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

Enzastaurin is a selective protein kinase C β inhibitor which is shown to have direct antitumor effect as well as suppress glycogen synthase kinase-3 β (GSK-3 β) 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-3 β by inhibition of GSK-3 β phosphorylation. Treatment of NK cells with GSK-3 β -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.

Keywords: Antibody-dependent cellular cytotoxicity, natural cytotoxicity, NKG2D, protein kinase C β , glycogen synthase kinase-3 β

1. INTRODUCTION

Enzastaurin (LY317615.HCl), an acyclic bisindolylmaleimide, is a selective orally administered protein kinase C β (PKC β) inhibitor. Although initially developed to selectively inhibit PKC β at nanomolar concentrations (IC₅₀ of 6 nmol/L), enzastaurin was demonstrated to also inhibit other PKC isoforms including PKC γ , PKC δ , PKC θ , PKC ϵ , PKC ξ , PKC η , PKC ζ and PKC α 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

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4 armed with an array of activating receptors that trigger cytolytic granules and cytokine
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6 production. Besides expression of several receptors that mediate natural cytotoxicity, NK cells
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8 also express CD16 which mediates antibody-dependent cellular cytotoxicity (ADCC) against
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10 IgG-coated target cells [10-13]. While NK cell activating receptors like NKp30, NKp44, NKp46,
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12 and CD16 associate with the transmembrane adaptors CD3 ζ , FcR γ , or DAP12 that contain a
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14 cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) [14], NKG2D associates
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16 with the adaptor protein DAP10 which contains a cytoplasmic YINM motif [15-19]. The ligation
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18 or antibody dependent cross linking of the ITAM-dependent receptors initiates NK cell signal
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20 transduction cascade by phosphorylation of tyrosine residues located within conserved ITAM
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22 sequences through Src protein tyrosine kinase (PTK) family [20]. These in turn allows the
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24 consequent recruitment and activation of Syk and/or ZAP70 PTK and the transmembrane
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26 adaptor proteins, linker for activation of T cells, LAT and LAT2 [20, 21], further leading to the
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28 recruitment and activation of downstream effector molecules such as PI3K [5, 22, 23], the
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30 phospholipases PLC γ 1 and PLC γ 2 [24-27], the guanosine triphosphate-guanosine diphosphate
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32 exchange factors Vav2 and Vav3, [28], the extracellular signal-regulated kinase (ERK) [29], and
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34 finally triggers both cytotoxicity and cytokine production [20]. On the other hand, ligation of
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36 DAP10 dependent receptor (NKG2D) directly recruits PI3K and Grb2-Vav1 complex [15-19],
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38 triggering cytotoxicity but not cytokine production [30, 31].
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49 Since enzastaurin effectively inhibits phosphorylation of PKC, AKT and GSK3 [1], all
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51 pathways necessary for NK cell activation, we therefore investigated the direct effect of
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53 enzastaurin in NK-mediated cytotoxicity (natural and antibody-dependent) and cytokine
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55 secretion as well as the influence of enzastaurin on signaling pathways involved in NK cell
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4 activity. We observed that enzastaurin 1) inhibits NK cell cytotoxicity (both natural and
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6 antibody-dependent), 2) specifically suppresses the expression of NKG2D and CD16 in primary
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8 NK cells, 3) impairs perforin release without affecting levels of intracellular perforin expression
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10 in NK cells and 4) activates GSK-3 β in IL-2-stimulated NK cells by inhibition of GSK-3 β
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12 phosphorylation.
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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 (Erbix) 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-VIVOTM 10 Medium (Lonza Verviers, Belgium) supplemented with 5% human plasma and 100U/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

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4 (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol. Flow
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6 cytometric analysis to determine purity of NK cells showed that more than 95% of the cells were
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8 CD56+CD3- (not shown). NK cells were cultured using IMDM containing 10% FCS, with or
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10 without 100 U/ml recombinant IL-2. For all experiments (unless otherwise indicated), IL-2-
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12 activated NK cells were simultaneously treated with or without enzastaurin at indicated
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14 concentration for 120 h.
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20 **2.4. Cytotoxicity assay and flow cytfluorometric analysis**

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22 Cytotoxicity of NK cells was determined by a 4 h coupled luminescent method using the
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24 "ACELLA-TOXTM" kit (Cell Technology, Mountain View, CA), as described [32]. K562 and
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26 A431 cell lines were used as target cells. For ADCC experiments, target cells were pre-coated
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28 with 0.5 µg/ml cetuximab mAb for 30 min before coculture with NK cells. Flow cytometry
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30 (FACS Calibur; Becton Dickinson, Mountain View, CA) was used for cell surface and
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32 intracellular molecule expression as well as for DNA content analyses. Controls for all
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34 experiments performed contained vehicle (maximum 0.1% DMSO) which did not influence NK
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36 cell activity when compared with cultures without vehicle (not shown).
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43 **2.5. Cell cycle analysis**

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45 Cell cycle was determined using a commercial kit (BD Biosciences, San Jose, CA)
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47 according to the manufacturer's instructions as described previously [33].
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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-3 β , phospho-GSK-3 β ^{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

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4 determined using the Quantikine Human IFN- γ ELISA kit (R&D Systems, Wiesbaden,
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6 Germany) while perforin and granzyme B release were measured using Perforin/Granzyme B-
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8 ELISA kit (Diaclone Research, Besancon Cedex, France) according to manufacturers protocol.
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11 **2.8. Statistics**

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15 Values presented are the mean \pm standard error of mean (S.E.M) of at least three
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17 experiments. Comparisons between two groups were performed using Student's t-test. P-values
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19 lower than 0.05 were considered to be significant.
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23 **2.9. Ethics Statement**

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

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4 treatment. Here, primary NK cells were stimulated for 96 h with IL-2 (100 U/ml) prior to
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6 addition of 2.5 μ M enzastaurin. Results show a 58% reduction in lysis of K562 target cells in
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8 enzastaurin-treated primary NK cells compared to untreated control (E/T ratio 4:1). The
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10 production of INF- γ by IL-2-activated NK cells was also measured upon coculture of NK cells
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12 and enzastaurin-treated NK cells with K562 target cells. Enzastaurin did not suppress IFN- γ
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14 production in IL-2-activated NK cells (not shown).
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20 **3.3. Enzastaurin interferes with NK-mediated ADCC**

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22 Apart from natural cytotoxicity, primary NK cells also mediate ADCC since they express
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24 high amounts of CD16 on their cell surface. We investigated whether enzastaurin has any
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26 influence on ADCC. For this purpose, primary NK cells treated simultaneously for 120 h with
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28 IL-2 and 2.5 μ M enzastaurin, were used in a cytolytic experiment against epidermal growth
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30 factor receptor (EGFR)-expressing tumor cell line A431 [36]. The cell line was coated with the
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32 EGFR-specific antibody cetuximab before coculture with NK cells. The result obtained show
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34 that while A431 cells were resistant to natural cytotoxicity mediated by IL-2-activated NK cells,
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36 the cells were sensitive to IL-2-activated NK-mediated ADCC. Enzastaurin however caused
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38 40% inhibition of NK cell-mediated ADCC of A431 cell line (Fig. 2C).
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45 **3.4. Enzastaurin down-modulates NK cell activating receptor expression**

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47 NK cell cytotoxicity is critically dependent on signaling through its receptors, leading to
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49 granule polarization and exocytosis. Accordingly, it is conceivable that enzastaurin might
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51 interfere with NK cell receptor expression pattern. To address this, we investigated the surface
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53 expression patterns of NK cell triggering receptors CD16, NKp30, NKp44, NKp46, NKG2D and
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4 DNAM-1, as well as NK cell inhibitory receptors KIR3DL1 and KIR2DL2/DL3 in non-treated
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6 and enzastaurin-treated NK cells. A correlation between NK cell cytotoxicity and NK cell
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8 receptor expression pattern was observed. The lytic capacity of NK cells treated with enzastaurin
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10 was associated with a specific inhibition of CD16 (involved in ADCC) and NKG2D (involved in
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12 natural cytotoxicity) surface expression (Fig. 3A). The surface expression of NKp30, NKp44,
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14 NKp46, KIR3DL1 and KIR2DL2/DL3 were not changed (not shown). Enzastaurin also did not
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16 suppress the surface expression of triggering and inhibitory receptors in NK cells cultured
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18 simultaneously for 18 h with IL-2 and enzastaurin (not shown). Basically NK cell activating or
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20 inhibitory receptor expression levels were 2-3 folds higher in NK cells cultured with IL-2 for 120
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22 h than in NK cells cultured with IL-2 for 18 h.
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29 **3.5. Enzastaurin impairs perforin release by NK cells**

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32 The cytotoxic granules of NK cells are final mediators of NK cytotoxicity. They become
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34 polarized at the interface with target cells and are released into the target cells. We studied the
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36 effect of enzastaurin on intracellular perforin and granzyme B expression and release in NK
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38 cells. We observed that while the intracellular perforin and granzyme B expression did not
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40 change (relative expression: for perforin, 38.82 ± 3 vs. 39.96 ± 4.2 ; for granzyme B, 154.41 ± 6.3 vs.
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42 146.43 ± 4.2 respectively for IL-2-treated NK cells and NK cells treated simultaneous for 120 h
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44 with IL-2 and $2.5 \mu\text{M}$ enzastaurin), enzastaurin significantly impaired release of perforin by
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46 2.36-fold but had no influence on granzyme B release in cocultures of enzastaurin-treated IL-2-
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48 activated NK and K562 compared to non-treated IL-2-activated NK and K562 coculture (Fig.
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50 3B).
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3.6. Enzastaurin directly suppresses GSK-3 β (ser⁹) 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-3 β 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).

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-3 β -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

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4 significantly influence target cell lysis by NK cells, combined treatment of NK cells with TDZD-
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7 8 and enzastaurin prevented the lytic inhibitory role of enzastaurin (**Fig. 6A and B**). Also,
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9 combined treatment enhanced granzyme B release (from 79 pg/ml in non-treated NK cells to 142
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11 pg/ml in enzastaurin-treated NK cells), and partially restored perforin release to levels higher
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13 than in NK cells treated with enzastaurin alone (from 697 pg/ml to 1002 pg/ml). The inhibition
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15 of NKG2D and CD16 expression were however not prevented by the combined treatment (not
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18 shown).
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4. DISCUSSION

Enzastaurin is a selective PKC β 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]. PKC α , PKC β , and PKC η can also directly phosphorylate AKT at Ser⁴⁷³, which is essential for AKT activity [48-50]. Moreover, both PKC [51, 52] and AKT activity [53] can phosphorylate GSK-3 β at Ser⁹, indicating an overlap in these signaling pathways. The phosphorylation of GSK-3 β 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-3 β in human NK cells by specifically suppressing GSK-3 β 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 PKC β may directly phosphorylate GSK-3 β without involvement of AKT [54]. 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-3 β as well as of AKT and sole inhibition GSK-3 β may not be sufficient to affect p70S6K signalling 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 [55]. In non-

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4 stimulated preparations of normal human PBMCs (of which NK cells are constituents) and in T
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6 cells, enzastaurin was found to be non-toxic in the investigated concentrations up to 50 μM [40,
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8 56, 57]. Stimulation of lymphocytes with cytokines or antigens may activate PKC β and render
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10 them more sensitive to cytotoxic and antiproliferative effects of the drug. However, we did not
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12 observe antiproliferative or toxic effects of enzastaurin on IL-2-stimulated NK cells at
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14 therapeutic relevant concentrations ranging from 0.625 μM to 2.5 μM . Notably, enzastaurin
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16 concentrations higher than 2.5 μM were required to affect cell proliferation and viability of some
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18 tumour cell types with highly activated PKC β [38,40]. However, enzastaurin concentrations
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20 between 0.625 μM and 2.5 μM inhibited dose-dependently GSK3 β phosphorylation in NK cells
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22 indicating an uncoupling of anti-proliferative effects of enzastaurin from effects on GSK3 β
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24 signalling. Similar observations were made in lymphoma cells treated with 2.5 μM enzastaurin
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26 which nearly completely abrogated GSK-3 β phosphorylation but did not influence cell
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28 proliferation [40].
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37 Our results further show that enzastaurin inhibits NK-mediated natural cytotoxicity and
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39 NK-mediated ADCC of different tumor targets. Additionally, enzastaurin impaired NK cell
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41 potential in releasing perforin but had no effect on granzyme B release. Combined treatment of
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43 NK cells with a GSK-3 β -specific inhibitor TDZD-8 and enzastaurin prevented the lytic
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45 inhibitory role of enzastaurin by enhancing granzyme B release and partially restoring perforin
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47 release when compared with non-treated or enzastaurin-treated NK cells. In contrast to a report
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49 by Aoukaty and Tan [9] demonstrating that inactivation (phosphorylation) of GSK-3 with
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51 TDZD-8 increased NK cytotoxicity and IFN- γ secretion, we did not observe any influence of
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53 TDZD-8 on NK cell cytotoxicity. The reasons for this difference observed by Aoukaty and Tan
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4 [9] and us remain obscure. However, there is a distinct difference in the experimental procedure
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6 applied that may contribute to the varying results. While Aoukaty and Tan [9] treated IL-2-
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8 activated NK cells with TDZD-8 for only 30 min in serum free medium, we treated NK cells
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10 simultaneously with IL-2 and TDZD-8 for 120 h and in a medium that contained serum.
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15 The activity of enzastaurin has been profiled in tumor cells using in vitro kinase assays
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17 [1]. The authors demonstrated that enzastaurin inhibited PKC β and other PKC isoforms
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19 including PKC γ , PKC δ , PKC θ , PKC ϵ , PKC ξ , by approximately 90% or more but showed no
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21 substantial inhibition of PKC μ and PKC ι . PKC η and PKC ζ were also inhibited by nearly 50%
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23 while PKC α was inhibited by 77% [1]. Moreover, different PKC isoforms were shown to
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25 phosphorylate GSK3 [54, 58-60]. Therefore, it is conceivable that PKC isoforms other than
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27 PKC β are also inhibited by enzastaurin and contribute to impaired NK functions through effects
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29 on GSK3.
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35 Also, specific PKC isoenzymes are known to regulate different cellular functions in
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37 stimulated lymphocytes: PKC α and PKC θ are involved in the expression of the IL-2 receptor
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39 [61, 62], whereas PKC β , PKC δ and PKC ϵ are involved in IL-2 synthesis and in the IL-2-
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41 mediated proliferation of T cells [63, 64]. In NK cells, the rapid activation of PKC enables them
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43 to mediate natural cytotoxicity towards tumor cells [65, 66], and PKC activation is essential for
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45 triggering lysis in IL-2-activated NK cells [6]. PKC θ was also demonstrated to be required for
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47 NK cell activation and in vivo control of tumor progression [44]. Some other report described the
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49 absolute requirement of PKC θ for ITAM-mediated IFN- γ secretion, but without a marked
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51 influence on the release of cytolytic mediators [20]. Specifically, the authors showed using
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4 PKC θ -deficient murine NK cells that NKG2D engagement does not influence target cell lysis
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6 and that CD16-mediated ADCC was only slightly affected [20]. This means that PKC θ is
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8 necessarily required for NKG2D and CD16-mediated lysis. Also, PKC β deficiency was shown to
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10 have no influence on LPS-induced cytokine production in murine bone marrow-derived mast
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12 cells [67]. In our study, enzastaurin did not suppress IFN- γ secretion but specifically down-
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14 regulated the surface expression of NKG2D, an NK activating receptor that is described to
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16 trigger cytotoxicity but not cytokine production [30, 31]. Taken together, NKG2D-mediated
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18 natural cytotoxicity and CD16-mediated ADCC in NK cells appear to require PKC β for their
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20 cytolytic function. Although we did not study how engagement of NKG2D receptor or CD16
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22 activates PKC β and/or PI3K/AKT signaling or downstream events, signaling via 2B4 (another
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24 NK cell activating receptor) was shown to be mediated by GSK-3 [9].
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32 Some studies on the effect of other PKC inhibitors on NK cell activity have been
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34 reported. While the PKC inhibitors Gö6976, Rottlerin, and Bisindolylmaleimide III were shown
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36 to have no effect on NCR (NKp30, NKp44 and NKp46) expression and NK cell-mediated
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38 cytotoxicity, Gö6983 induced a remarkable increase of NCR expression and strongly up-
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40 regulated the death ligands FasL and TRAIL on primary human NK cells, leading to enhanced
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42 NK cell lysis of target cells [68]. The authors therefore speculated that NK cell activation by
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44 Gö6983 occurs via an unknown mechanism other than PKC inhibition. Furthermore, the PKC
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46 inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride was shown to inhibit
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48 both natural cytotoxicity and ADCC of human NK cells [45]. Similar to some data in these
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50 reports, enzastaurin had no effect on NCR expression but impaired NK cell cytotoxicity.
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4 In conclusion, we show for the first time that the PKC β -specific inhibitor enzastaurin
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6 suppresses both ADCC and natural cytotoxicity of human NK cells by inhibiting the expression
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8 of NKG2D and CD16 as well as impairment of perforin release. In addition, we show that
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10 enzastaurin selectively inhibits GSK-3 β phosphorylation (known to be involved in NK cell
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12 cytotoxicity) without affecting signaling molecules responsible for cell proliferation and survival
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14 (AKT, S6, p70S6K). Although enzastaurin plays vital roles in inhibition of tumor-induced
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16 angiogenesis, inhibition of tumor cell proliferation, and induces apoptosis in tumor cells, our data
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18 suggest that it also has a negative impact on two crucial NK cell-mediated functions, namely
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20 natural cytotoxicity and ADCC. This implies that treatment of tumor patients with enzastaurin
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22 may impair their NK cell cytotoxic potential. Careful administration and control of patients
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24 undergoing enzastaurin therapy is therefore required.
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FIGURE LEGEND**Figure 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.

Figure 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 μ M enzastaurin for 120 h; EGFR-expressing A431 cells and A431 cells coated with 0.5 μ g/ml cetuximab or IgG were used as target cells. Columns represent means of triplicate of one representative experiment; error bars indicate \pm S.E.M; * $P < 0.05$ relative to control.

Figure 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 μ M 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

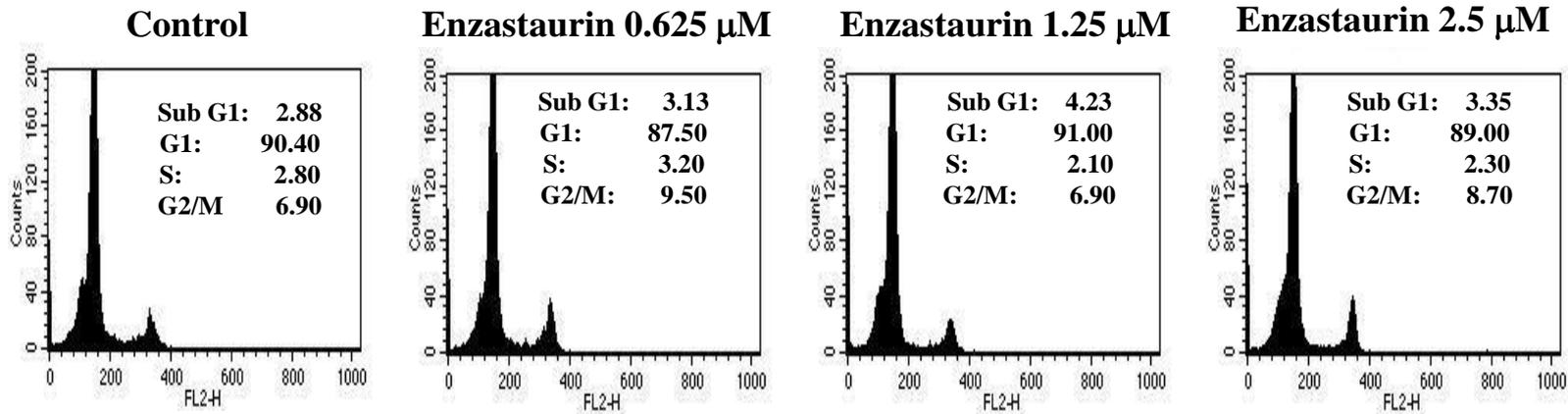
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4 cocultured with K562 target cells. Afterwards, supernatant was collected and analyzed by ELISA
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6 for granule release. Columns represent means of triplicate of one representative experiment;
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8 error bars indicate \pm S.E.M; * $P < 0.05$ relative to control.
9

10
11 **Figure 4. Enzastaurin inhibits phosphorylation of GSK-3 β .** A) Primary natural killer (NK)
12
13 cells and B) NK-92 cells were treated simultaneously with/without IL-2 and enzastaurin for 120
14
15 h. The same amount of protein extracts prepared from enzastaurin-treated and non-treated NK
16
17 cells was used for western blot analysis of the indicated signaling proteins. Data are
18
19 representative of at least three experiments.
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24 **Figure 5. Enzastaurin dose-dependently inhibits phosphorylation of GSK-3 β without**
25
26 **affecting ERK1/2 and p38 phosphorylation.** Primary natural killer (NK) cells (A and C) and
27
28 NK-92 cells (B and D) were treated simultaneously with/without IL-2 and enzastaurin for 120 h.
29
30 The same amount of protein extracts prepared from enzastaurin-treated and non-treated NK cells
31
32 was used for western blot analysis of the indicated signaling proteins. Data are representative of
33
34 at least three experiments.
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40 **Figure 6. GSK-3 β -inhibitor TDZD-8 prevents enzastaurin-induced inhibition of natural**
41
42 **killer (NK) cell lysis.** Primary NK cells (A) and NK-92 cells (B) were treated with IL-2 alone or
43
44 in single combination with either 2.5 μ M enzastaurin or 1 μ M TDZD-8 or both. Cytotoxicity
45
46 experiment was performed using K562 as target cells. Columns represent means of triplicate of
47
48 one representative experiment; error bars indicate \pm S.E.M; * $P < 0.05$ relative to control.
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Figure 1

Primary
NK cells

NK-92

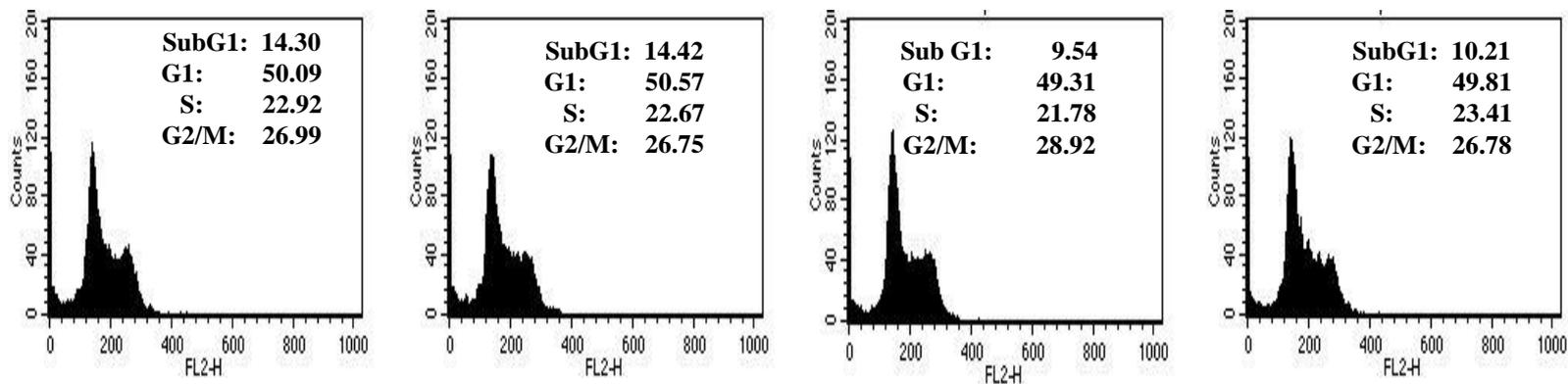


Figure 2

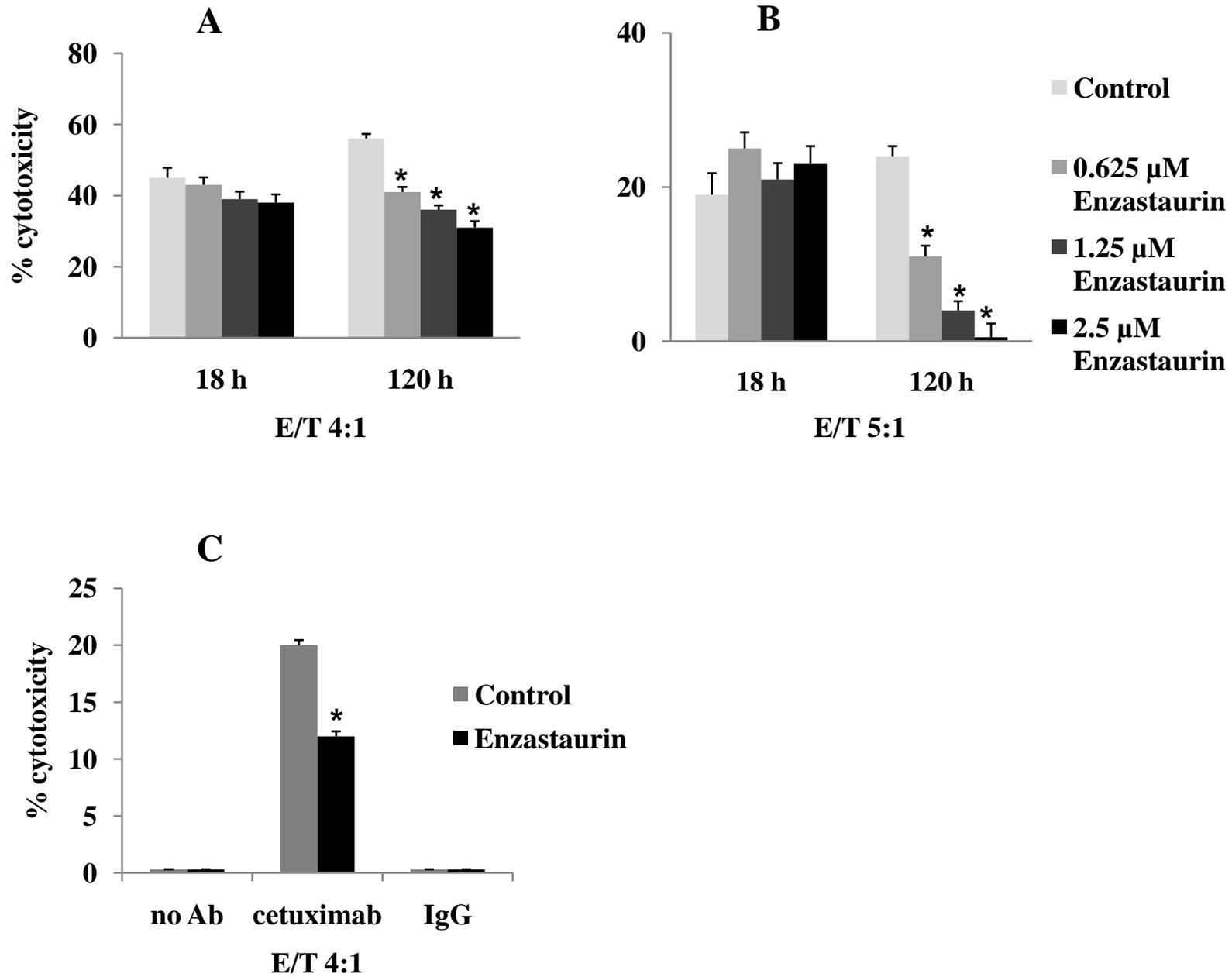
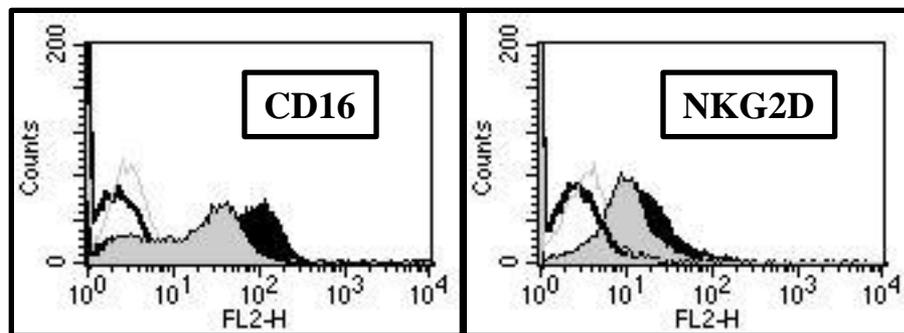


Figure 3

A



B

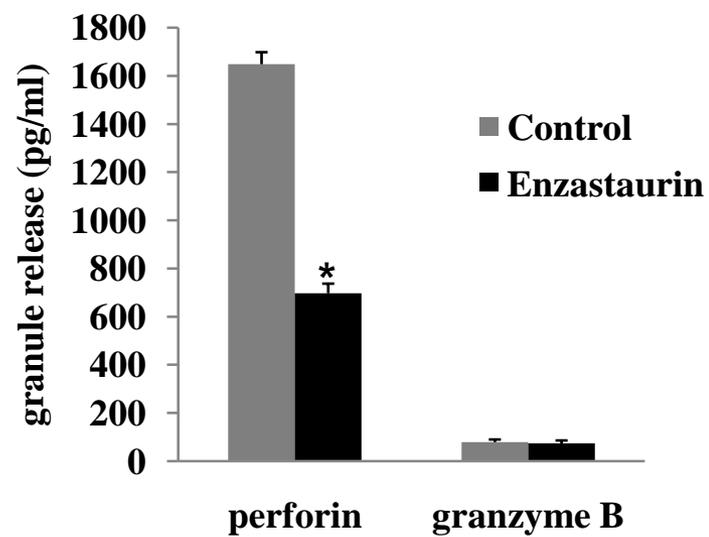


Figure 4

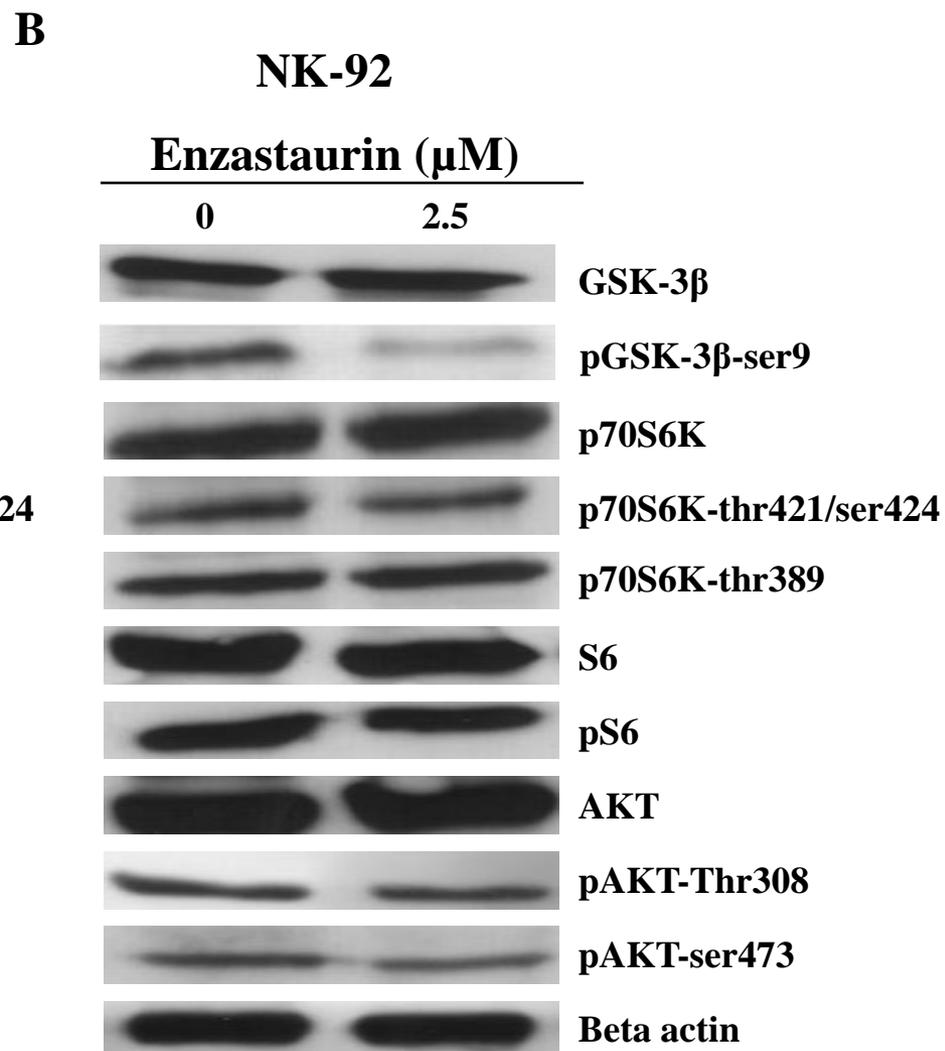
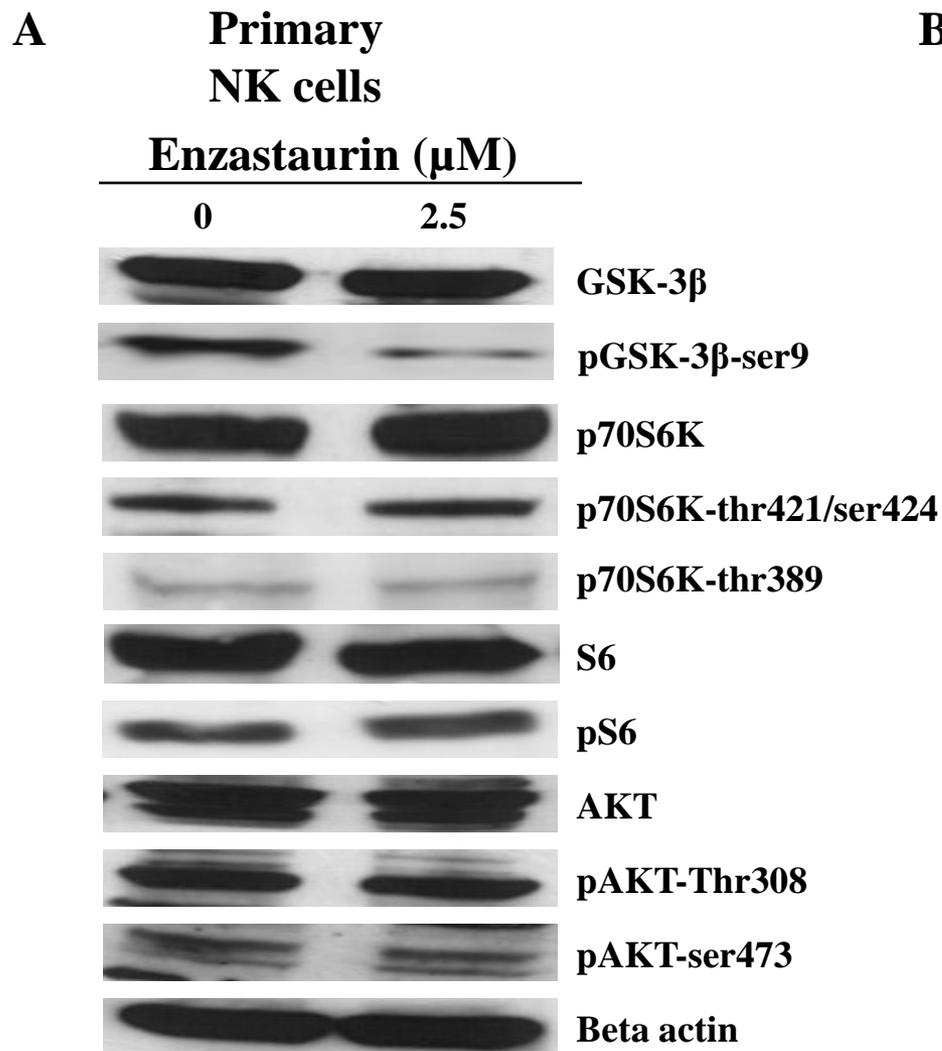
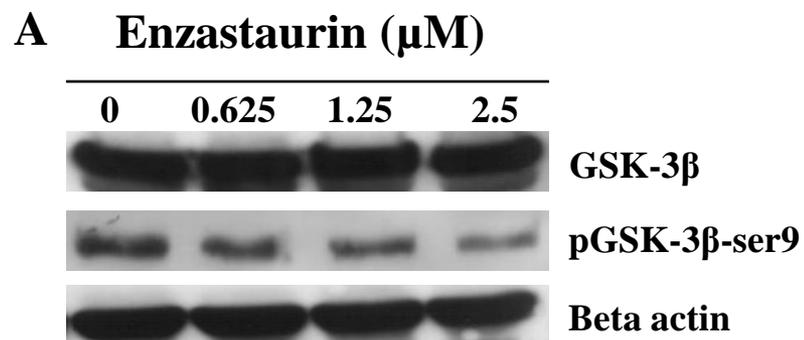


Figure 5

Primary
NK cells

NK-92

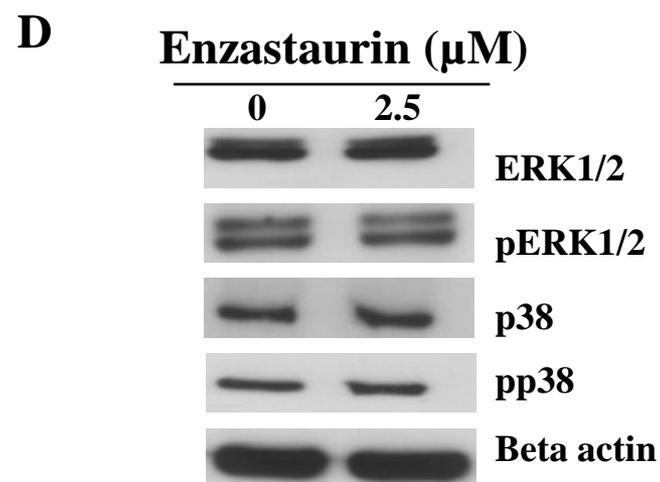
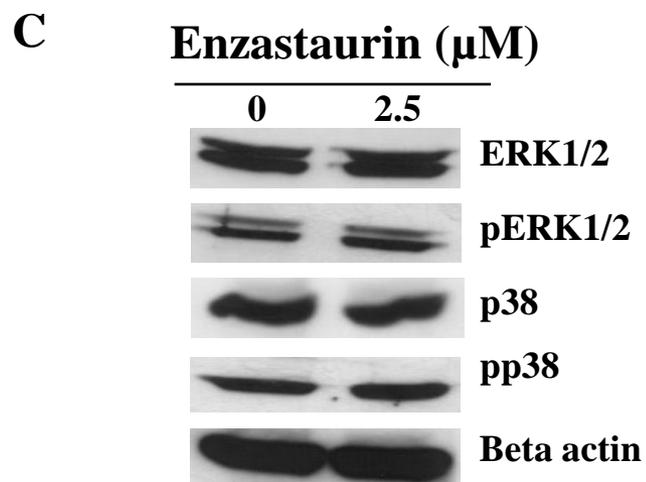
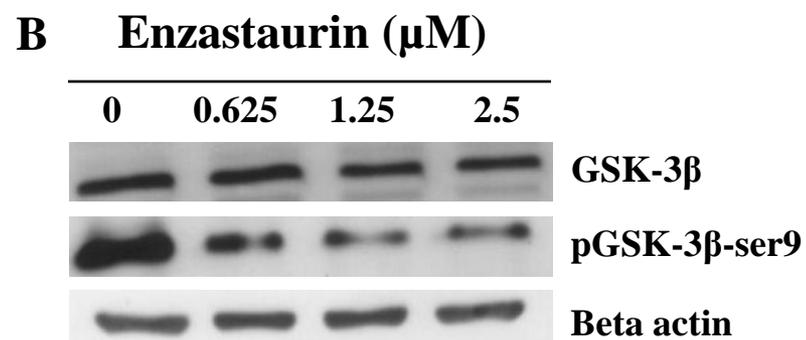
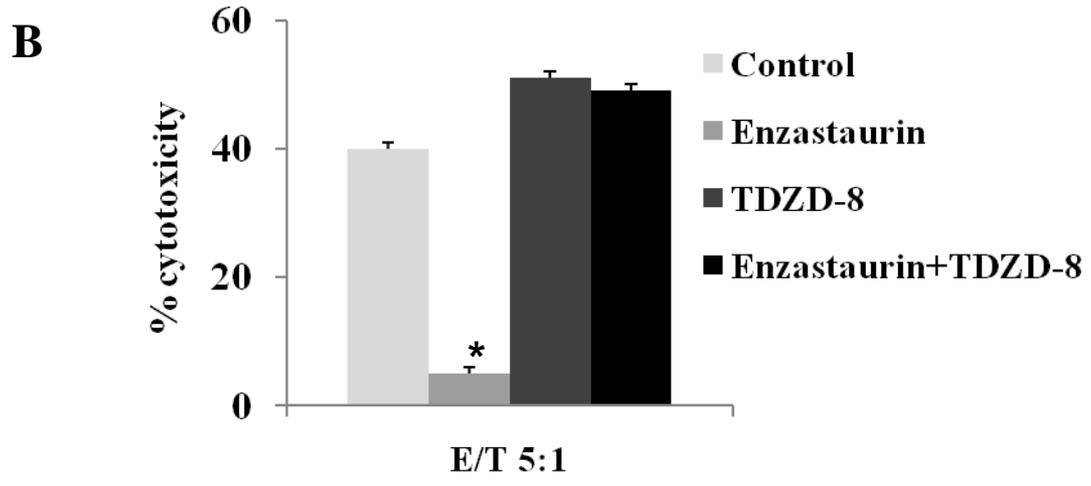
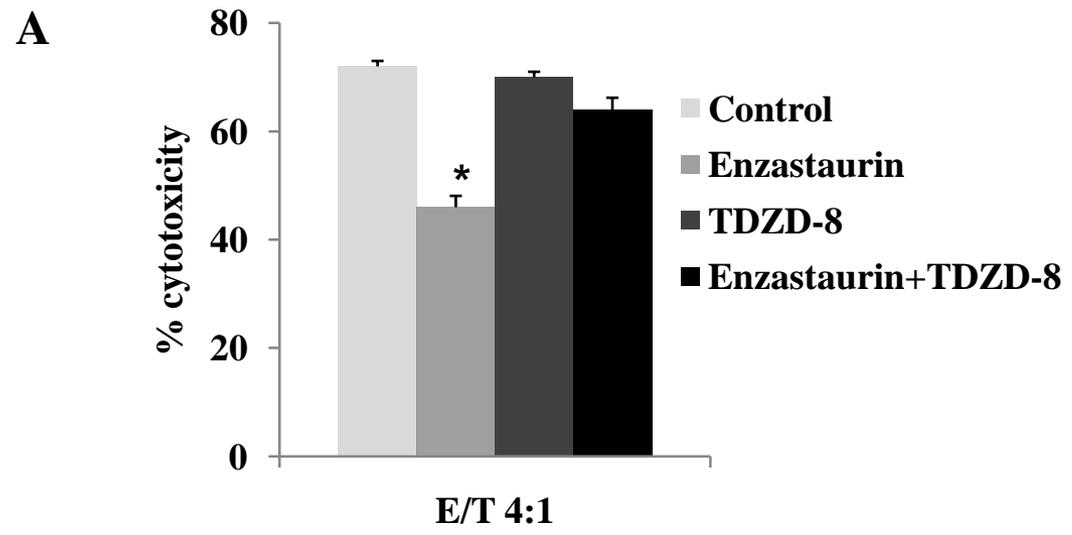


Figure
Figure 6



Enzastaurin inhibits both natural and antibody-dependent cellular cytotoxicity (ADCC) of NK cells.

