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ARTICLE

Inhibition of Novel Protein Kinase Cε Augments TRAIL-induced Cell Death in A549 Lung Cancer Cells

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has great potential for cancer treatment since it provokes cell death in most tumor cells while leaving most normal cells unscathed. Some cancers, however, show resistance to TRAIL, indicating that TRAIL alone may be insufficient for cancer therapy. Here we studied whether the apoptotic susceptibility of A549 non-small cell lung cancer cells could be modulated by inhibiting protein kinase C (PKC). We show that an inhibitor with preference for novel PKC isozymes, NPC 15437, significantly augmented TRAIL sensitivity of A549 cells, as judged by assessing cell death and mitochondrial membrane potential. Likewise, NPC 15437 also significantly potentiated the responsiveness of DAOY medulloblastoma cells to TRAIL. In contrast, an inhibitor with preference for conventional PKC isozymes, Gö6976, did not augment TRAIL sensitivity of A549 cells. To further specify the PKC isozyme responsible for TRAIL sensitization, we used a peptide inhibitor with selectivity for the novel PKC isozyme ε, myr-PKCε V1-2. The inhibition of PKCε resulted in a significant amplification of the cytotoxic activity of TRAIL in A549 cells. Altogether, our study provides evidence for a considerable role of PKCε in the apoptotic responsiveness of A549 lung cancer cells, and possibly other malignancies, to TRAIL. (Pathology Oncology Research Vol 13, No 4, 295–301)

Key words: lung cancer, medulloblastoma, NPC 15437, protein kinase Cε, TNF, TRAIL

Introduction

Lung cancer is the leading cause of cancer-related death, with non-small cell lung cancer (NSCLC) accounting for about 80% of all cases.[16] Most patients with NSCLC are diagnosed with advanced disease at presentation and require systemic chemotherapy. However, survival times for patients receiving such therapy are devastatingly low, due mainly to disposition to metastasize and resistance to chemotherapy. Although combination chemotherapy regimens have been developed, recent studies indicate that systemic chemotherapy has reached a plateau of its clinical efficacy, with a median survival of approximately eight months.[21,36] New treatment strategies to improve survival, thus, are urgently needed.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; also called Apo-2L), a member of the tumor necrosis factor family of cytokines, is a highly promising candidate for cancer treatment, as it induces cell death in a wide variety of cancer cells while leaving most normal cells unscathed.[8,10,22,33] Some cancers, however, fail to respond to the cytotoxic effects of TRAIL; for example, in a recent study on cells from 53 primary leukemias, TRAIL induced relevant apoptosis in only 25% of samples.[1] Therefore, the cytotoxic activity of TRAIL alone may be insufficient for cancer therapy, but may be augmented by coadministration of chemotherapeutic drugs.[14] In NSCLC cell lines, cytostatics[9,28] as well as histone deacetylase inhibitors[41] have been shown to synergize with TRAIL to induce cell death, and TRAIL activity could also be enhanced by chemotherapeutics in NSCLC xenograft models.[7,17] On the other hand, the combination of TRAIL and chemotherapy bears the risk of eliciting apoptosis in otherwise TRAIL-resistant normal cells.[11,47] A more spe-

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cific sensitization to TRAIL may be achieved by targeting TRAIL resistance factors.

Various factors have been proposed for TRAIL resistance, including the downregulation of receptors TRAIL-R1 or -R2, the expression of the so-called "decoy" receptors TRAIL-R3 or -R4, silencing of caspase-8, upregulation of c-FLIP, overexpression of antiapoptotic Bcl-2 family members, or the TRAIL-induced activation of NF-κB.[8,10,22,33] In addition, protein kinase C (PKC) isoforms have been implicated in the susceptibility of cancer cells to TRAIL.[12,13,26,29,30,32,35,37,39,40,42,45,46,49] Yet, the importance of PKC for TRAIL-mediated apoptosis in NSCLC cells has not been studied. PKC is a family of ten serine/threonine protein kinases, which can be classified into three groups, based on their structural and biochemical properties:[15] phorbol ester-responsive and Ca^{++}-dependent conventional PKCs (cPKCs; α, β1, β2, γ), phorbol ester-responsive but Ca^{++}-independent novel PKCs (nPKCs; δ, ε, η, θ), and TPA-unresponsive and Ca^{++}-independent but still phosphatidylserine-activated atypical PKCs (ι, ζ). In the present study, we tested the effect of TRAIL in combination with PKC inhibitors on the NSCLC cell line A549, which expresses multiple PKC isozymes.[6,27] We show that NPC 15437, a PKC inhibitor interacting at the regulatory region of the enzyme[44] and, more specifically, a peptide inhibitor selective for PKCε[18] potently augmented TRAIL-induced cell death.

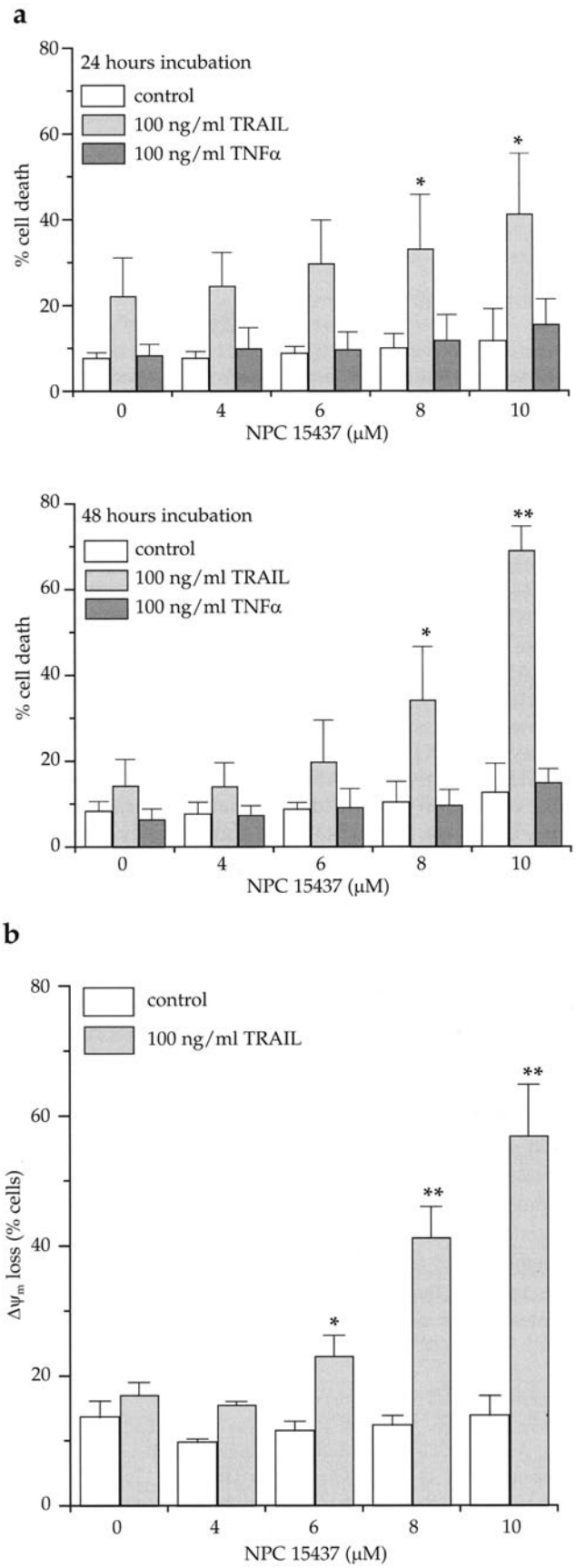
Materials and Methods

Reagents

TRAIL and TNFα were purchased from Peprotech (Rocky Hill, NJ, USA). NPC 15437 was purchased from Sigma (Deisenhofen, Germany). z-VAD-fmk and Gö6976 were purchased from Alexis (Grünberg, Germany). myr-PKCε V1-2 (εV1-2) was purchased from Biomol (Hamburg, Germany).

Cell culture

A549 NSCLC cells were obtained from ATCC (Rockville, MD, USA). DAOY medulloblastoma cells were a gift from Dr. M. Grotzer (Zurich, Switzerland). Cells were maintained in Ham's F12K or Improved MEM Zinc Option (Invitrogen, Karlsruhe, Germany), respectively, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (Ham's F12K and supplements were purchased from Biochrom, Berlin, Germany). Cells were cultivated at 37°C in a humidified 5% CO_2 incubator and routinely passaged when 90-95% confluent. Cell viability was determined by the trypan blue exclusion test. Cells were regularly inspected to be free of mycoplasma with mycoplasma detection reagents from Roche (Mannheim, Germany).



Flow cytofluorometric analysis of cell death

To determine cell death, cells were harvested after a 24-h or 48-h cultivation in the presence of TRAIL or TNFα, followed by a 5-min incubation in 2 μg/ml propidium iodide (PI) (Sigma, St. Louis, MO) in PBS at 4°C in the dark. PI uptake was assessed by flow cytometry analysis on a Becton Dickinson (Heidelberg, Germany) FACSCalibur flow cytometer. Ten thousand cells were analyzed in each sample; data were gated to exclude debris.

Flow cytofluorometric analysis of mitochondrial transmembrane potential ($\Delta\psi_m$)

$\Delta\psi_m$ was determined by assessing the accumulation of the cationic lipophilic fluorochrome 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] in the mitochondrial matrix. Thirty-six or 48 h after treatment with TRAIL or TNFα, cells were incubated with 50 nM $DiOC_6(3)$ (Molecular Probes, Eugene, OR, USA) at 37°C for 30 min. After washing, 10,000 cells were analyzed using a FACSCalibur flow cytometer. Data were gated to exclude debris.

Statistical analysis

Statistical significance of differences between experimental groups was determined using the paired two-tailed Student's t test.

Results

NPC 15437 interacts synergistically with TRAIL, but not with TNFα, in A549 cells

To assess possible effects of NPC 15437 on TRAIL- or TNFα-induced cell death, we initially monitored cell killing by determining the integrity of the cell membrane by cytofluorometric analysis of PI uptake. One hour after treatment with varying concentrations of NPC 15437, A549 cells were exposed to 100 ng/ml of TRAIL or TNFα for another 24 or 48 h. As depicted in *Fig. 1a*, NPC 15437 in itself was not cytotoxic. Likewise non-pretreated cells showed only weak susceptibility to TRAIL and no susceptibility to TNFα. However, NPC 15437 caused a dose-dependent sensitization to TRAIL: in cells pretreated with NPC 15437, TRAIL evoked cell killing in up to 41% of cells after 24 h and up to 69% of cells after 48 h. In contrast, NPC 15437 had no effect on the responsiveness of A549 cells to TNFα.

To evaluate the favorable cooperation of NPC 15437 and TRAIL by an independent read-out, we examined the effect of TRAIL on the mitochondrial membrane potential ($\Delta\psi_m$). A549 cells were pretreated with NPC 15437 for 1 h, cultured with TRAIL for additional 48 h, and the loss of $\Delta\psi_m$ was determined by $DiOC_6(3)$ staining. Treatment with either NPC 15437 or TRAIL alone was of little effect on $\Delta\psi_m$, whereas combined treatment led to significant supra-additive $\Delta\psi_m$ dissipation (*Fig. 1b*).

To determine whether the NPC 15437-enhanced TRAIL response would depend on caspase activation, we applied the pan-caspase inhibitor z-VAD-fmk. A549 cells were treated with 20 μM z-VAD-fmk 1 h before application of NPC 15437, 100 ng/ml TRAIL was administered 1 h later, and cell death was monitored by PI uptake analysis after a further 48-h incubation. As shown in *Fig. 1c*, TRAIL-induced cell killing was fully abolished by z-VAD-fmk.

NPC 15437 interacts synergistically with TRAIL, but not with TNFα, in the medulloblastoma cell line DAOY

In order to find out whether NPC 15437 would also sensitize other cancer cells to TRAIL, we extended our study to medulloblastoma, another tumor entity in which the role of PKC in the responsiveness to TRAIL has not yet been investigated. As presented in *Fig. 2a*, non-pretreated DAOY cells displayed some susceptibility to TRAIL (i.e.

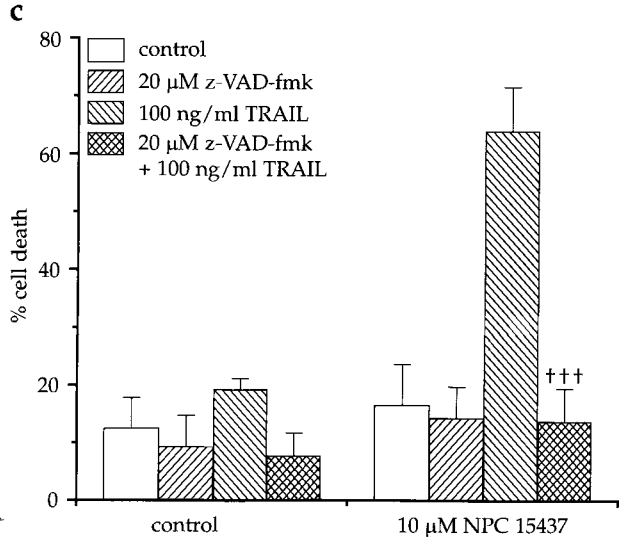


Figure 1. *NPC 15437 augments TRAIL-induced cell death in A549 cells.* ***(a)*** *One h after treatment with NPC 15437, cells were exposed to TRAIL or TNFα for 24 or 48 h. Cell death was determined by cytofluorometric analysis of PI uptake.* ***(b)*** *1 h after treatment with NPC 15437, cells were exposed to TRAIL or TNFα for 48 h. $\Delta\psi_m$ was assessed by cytofluorometric analysis of $DiOC_6(3)$ staining. Mean ± SD of 4 independent experiments are shown (NPC 15437 + TRAIL vs. TRAIL: *p<0.05, **p<0.01).* ***(c)*** *z-VAD-fmk was applied 1 h before treatment with NPC 15437. Mean ± SD of 3 independent experiments are shown (z-VAD-fmk + NPC 15437 + TRAIL vs. NPC 15437 + TRAIL: $^{+++}p<0.001$).*

24% cell death at 100 ng/ml TRAIL for 48 h). In cells pretreated with NPC 15437, TRAIL evoked a strongly increased cytotoxic effect, with up to 76% cell killing. Interestingly, a fully nontoxic dose of NPC 15437 (8 μM) significantly enhanced TRAIL-induced cell death. As observed for A549 cells, NPC 15437 had no effect on the susceptibility of DAOY cells to TNFα. Again, we verified the data obtained by PI uptake analysis by assessing $\Delta\psi_m$, which also revealed a synergistic interaction of NPC 15437 with TRAIL, but not with TNFα (*Fig. 2b*).

Gö6976 does not sensitize A549 cells to TRAIL

NPC 15437 has been described as a PKC inhibitor with some selectivity for nPKC isoform η.[34] However, it also inhibits cPKCs, with an IC_{50} of 19 μM being reported for PKCα.[44] In order to distinguish between cPKCs and nPKCs in the observed sensitization to TRAIL, we made use of the PKC inhibitor Gö6976, which inhibits cPKCs at nanomolar concentrations but does not affect nPKCs even at concentrations up to 3 μM.[24] A549 cells were pretreated with Gö6976 for 1 h, exposed to 100 ng/ml TRAIL for further 48 h, and cell death measured by PI uptake analysis. *Fig. 3* shows that the combination of Gö6976 and TRAIL resulted in not more than additive cytotoxicity.

The PKCε inhibitory peptide myr-PKCε V1-2 synergizes with TRAIL to induce cell death in A549 cells

The above results suggested that the sensitization to TRAIL was based on the inhibition of nPKCs, however, they did not indicate the specific isozyme involved. It has been reported that the nPKC isoform ε protects lung cancer cells against ionizing radiation-[20] and chemotherapy-induced cell death.[6] In order to examine whether PKCε may also be involved in the responsiveness to TRAIL, we engaged a cell-permeable inhibitory peptide specific for PKCε, εV1-2.[18] A549 cells were pretreated with 100 μM εV1-2 for 1 h and exposed to 100 ng/ml TRAIL for additional 48 h. *Fig. 4* demonstrates that εV1-2 strongly amplified TRAIL-triggered cell death: εV1-2 or TRAIL alone elicited cell death in 12% or 15% of cells, respectively, whereas combined treatment resulted in cell killing in 87% of cells.

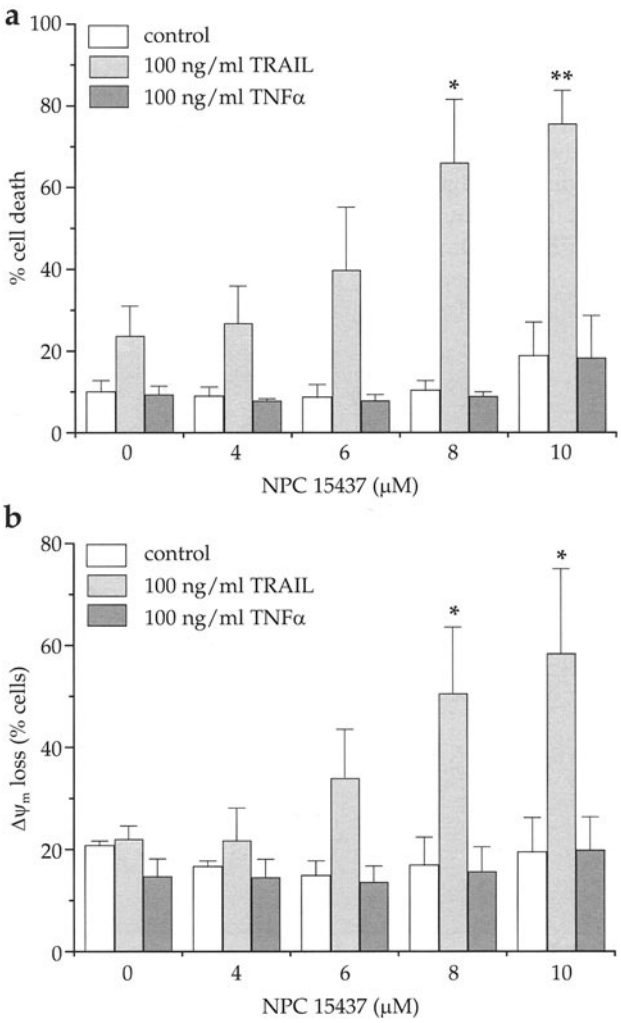


Figure 2. *NPC 15437 augments TRAIL-induced cell death in DAOY cells.* ***(a)*** *One h after treatment with NPC 15437, cells were exposed to TRAIL or TNFα for 48 h. Cell death was determined by cytofluorometric analysis of PI uptake.* ***(b)*** *One h after treatment with NPC 15437, cells were exposed to TRAIL or TNFα for 36 h.* $\Delta\psi_m$ *was assessed by cytofluorometric analysis of* $DiOC_6(3)$ *staining. Mean ± SD of each 4 independent experiments are shown (NPC 15437 + TRAIL vs. TRAIL: *p<0.05, **p<0.01).*

Discussion

Resistance to chemotherapy is the primary cause for treatment failure in advanced, inoperable lung cancer. Recent studies indicate that novel combination-chemotherapy regimes are not likely to make substantial improvements.[4] Resistance to chemotherapy is considered the result of curtailed apoptosis.[19] Chemotherapy activates the apoptotic machinery only indirectly; more effective outcomes might be accomplished by direct induction of apoptosis. In this respect, TRAIL is a very promising candidate since it kills cancer cells while sparing most normal cells.[8,10,22,33] On the other hand, the clinical application of TRAIL may be limited, as many cancer cells display resistance to TRAIL. TRAIL resistance may be overcome by combining TRAIL with chemotherapeutics, which, however, bears the risk of sensitizing otherwise TRAIL-resistant normal cells, or by targeting TRAIL resistance factors. Therefore, the goal is to pinpoint such factors and to develop methods by which they could be directly targeted.

A number of studies have implicated several PKC isoforms in regulation of apoptosis: for example, it has been shown that inactivation of PKCs sensitizes cancer cells to drug-induced apoptosis and, vice versa, that overexpression of PKCs confers protection against apoptosis.[15] In the present study, we demonstrated that inhibition of the PKC isoform ε is accompanied by a substantial sensitization of A549 NSCLC cells to TRAIL. In the beginning, we showed that the PKC inhibitor NPC 15437 significantly enhanced TRAIL-induced cell death. NPC 15437 appeared to be especially suitable for elucidating the potential role of PKC in TRAIL-mediated apoptosis in A549 cells, as it has been described as a relatively selective inhibitor of PKC[44] with an only weak cytostatic effect in A549 cells.[5] It thus could be expected that a potential sensitizing effect of NPC 15437 would not be confounded by its cytotoxic action. Indeed, even at the highest concentration applied, it was scarcely cytotoxic but potently augmented TRAIL-triggered cell death. To discriminate between the PKC isoforms involved, we compared the effect of NPC 15437 to that of another PKC inhibitor, Gö6976. The former has been reported to preferentially inhibit nPKCs[34] while the latter is selective for cPKCs.[24] Treatment with Gö6976 was at most additive to the effect of TRAIL, indicating that the NPC 15437-mediated sensitization to TRAIL was not due to the inhibition of cPKCs.

It is generally acknowledged that there are two major apoptotic pathways, which are functionally separable but molecularly linked: one relies on death signals transduced through death receptors and the other involves the engagement of mitochondria.[19] Although both pathways are originally centered around unique events, they eventually converge on the activation of caspases. TRAIL triggers apoptosis by binding to its receptors but can also harness the mitochondrial pathway of apoptosis.[22] We found that NPC 15437 and TRAIL synergistically affected mitochondrial function, as evidenced by assessing $\Delta\psi_m$, suggesting that PKC acts upstream of mitochondria. In addition, we found that the broad-spectrum caspase inhibitor z-VAD-fmk significantly prevented NPC 15437/TRAIL-induced cell death, indicating that the combinatorial effect of NPC 15437 and TRAIL crucially relied on the activation of caspases.

Consistent with previous studies, we observed complete resistance of A549 cells to TNFα-induced apoptosis. The unresponsiveness of A549 cells to TNFα has been reported to be the result of low TNF receptor expression[38] and has been shown to be overcome by the retinoid-induced upregulation of TNF receptors.[23] NPC 15437 failed to sensitize A549 cells to TNFα, suggesting that death receptor deficiency cannot be overcome by inhibiting PKC. Regarding TRAIL, this observation raises the issue as to whether targeting PKC might also be insufficient if TRAIL resistance is due to TRAIL receptor deficiency. However, this concern is not likely to be relevant with respect to NSCLC cells: in a TRAIL receptor expression study on samples from 87 stage III NSCLC patients, the vast majority expressed at least one of the two death receptors, while only one NSCLC expressed neither TRAIL-R1 nor -R2.[43]

To this point, our results indicated that nPKCs but not cPKCs were important in regulating sensitivity of A549 cells to TRAIL, but the question remained: which of the four nPKC isozymes δ, ε, η, or θ is the crucial one. Evidence suggests that the δ isoform usually functions as a promoter of

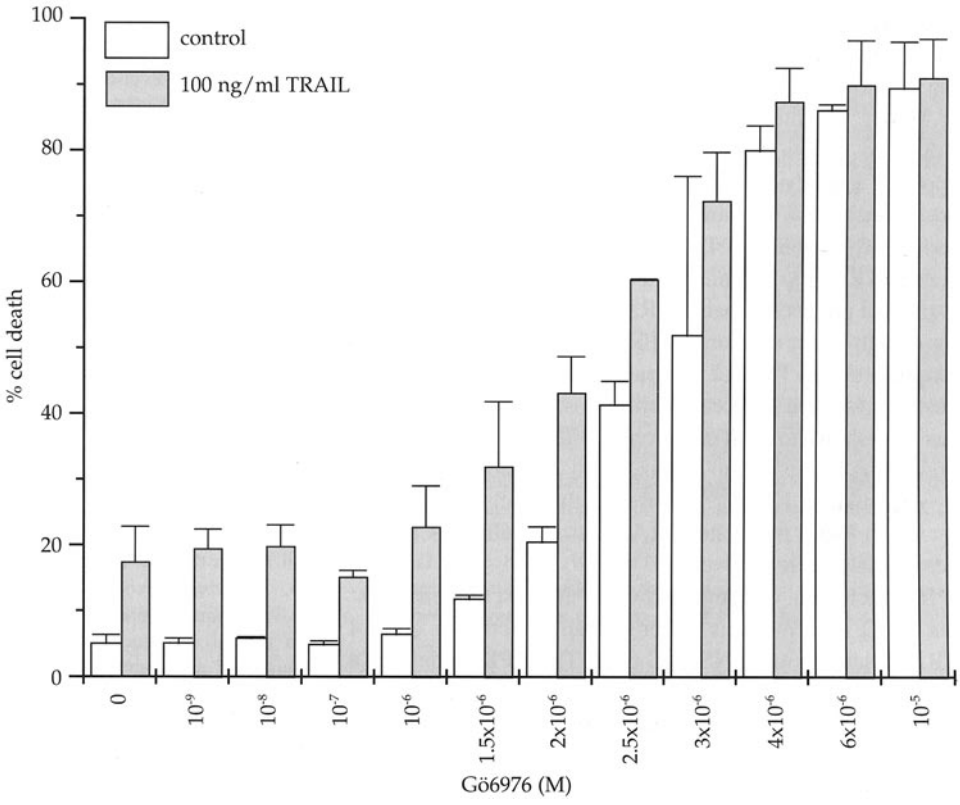


Figure 3. *Gö6976 does not sensitize A549 cells to TRAIL. One h after treatment with Gö6976, cells were exposed to TRAIL for 48 h. Cell death was determined by cytofluorometric analysis of PI uptake. Mean ± SD of 3 independent experiments are shown.*

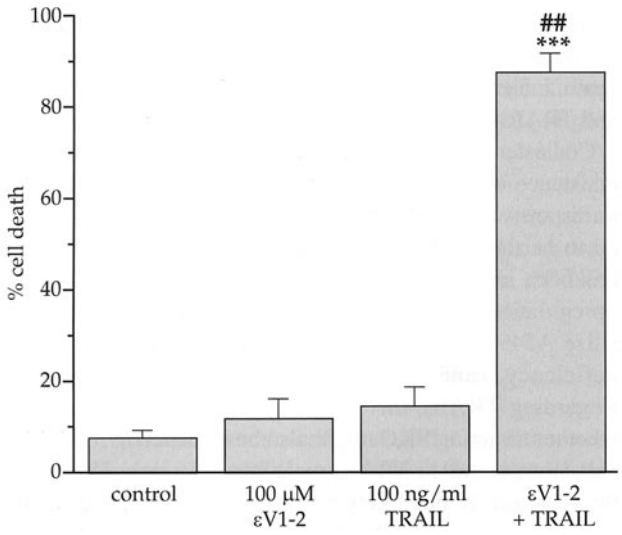


*Figure 4. myr-PKCε V1-2 augments TRAIL-induced cell death in A549 cells. One h after treatment with εV1-2, cells were exposed to TRAIL for 48 h. Cell death was determined by cytofluorometric analysis of PI uptake. Mean ± SD of 3 independent experiments are shown (εV1-2 + TRAIL vs. εV1-2: ##p<0.01; εV1-2 + TRAIL vs. TRAIL: ***p<0.001).*

apoptosis,[15] and the θ isoform could not be detected in A549 cells;[27] therefore, these isoforms did not appear to be plausible candidates. However, the ε isozyme has been reported to protect cancer cells against different cytotoxic insults, including ionizing radiation,[20] cytostatics[3,6] and TNFα.[2] With regard to TRAIL, a protective function of PKCε has been demonstrated in glioma,[29,39] breast cancer[40] and melanoma.[12] By applying a highly selective antagonist for PKCε, the peptide inhibitor εV1-2,[18] we showed here that PKCε is also a TRAIL resistance factor in NSCLC. Because εV1-2 sensitized A549 cells to TRAIL to a similar extent as NPC 15437, these data argue that protection against TRAIL is primarily the result of the activity of the ε isoform of PKC. But how may PKCε confer protection to TRAIL? Several potential explanations have been put forward in recent publications. For example, PKCε has been shown to exert pro-survival effects in cancer cells by preventing the activation of Bax,[12,25] by activating Akt,[29,48] or by upregulating XIAP.[31,37] However, the precise mechanism by which PKCε modulates TRAIL susceptibility has not yet been conclusively resolved.

In conclusion, our study provides the first evidence that the PKC isozyme ε is a considerable factor to control TRAIL sensitivity in NSCLC cells. Thus, PKCε defines a promising target in an adjuvant treatment to exploit the therapeutic potential of TRAIL in NSCLC.

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