UK). Cetuximab (Erbitux) was from Merck Pharma GmbH (Darmstadt, Germany).

2.2. Cells

A431, K562 and NK-92 cell lines were obtained from American type culture collection (ATCC; Manassas, VA). NK-92 cell line was grown in X-VIVO™ 10 Medium (Lonza Verviers, Belgium) supplemented with 5% human plasma and 100 U/ml recombinant IL-2. K562 cell line was grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% fetal calf serum (FCS), while A431 cell line was grown in IMDM supplemented with 10% FCS. Media and supplements were from Seromed (Berlin, Germany).

2.3. Polyclonal NK cell preparation

Buffy coats from healthy blood donors were obtained from the Frankfurt Red Cross Blood Bank (Frankfurt, Germany). PBMC were isolated from buffy coats by Ficoll-Hypaque density gradient centrifugation followed by separation using the MACS NK cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol. Flow cytometric analysis to determine purity of NK cells showed that more than 95% of the cells were CD56+ CD3− (not shown). NK cells were cultured using IMDM containing 10% FCS, with or without 100 U/ml recombinant IL-2. For all experiments (unless otherwise indicated), IL-2-activated NK cells were simultaneously treated with or without enzastaurin at indicated concentration for 120 h.

2.4. Cytotoxicity assay and flow cytofluorometric analysis

Cytotoxicity of NK cells was determined by a 4 h coupled luminescent method using the "ACELLA-TOX™" kit (Cell Technology, Mountain View, CA), as described [32]. K562 and A431 cell lines were used as target cells. For ADCC experiments, target cells were pre-coated with 0.5 μg/ml cetuximab mAb for 30 min before coculture with NK cells. Flow cytometry (FACS Calibur; Becton Dickinson, Mountain View, CA) was used for cell surface and intracellular molecule expression as well as for DNA content analyses. Controls for all experiments performed contained vehicle (maximum 0.1% DMSO) which did not influence NK cell activity when compared with cultures without vehicle (not shown).

2.5. Cell cycle analysis

Cell cycle was determined using a commercial kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions as described previously [33].

2.6. Western blot

Cells were cultured in IL-2 and in the presence or absence of enzastaurin for 120 h. Afterwards, Western blot analysis was performed as previously described [34]. Briefly, cell lysates were subjected to SDS-PAGE before transfer to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using the Mini-Protean II system (Bio-Rad, Munich, Germany). After transfer, blots were blocked in tris-buffered saline (TBS) blocking buffer containing 3% bovine serum albumin for 1 h at room temperature to saturate the non-specific protein-binding sites on the nitrocellulose membrane. The following primary rabbit polyclonal Abs were used: GSK-3β, phospho-GSK-3β^{ser9}, AKT, phospho-AKT^{Ser473}, phospho-AKT^{Thr308}, ribosomal protein S6, phospho-S6$^{S240/244}$, ribosomal p70S6 kinase (p70S6K), phospho-p70S6K$^{Ser424/Thr421}$, phospho-p70S6K^{Thr389}, ERK1/2, phospho-ERK1/2, p38, phospho-p38—all from Cell Signaling (Beverly, MA), mouse polyclonal beta actin Ab was from Sigma–Aldrich (Taufkirchen, Germany). The blots were incubated overnight with the primary Ab diluted in TBS at 4 °C with gentle agitation. Following a 1 h incubation period with peroxidase-conjugated secondary Ab at room temperature visualization was performed by enhanced chemiluminescence using a commercially available kit (Amersham, Liverpool, UK).

2.7. Measurement of IFN-γ production, perforin and granzyme B release

A total of 2×10^4 K562 cells were cocultured for 24 h with 1×10^5 NK cells cultured in IL-2 and in the presence or absence of enzastaurin. Both enzastaurin-treated and non-treated IL-2-activated NK cells alone or K562 cells were used as control. Supernatants were collected and tested for production of IFN-γ, perforin and granzyme B release. The amounts of IFN-γ were determined using the Quantikine Human IFN-γ ELISA kit (R&D Systems, Wiesbaden, Germany) while perforin and granzyme B release were measured using Perforin/Granzyme B-ELISA kit (Diaclone Research, Besancon Cedex, France) according to manufacturers protocol.

2.8. Statistics

Values presented are the mean ± standard error of mean (S.E.M.) of at least three experiments. Comparisons between two groups were

performed using Student's *t*-test. *P*-values lower than 0.05 were considered to be significant.

2.9. Ethics statement

For this study, ethical approval/ethics statement and informed consent is not required (not applicable).

3. Results

3.1. Effect of enzastaurin on viability of NK cells

We first studied effect of enzastaurin at clinically relevant concentrations [35] (0.625–2.5 μM) on viability of NK cells. For this purpose, NK cells were cultured simultaneously for 120 h with IL-2 and enzastaurin. Dead cells were identified by fractional DNA content (''sub-G1 fraction''). NK cells treated with IL-2 alone were used as control. Results reveal enzastaurin to be non-toxic to both primary NK cells and NK-92 cell line in the range of therapeutic concentrations (Fig. 1). Also, NK cell and NK-92 cell proliferation were not affected by enzastaurin concentrations up to 2.5 μM (not shown).

3.2. Enzastaurin suppresses NK cell cytotoxicity without interfering with IFN-γ production

The role of enzastaurin on NK cell cytotoxicity was next investigated. NK cells were treated simultaneously for either 18 h or 120 h with IL-2 and enzastaurin concentrations ranging from 0.625 μM to 2.5 μM, after which the cytotoxicity experiment against K562 cells was performed. The results obtained show that enzastaurin significantly suppressed lysis of K562 in a dose-dependent manner only in 120 h treated NK cells (Fig. 2A). The % inhibition of primary NK in comparison to control were 26.8%, 35.7%, and 44.7% respectively for 0.625, 1.25, and 2.5 μM enzastaurin at an effector/target (E/T) ratio of 4:1. A similar trend of inhibition was observed for NK-92 cell line in comparison to control (54.2%, 83% and 100% respectively for concentration of 0.625, 1.25, and 2.5 μM enzastaurin at E/T ratio of 5:1) (Fig. 2B). Since NK cell cytotoxicity was inhibited upon simultaneous addition of IL-2 and enzastaurin for 120 h, we tested the effect of enzastaurin on pre-activated primary NK cells after 18 h enzastaurin treatment. Here, primary NK cells were stimulated for 96 h with IL-2 (100 U/ml) prior to addition of 2.5 μM enzastaurin. Results show a 58% reduction in lysis of K562 target cells in enzastaurin-treated primary NK cells compared to untreated control (E/T ratio 4:1). The production of INF-γ by IL-2-activated NK cells was also measured upon coculture of NK cells and enzastaurin-treated NK cells with K562 target cells. Enzastaurin did not suppress IFN-γ production in IL-2-activated NK cells (not shown).

3.3. Enzastaurin interferes with NK-mediated ADCC

Apart from natural cytotoxicity, primary NK cells also mediate ADCC since they express high amounts of CD16 on their cell surface. We investigated whether enzastaurin has any influence on ADCC. For this purpose, primary NK cells treated simultaneously for 120 h with IL-2 and 2.5 μM enzastaurin, were used in a cytolytic experiment against epidermal growth factor receptor (EGFR)-expressing tumor cell line A431 [36]. The cell line was coated with the EGFR-specific antibody cetuximab before coculture with NK cells. The result obtained show that while A431 cells were resistant to natural cytotoxicity mediated by IL-2-activated NK cells, the cells were sensitive to IL-2-activated NK- mediated ADCC. Enzastaurin however caused 40% inhibition of NK cell-mediated ADCC of A431 cell line (Fig. 2C).

3.4. Enzastaurin down-modulates NK cell activating receptor expression

NK cell cytotoxicity is critically dependent on signaling through its receptors, leading to granule polarization and exocytosis. Accordingly, it is conceivable that enzastaurin might interfere with NK cell receptor expression pattern. To address this, we investigated the surface expression patterns of NK cell triggering receptors CD16, NKp30, NKp44, NKp46, NKG2D and DNAM-1, as well as NK cell inhibitory receptors KIR3DL1 and KIR2DL2/DL3 in non-treated and enzastaurin-treated NK cells. A correlation between NK cell cytotoxicity and NK cell receptor expression

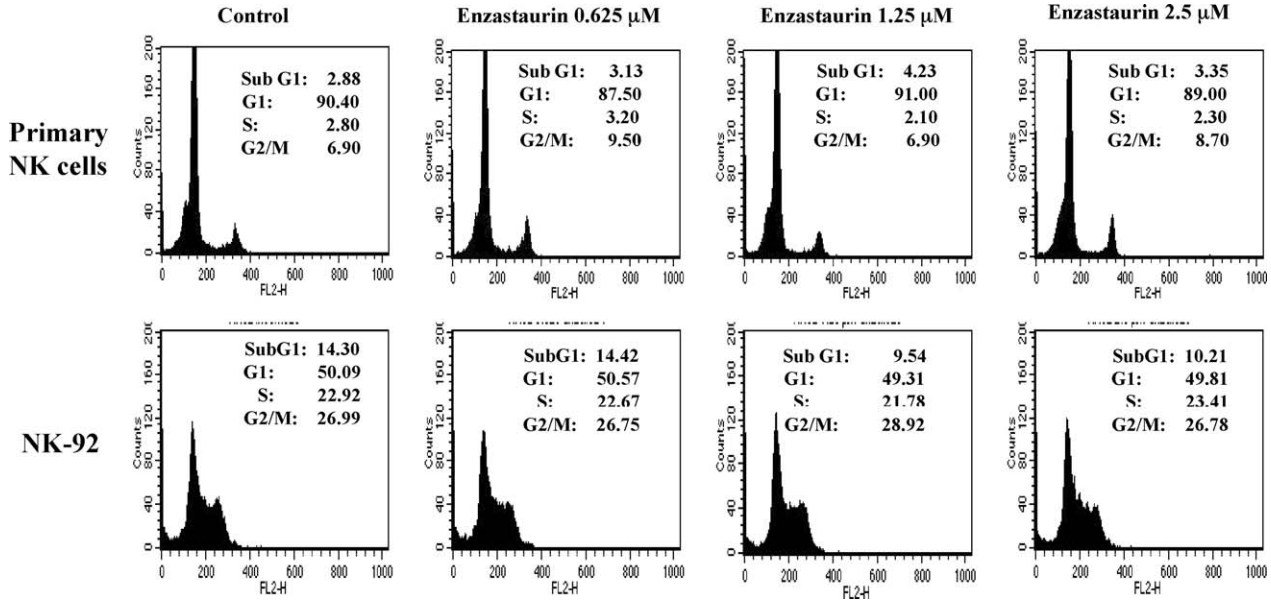

Fig. 1. Effect of enzastaurin on primary natural killer (NK) cell and NK-92 cell cycle. Values represent percentage of cells in the different phases. The percentages of cells in G1, S and G2/M phases were deduced from the number of viable cells (set to 100%) after subtracting the dead cells (sub G1) from total gated cells. One representative of 3 different experiments is shown.

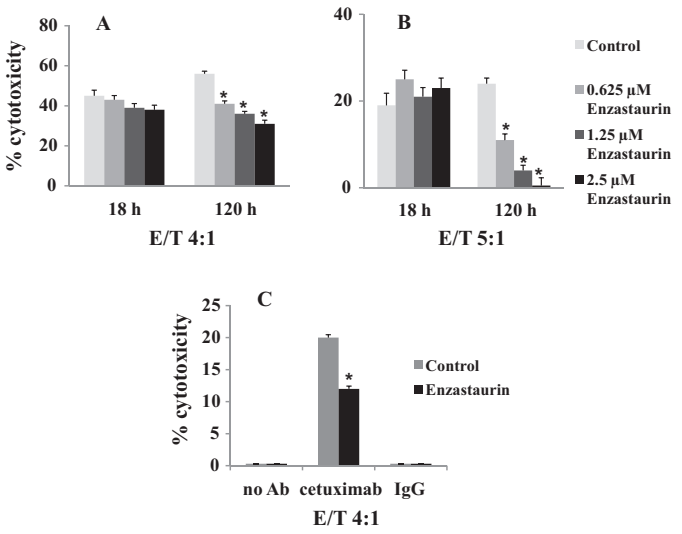

Fig. 2. Effect of enzastaurin on primary natural killer (NK) cell and NK-92 cytotoxicity. NK cell cytotoxicity of A) primary NK cells and B) NK-92 cells treated with enzastaurin at indicated time points against 5000 K562 target cells was determined in a 4 h cytolytic assay. C) IL-2-activated NK cell-mediated ADCC of non-treated (control) NK cells as well NK cells treated simultaneously with IL-2 and 2.5 μM enzastaurin for 120 h; EGFR-expressing A431 cells and A431 cells coated with 0.5 μg/ml cetuximab or IgG were used as target cells. Columns represent means of triplicate of one representative experiment; error bars indicate ± S.E.M.; $^{*}P < 0.05$ relative to control.

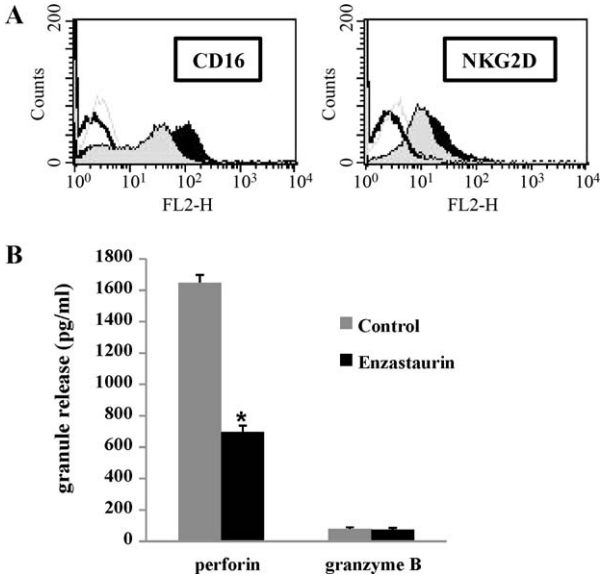

Fig. 3. Enzastaurin suppresses natural killer (NK) cell receptor expression and granule release. NK cells were treated with IL-2 and in the presence or absence of 2.5 μM enzastaurin for 120 h. A) Flow cytometric analysis for the expression of NK triggering receptors NKG2D and CD16. Grey line and grey filled histogram indicate isotype control and antibody staining respectively for enzastaurin-treated NK cells; black line and black filled histogram indicate isotype and antibody staining respectively for non-treated NK cells. One representative of at least 3 separate experiments is shown. B) Enzastaurin-treated and non-treated NK cells were cocultured with K562 target cells. Afterwards, supernatant was collected and analyzed by ELISA for granule release. Columns represent means of triplicate of one representative experiment; error bars indicate ± S.E.M.; $^{*}P < 0.05$ relative to control.

pattern was observed. The lytic capacity of NK cells treated with enzastaurin was associated with a specific inhibition of CD16 (involved in ADCC) and NKG2D (involved in natural cytotoxicity) surface expression (Fig. 3A). The surface expression of NKp30, NKp44, NKp46, KIR3DL1 and KIR2DL2/DL3 were not changed (not shown). Enzastaurin also did not suppress the surface expression of triggering and inhibitory receptors in NK cells cultured simultaneously for 18 h with IL-2 and enzastaurin (not shown). Basically NK cell activating or inhibitory receptor expression levels were 2–3 folds higher in NK cells cultured with IL-2 for 120 h than in NK cells cultured with IL-2 for 18 h.

3.5. Enzastaurin impairs perforin release by NK cells

The cytotoxic granules of NK cells are final mediators of NK cytotoxicity. They become polarized at the interface with target cells and are released into the target cells. We studied the effect of enzastaurin on intracellular perforin and granzyme B expression and release in NK cells. We observed that while the intracellular perforin and granzyme B expression did not change (relative

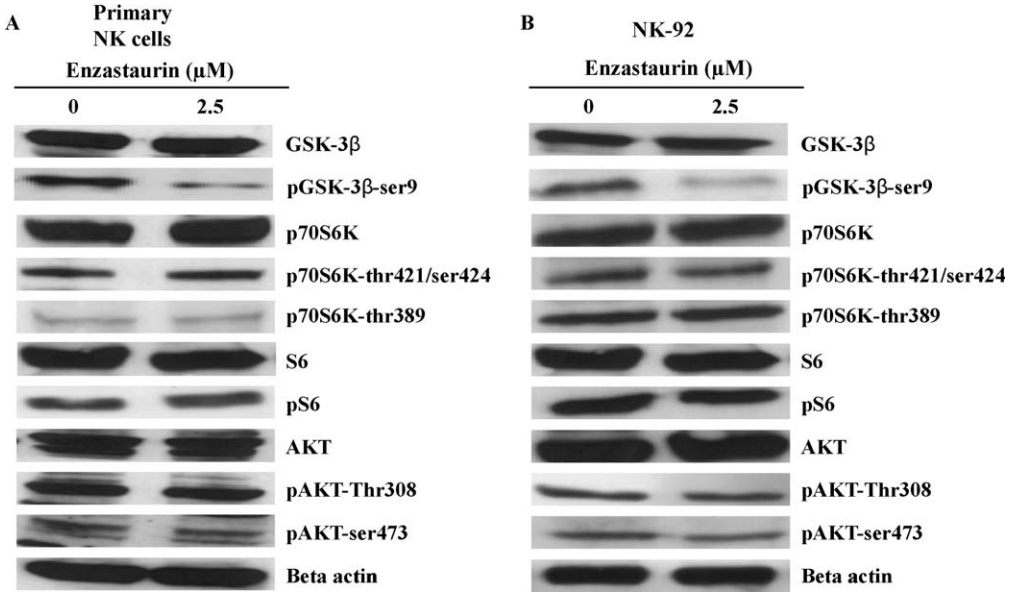

Fig. 4. Enzastaurin inhibits phosphorylation of GSK-3β. A) Primary natural killer (NK) cells and B) NK-92 cells were treated simultaneously with/without IL-2 and enzastaurin for 120 h. The same amount of protein extracts prepared from enzastaurin-treated and non-treated NK cells was used for western blot analysis of the indicated signaling proteins. Data are representative of at least three experiments.

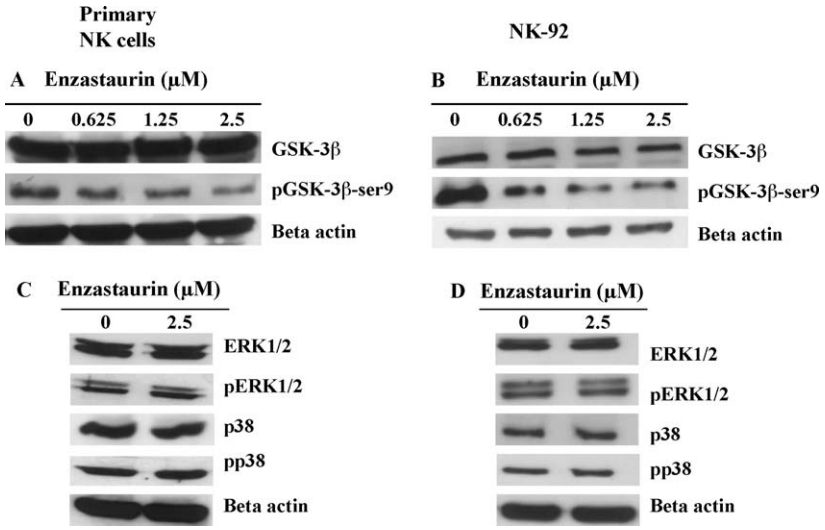

Fig. 5. Enzastaurin dose-dependently inhibits phosphorylation of GSK-3β without affecting ERK1/2 and p38 phosphorylation. Primary natural killer (NK) cells (A and C) and NK-92 cells (B and D) were treated simultaneously with/without IL-2 and enzastaurin for 120 h. The same amount of protein extracts prepared from enzastaurin-treated and non-treated NK cells was used for western blot analysis of the indicated signaling proteins. Data are representative of at least three experiments.

expression: for perforin, 38.82 ± 3 vs. 39.96 ± 4.2; for granzyme B, 154.41 ± 6.3 vs. 146.43 ± 4.2 respectively for IL-2-treated NK cells and NK cells treated simultaneous for 120 h with IL-2 and 2.5 μM enzastaurin), enzastaurin significantly impaired release of perforin by 2.36-fold but had no influence on granzyme B release in cocultures of enzastaurin-treated IL-2-activated NK and K562 compared to non-treated IL-2-activated NK and K562 coculture (Fig. 3B).

3.6. Enzastaurin directly suppresses GSK-3β (ser^9) phosphorylation

Enzastaurin has been reported to induce apoptosis in several tumors by interfering with signaling through the PI3K/AKT pathway [1,37,38]. We investigated by western blot the influence of enzastaurin on AKT activity and its downstream effectors in NK cells. We observed that although enzastaurin did not influence the phosphorylation of AKT, ribosomal protein S6, and the ribosomal p70S6K (Fig. 4A and B) it specifically and dose-dependently suppressed GSK-3β phosphorylation in IL-2-activated primary NK cells (Fig. 5A) and NK-92 cell lines (Fig. 5B). MAPK pathways have also been shown to be affected by enzastaurin. Depending on the cell type used contrasting reports exist for the effect of enzastaurin on MAPK signaling. While Guo et al. 2008 [39] revealed that enzastaurin suppressed ERK1/2 and p38 but not JNK phosphorylation in hepatocellular carcinoma, Moreau et al. 2007 [40] showed an induction of ERK1/2 phosphorylation by enzastaurin in Waldenström macroglobulinemia. Since ERK1/2 and p38 may play a role in NK cell activation [41,42] we tested by western blot whether enzastaurin treatment has influence on ERK1/2 and p38 activation in primary NK cells and NK-92 cell line. Results show that enzastaurin does not affect the phosphorylation of ERK1/2 and p38 in both types of NK cells (Fig. 5C and D).

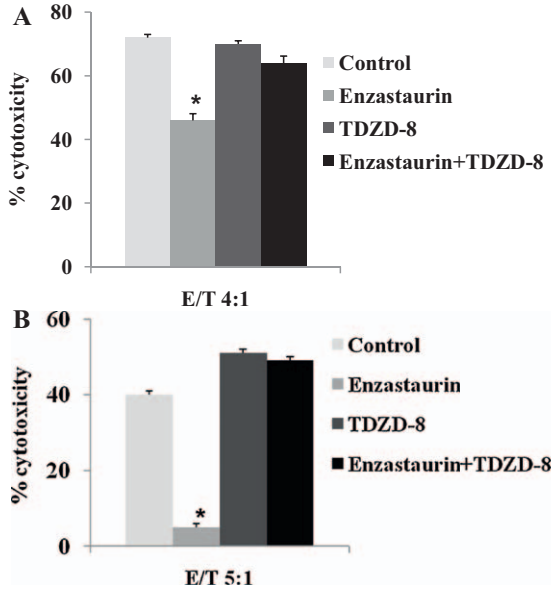

Fig. 6. GSK-3β-inhibitor TDZD-8 prevents enzastaurin-induced inhibition of natural killer (NK) cell lysis. Primary NK cells (A) and NK-92 cells (B) were treated with IL-2 alone or in single combination with either 2.5 μM enzastaurin or 1 μM TDZD-8 or both. Cytotoxicity experiment was performed using K562 as target cells. Columns represent means of triplicate of one representative experiment; error bars indicate ± S.E.M.; $^*P < 0.05$ relative to control.

3.7. TDZD-8 prevents enzastaurin-induced inhibition of NK cell lysis

A previous report indicated that inactivation of GSK-3 using the specific GSK-3β-inhibitor TDZD-8 increased the cytotoxicity of NK cells [9]. We therefore simultaneously treated NK cells with IL-2, TDZD-8 and enzastaurin for 120 h and performed cytolytic experiments using K562 as target cells. Although treatment with TDZD-8 alone did not significantly influence target cell lysis by NK cells, combined treatment of NK cells with TDZD-8 and enzastaurin prevented the lytic inhibitory role of enzastaurin (Fig. 6A and B). Also, combined treatment enhanced granzyme B release (from 79 pg/ml in non-treated NK cells to 142 pg/ml in enzastaurin-treated NK cells), and partially restored perforin release to levels higher than in NK cells treated with enzastaurin alone (from 697 pg/ml to 1002 pg/ml). The inhibition of NKG2D and CD16 expression were however not prevented by the combined treatment (not shown).

4. Discussion

Enzastaurin is a selective PKCβ inhibitor that is under clinical investigation as anti-cancer drug in phase II trials [1–3]. The PKC family of serine-threonine protein kinases have not only been implicated in the processes that control tumor cell growth,

survival, and progression [43], but also in the processes essential for NK cell function [4–6,20,44–46]. PKC activation can trigger signaling through the ras/ERK pathway [47] and/or the PI3K/AKT pathway [7]. PKCα, PKCβ, and PKCη can also directly phosphorylate AKT at Ser^{473}, which is essential for AKT activity [48–50]. Moreover, both PKC [51,52] and AKT activity [53] can phosphorylate GSK-3β at Ser^{9}, indicating an overlap in these signaling pathways. The phosphorylation of GSK-3β has been described to serve as a reliable pharmacodynamic marker for enzastaurin activity [1].

In this report, we demonstrate for the first time that clinically achievable enzastaurin concentrations activate GSK-3β in human NK cells by specifically suppressing GSK-3β phosphorylation. Interestingly, enzastaurin had no effect on the phosphorylation of cell proliferation- and cell survival-molecules including AKT, S6, and p70S6K. This finding is in line with data showing that PKCβ may directly phosphorylate GSK-3β without involvement of AKT [51]. In this context, it appears also not to be too much surprising that phosphorylation of p70S6K and its downstream S6 is not modulated after enzastaurin treatment. P70S6K is a substrate of GSK-3β as well as of AKT and sole inhibition GSK-3β may not be sufficient to affect p70S6K signaling as long as AKT remains unaffected.

NK cell proliferation and viability was not affected by enzastaurin treatment. Enzastaurin exerts its antitumoral effects mainly through cytotoxic and antiproliferative activity [54]. In non-stimulated preparations of normal human PBMCs (of which NK cells are constituents) and in T cells, enzastaurin was found to be non-toxic in the investigated concentrations up to 50 μM [40,55,56]. Stimulation of lymphocytes with cytokines or antigens may activate PKCß and render them more sensitive to cytotoxic and antiproliferative effects of the drug. However, we did not observe antiproliferative or toxic effects of enzastaurin on IL-2-stimulated NK cells at therapeutic relevant concentrations ranging from 0.625 μM to 2.5 μM. Notably, enzastaurin concentrations higher than 2.5 μM were required to affect cell proliferation and viability of some tumor cell types with highly activated PKCβ [38,40]. However, enzastaurin concentrations between 0.625 μM and 2.5 μM inhibited dose-dependently GSK3β phosphorylation in NK cells indicating an uncoupling of anti-proliferative effects of enzastaurin from effects on GSK3β signaling. Similar observations were made in lymphoma cells treated with 2.5 μM enzastaurin which nearly completely abrogated GSK-3β phosphorylation but did not influence cell proliferation [40].

Our results further show that enzastaurin inhibits NK-mediated natural cytotoxicity and NK-mediated ADCC of different tumor targets. Additionally, enzastaurin impaired NK cell potential in releasing perforin but had no effect on granzyme B release. Combined treatment of NK cells with a GSK-3β-specific inhibitor TDZD-8 and enzastaurin prevented the lytic inhibitory role of enzastaurin by enhancing granzyme B release and partially restoring perforin release when compared with non-treated or enzastaurin-treated NK cells. In contrast to a report by Aoukaty and Tan [9] demonstrating that inactivation (phosphorylation) of GSK-3 with TDZD-8 increased NK cytotoxicity and IFN-γ secretion, we did not observe any influence of TDZD-8 on NK cell cytotoxicity. The reasons for this difference observed by Aoukaty and Tan [9] and us remain obscure. However, there is a distinct difference in the experimental procedure applied that may contribute to the varying results. While Aoukaty and Tan [9] treated IL-2-activated NK cells with TDZD-8 for only 30 min in serum free medium, we treated NK cells simultaneously with IL-2 and TDZD-8 for 120 h and in a medium that contained serum.

The activity of enzastaurin has been profiled in tumor cells using in vitro kinase assays [1]. The authors demonstrated that enzastaurin inhibited PKCβ and other PKC isoforms including PKCγ, PKCδ, PKCθ, PKCε, PKCξ, by approximately 90% or more but showed no substantial inhibition of PKCμ and PKCι. PKCη and PKCζ were also inhibited by nearly 50% while PKCα was inhibited by 77% [1]. Moreover, different PKC isoforms were shown to phosphorylate GSK3 [51,57–59]. Therefore, it is conceivable that PKC isoforms other than PKCβ are also inhibited by enzastaurin and contribute to impaired NK functions through effects on GSK3.

Also, specific PKC isoenzymes are known to regulate different cellular functions in stimulated lymphocytes: PKCα and PKCθ are involved in the expression of the IL-2 receptor [60,61], whereas PKCβ, PKCδ and PKCε are involved in IL-2 synthesis and in the IL-2-mediated proliferation of T cells [62,63]. In NK cells, the rapid activation of PKC enables them to mediate natural cytotoxicity towards tumor cells [64,65], and PKC activation is essential for triggering lysis in IL-2-activated NK cells [6]. PKCθ was also demonstrated to be required for NK cell activation and in vivo control of tumor progression [44]. Some other report described the absolute requirement of PKCθ for ITAM-mediated IFN-γ secretion, but without a marked influence on the release of cytolytic mediators [20]. Specifically, the authors showed using PKCθ-deficient murine NK cells that NKG2D engagement does not influence target cell lysis and that CD16-mediated ADCC was only slightly affected [20]. This means that PKCθ is necessarily required for NKG2D and CD16-mediated lysis. Also, PKCβ deficiency was shown to have no influence on LPS-induced cytokine production in murine bone marrow-derived mast cells [66]. In our study, enzastaurin did not suppress IFN-γ secretion but specifically down-regulated the surface expression of NKG2D, an NK activating receptor that is described to trigger cytotoxicity but not cytokine production [30,31]. Taken together, NKG2D-mediated natural cytotoxicity and CD16-mediated ADCC in NK cells appear to require PKCβ for their cytolytic function. Although we did not study how engagement of NKG2D receptor or CD16 activates PKCβ and/or PI3K/AKT signaling or downstream events, signaling via 2B4 (another NK cell activating receptor) was shown to be mediated by GSK-3 [9].

Some studies on the effect of other PKC inhibitors on NK cell activity have been reported. While the PKC inhibitors Gö6976, Rottlerin, and Bisindolylmaleimide III were shown to have no effect on NCR (NKp30, NKp44 and NKp46) expression and NK cell-mediated cytotoxicity, Gö6983 induced a remarkable increase of NCR expression and strongly up-regulated the death ligands FasL and TRAIL on primary human NK cells, leading to enhanced NK cell lysis of target cells [67]. The authors therefore speculated that NK cell activation by Gö6983 occurs via an unknown mechanism other than PKC inhibition. Furthermore, the PKC inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride was shown to inhibit both natural cytotoxicity and ADCC of human NK cells [45]. Similar to some data in these reports, enzastaurin had no effect on NCR expression but impaired NK cell cytotoxicity.

In conclusion, we show for the first time that the PKCβ-specific inhibitor enzastaurin suppresses both ADCC and natural cytotoxicity of human NK cells by inhibiting the expression of NKG2D and CD16 as well as impairment of perforin release. In addition, we show that enzastaurin selectively inhibits GSK-3β phosphorylation (known to be involved in NK cell cytotoxicity) without affecting signaling molecules responsible for cell proliferation and survival (AKT, S6, p70S6K). Although enzastaurin plays vital roles in inhibition of tumor-induced angiogenesis, inhibition of tumor cell proliferation, and induces apoptosis in tumor cells, our data suggest that it also has a negative impact on two crucial NK cell-mediated functions, namely natural cytotoxicity and ADCC. This implies that treatment of tumor patients with enzastaurin may impair their NK cell cytotoxic potential. Careful administration and control of patients undergoing enzastaurin therapy is therefore required.

Acknowledgements

The authors thank Kerstin Euler and Christina Matreux for technical assistance. The work was supported by the friendly society Hilfe für krebskranke Kinder Frankfurt e.V. and its foundation Frankfurter Stiftung für krebskranke Kinder.

References

[1] Graff JR, McNulty AM, Hanna KR, Konicek BW, Lynch RL, Bailey SN, et al. The protein kinase Cbeta-selective inhibitor, Enzastaurin (LY317615. HCl), suppresses signaling through the AKT pathway, induces apoptosis, and suppresses growth of human colon cancer and glioblastoma xenografts. Cancer Res 2005;65:7462–9.
[2] Glimelius B, Lahn M, Gawande S, Cleverly A, Darstein C, Musib L, et al. A window of opportunity phase II study of enzastaurin in chemonaive patients with asymptomatic metastatic colorectal cancer. Ann Oncol 2010;21:1020–6.
[3] Robertson MJ, Kahl BS, Vose JM, de Vos S, Laughlin M, Flynn PJ, et al. Phase II study of enzastaurin, a protein kinase C beta inhibitor, in patients with relapsed or refractory diffuse large B-cell lymphoma. J Clin Oncol 2007;25:1741–6.
[4] Ting AT, Schoon RA, Abraham RT, Leibson PJ. Interaction between protein kinase C-dependent and G protein-dependent pathways in the regulation of natural killer cell granule exocytosis. J Biol Chem 1992;267:23957–62.
[5] Bonnema JD, Karnitz LM, Schoon RA, Abraham RT, Leibson PJ. Fc receptor stimulation of phosphatidylinositol 3-kinase in natural killer cells is associated with protein kinase C-independent granule release and cell-mediated cytotoxicity. J Exp Med 1994;180:1427–35.
[6] Kvanta A, Nordstedt C, Fredholm B, Gullberg M, Jondal M. Evidence that protein kinase C activation is essential for killing by IL-2-activated lymphocytes. Scand J Immunol 1990;31:103–8.
[7] Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR. Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms. FEBS Lett 2000;484:217–23.
[8] Beurel E, Jope RS. Glycogen synthase kinase-3 promotes the synergistic action of interferon-gamma on lipopolysaccharide-induced IL-6 production in RAW264.7 cells. Cell Signal 2009;21:978–85.
[9] Aoukaty A, Tan R. Role for glycogen synthase kinase-3 in NK cell cytotoxicity and X-linked lymphoproliferative disease. J Immunol 2005;174:4551–8.
[10] Perussia B. Fc receptors on natural killer cells. Curr Top Microbiol Immunol 1998;230:63–88.
[11] Niwa R, Hatanaka S, Shoji-Hosaka E, Sakurada M, Kobayashi Y, Uehara A, et al. Enhancement of the antibody-dependent cellular cytotoxicity of low-fucose IgG1 is independent of FcgammaRIIIa functional polymorphism. Clin Cancer Res 2004;10:6248–55.
[12] Bryceson YT, March ME, Ljunggren HG, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. Immunol Rev 2006;214:73–91.
[13] Moretta A, Marcenaro E, Parolini S, Ferlazzo G, Moretta L. NK cells at the interface between innate and adaptive immunity. Cell Death Differ 2008;15:226–33.
[14] Vivier E, Nunès JA, Vély F. Natural killer cell signaling pathways. Science 2004;306:1517–9.
[15] Lanier LL. Natural killer cell receptor signaling. Curr Opin Immunol 2003;15:308–14.
[16] Upshaw JL, Arneson LN, Schoon RA, Dick CJ, Billadeau DD, Leibson PJ. NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. Nat Immunol 2006;7:524–32.
[17] Billadeau DD, Upshaw JL, Schoon RA, Dick CJ, Leibson PJ. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. Nat Immunol 2003;4:557–64.
[18] Graham DB, Cella M, Giurisato E, Fujikawa K, Miletic AV, Kloeppel T, et al. Vav1 controls DAP10-mediated natural cytotoxicity by regulating actin and microtubule dynamics. J Immunol 2006;177:2349–55.
[19] Giurisato E, Cella M, Takai T, Kurosaki T, Feng Y, Longmore GD, et al. Phosphatidylinositol 3-kinase activation is required to form the NKG2D immunological synapse. Mol Cell Biol 2007;27:8583–99.
[20] Tassi I, Cella M, Presti R, Colucci A, Gilfillan S, Littman DR, et al. NK cell-activating receptors require PKC-theta for sustained signaling, transcriptional activation, and IFN-gamma secretion. Blood 2008;112:4109–16.
[21] Chiesa S, Mingueneau M, Fuseri N, Malissen B, Raulet DH, Malissen M, et al. Multiplicity and plasticity of natural killer cell signaling pathways. Blood 2006;107:2364–72.
[22] Jiang K, Zhong B, Gilvary DL, Corliss BC, Hong-Geller E, Wei S, et al. Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. Nat Immunol 2000;1:419–25.
[23] Jiang K, Zhong B, Gilvary DL, Corliss BC, Vivier E, Hong-Geller E, et al. Syk regulation of phosphoinositide 3-kinase-dependent NK cell function. J Immunol 2002;168:3155–64.
[24] Tassi I, Presti R, Kim S, Yokoyama WM, Gilfillan S, Colonna M. Phospholipase C-gamma 2 is a critical signaling mediator for murine NK cell activating receptors. J Immunol 2005;175:749–54.
[25] Caraux A, Kim N, Bell SE, Zompi S, Ranson T, Lesjean-Pottier S, et al. Phospholipase C-gamma2 is essential for NK cell cytotoxicity and innate immunity to malignant and virally infected cells. Blood 2006;107:994–1002.
[26] Regunathan J, Chen Y, Kutlesa S, Dai X, Bai L, Wen R, et al. Differential and nonredundant roles of phospholipase Cgamma2 and phospholipase Cgamma1 in the terminal maturation of NK cells. J Immunol 2006;177:5365–76.
[27] Upshaw JL, Schoon RA, Dick CJ, Billadeau DD, Leibson PJ. The isoforms of phospholipase C-gamma are differentially used by distinct human NK activating receptors. J Immunol 2005;175:213–8.
[28] Cella M, Fujikawa K, Tassi I, Kim S, Latinis K, Nishi S, et al. Differential requirements for Vav proteins in DAP10- and ITAM-mediated NK cell cytotoxicity. J Exp Med 2004;200:817–23.
[29] Trotta R, Puorro KA, Paroli M, Azzoni L, Abebe B, Eisenlohr LC, et al. Dependence of both spontaneous and antibody-dependent, granule exocytosis-mediated NK cell cytotoxicity on extracellular signal-regulated kinases. J Immunol 1998;161:6648–56.
[30] Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 2008;9:495–502.
[31] Zompi S, Hamerman JA, Ogasawara K, Schweighoffer E, Tybulewicz VL, Di Santo JP, et al. NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases. Nat Immunol 2003;4:565–72.
[32] Ogbomo H, Michaelis M, Klassert D, Doerr HW, Cinatl Jr J. Resistance to cytarabine induces the up-regulation of NKG2D ligands and enhances natural killer cell lysis of leukemic cells. Neoplasia 2008;10:1402–10.
[33] Michaelis M, Suhan T, Michaelis UR, Beek K, Rothweiler F, Tausch L, et al. Valproic acid induces extracellular signal-regulated kinase 1/2 activation and inhibits apoptosis in endothelial cells. Cell Death Differ 2006;13:446–53.
[34] Blaheta RA, Weich E, Marian D, Bereiter-Hahn J, Jones J, Jonas D, et al. Human cytomegalovirus infection alters PC3 prostate carcinoma cell adhesion to endothelial cells and extracellular matrix. Neoplasia 2006;8:807–16.
[35] Carducci MA, Musib L, Kies MS, Pili R, Truong M, Brahmer JR, et al. Phase I dose escalation and pharmacokinetic study of enzastaurin, an oral protein kinase C beta inhibitor, in patients with advanced cancer. J Clin Oncol 2006;24:4092–9.
[36] Albanell J, Codony-Servat J, Rojo F, Del Campo JM, Sauleda S, Anido J, et al. Activated extracellular signal-regulated kinases: association with epidermal growth factor receptor/transforming growth factor alpha expression in head and neck squamous carcinoma and inhibition by anti-epidermal growth factor receptor treatments. Cancer Res 2001;61:6500–10.
[37] Querfeld C, Rizvi MA, Kuzel TM, Guitart J, Rademaker A, Sabharwal SS, et al. The selective protein kinase C beta inhibitor enzastaurin induces apoptosis in cutaneous T-cell lymphoma cell lines through the AKT pathway. J Invest Dermatol 2006;126:1641–7.
[38] Lee KW, Kim SG, Kim HP, Kwon E, You J, Choi HJ, et al. Enzastaurin, a protein kinase C beta inhibitor, suppresses signaling through the ribosomal S6 kinase and bad pathways and induces apoptosis in human gastric cancer cells. Cancer Res 2008;68:1916–26.
[39] Guo K, Liu Y, Zhou H, Dai Z, Zhang J, Sun R, et al. Involvement of protein kinase C beta-extracellular signal-regulating kinase 1/2/p38 mitogen-activated protein kinase-heat shock protein 27 activation in hepatocellular carcinoma cell motility and invasion. Cancer Sci 2008;99:486–96.
[40] Moreau AS, Jia X, Ngo HT, Leleu X, O'Sullivan G, Alsayed Y, et al. Protein kinase C inhibitor enzastaurin induces in vitro and in vivo antitumor activity in Waldenstrom macroglobulinemia. Blood 2007;109:4964–72.
[41] Chiossone L, Vitale C, Cottalasso F, Moretti S, Azzarone B, Moretta L, et al. Molecular analysis of the methylprednisolone-mediated inhibition of NK-cell function: evidence for different susceptibility of IL-2- versus IL-15-activated NK cells. Blood 2007;109:3767–75.
[42] Chini CC, Boos MD, Dick CJ, Schoon RA, Leibson PJ. Regulation of p38 mitogen-activated protein kinase during NK cell activation. Eur J Immunol 2000;30:2791–8.
[43] Goekjian PG, Jirousek MR. Protein kinase C inhibitors as novel anticancer drugs. Expert Opin Investig Drugs 2001;10:2117–21.
[44] Aguiló JI, Garaude J, Pardo J, Villalba M, Anel A. Protein kinase C-theta is required for NK cell activation and in vivo control of tumor progression. J Immunol 2009;182:1972–81.
[45] Steele TA, Brahmi Z. Inhibition of human natural killer cell activity by the protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine is an early but post-binding event. J Immunol 1988;141:3164–9.
[46] Page KM, Chaudhary D, Goldman SJ, Kasaian MT. Natural killer cells from protein kinase C theta-/- mice stimulated with interleukin-12 are deficient in production of interferon-gamma. J Leukoc Biol 2008;83:1267–76.
[47] Zhang J, Anastasiadis PZ, Liu Y, Thompson EA, Fields AP. Protein kinase C (PKC) betaII induces cell invasion through a Ras/Mek-, PKC iota/Rac 1-dependent signaling pathway. J Biol Chem 2004;279:22118–23.
[48] Partovian C, Simons M. Regulation of protein kinase B/Akt activity and Ser473 phosphorylation by protein kinase Calpha in endothelial cells. Cell Signal 2004;16:951–7.
[49] Kawakami Y, Nishimoto H, Kitaura J, Maeda-Yamamoto M, Kato RM, Littman DR, et al. Protein kinase C betaII regulates Akt phosphorylation on Ser-473 in a cell type- and stimulus-specific fashion. J Biol Chem 2004;279:47720–5.
[50] Aeder SE, Martin PM, Soh JW, Hussaini IM. PKC-eta mediates glioblastoma cell proliferation through the Akt and mTOR signaling pathways. Oncogene 2004;23:9062–9.
[51] Fang X, Yu S, Tanyi JL, Lu Y, Woodgett JR, Mills GB. Convergence of multiple signaling cascades at glycogen synthase kinase 3: Edg receptor-mediated

phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. Mol Cell Biol 2002;22:2099–110.
[52] Goode N, Hughes K, Woodgett JR, Parker PJ. Differential regulation of glycogen synthase kinase-3 beta by protein kinase C isotypes. J Biol Chem 1992;267:16878–82.
[53] Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995;378:785–9.
[54] Herbst RS, Oh Y, Wagle A, Lahn M. Enzastaurin, a protein kinase Cbeta-selective inhibitor, and its potential application as an anticancer agent in lung cancer. Clin Cancer Res 2007;13:s4641–6.
[55] Podar K, Raab MS, Zhang J, McMillin D, Breitkreutz I, Tai YT, et al. Targeting PKC in multiple myeloma: in vitro and in vivo effects of the novel, orally available small-molecule inhibitor enzastaurin (LY317615.HCl). Blood 2007;109:1669–77.
[56] Holler C, Piñón JD, Denk U, Heyder C, Hofbauer S, Greil R, et al. PKCbeta is essential for the development of chronic lymphocytic leukemia in the TCL1 transgenic mouse model: validation of PKCbeta as a therapeutic target in chronic lymphocytic leukemia. Blood 2009;113:2791–4.
[57] Jope RS, Johnson GV. The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 2004;29:95–102.
[58] De Servi B, Heramni A, Medunjanin S, Mayer D. Impact of PKCδ onj estrogen receptor localization and activity in breast cancer cells. Oncogene 2005;24:4946–55.
[59] Gruber T, Hermann-Kleiter N, Pfeifhofer-Obermair C, Lutz-Nicoladoni C, Thuille N, Letschka T, Barsig J, Baudler M, Li J, Metzler B, Nüsslein-Hildesheim B, Wagner J, Leitges M, Baier G. PKC theta cooperates with PKC alpha in alloimmune responses of T cells in vivo. Mol Immunol 2009;46:2071–9.
[60] Isakov N, Altman A. Protein kinase C (theta) in T cell activation. Annu Rev Immunol 2002;20:761–94.
[61] Szamel M, Ebel U, Uciechowski P, Kaever V, Resch K. T cell antigen receptor dependent signalling in human lymphocytes: cholera toxin inhibits interleukin-2 receptor expression but not interleukin-2 synthesis by preventing activation of a protein kinase C isotype. PKC-alpha. Biochim Biophys Acta 1997;1356:237–48.
[62] Gómez J, Pitton C, García A, Martinez de Aragón A, Silva A, Rebollo A. The zeta isoform of protein kinase C controls interleukin-2-mediated proliferation in a murine T cell line: evidence for an additional role of protein kinase C epsilon and beta. Exp Cell Res 1995;218:105–13.
[63] Szamel M, Appel A, Schwinzer R, Resch K. Different protein kinase C isoenzymes regulate IL-2 receptor expression or IL-2 synthesis in human lymphocytes stimulated via the TCR. J Immunol 1998;160:2207–14.
[64] Shenoy AM, Brahmi Z. Inhibition of the calpain-mediated proteolysis of protein kinase C enhances lytic activity in human NK cells. Cell Immunol 1991;138:24–34.
[65] Shenoy AM, Sidner RA, Brahmi Z. Signal transduction in cytotoxic lymphocytes: decreased calcium influx in NK cell inactivated with sensitive target cells. Cell Immunol 1993;147:294–301.
[66] Zorn CN, Keck S, Hendriks RW, Leitges M, Freudenberg MA, Huber M. Bruton's tyrosine kinase is dispensable for the Toll-like receptor-mediated activation of mast cells. Cell Signal 2009;21:79–86.
[67] Kim HR, Lee KH, Park SJ, Kim SY, Yang YK, Tae J, et al. Anti-cancer activity and mechanistic features of a NK cell activating molecule. Cancer Immunol Immunother 2009;58:1691–700.