

The Phosphatidylinositol 3'-Kinase/Akt Survival Pathway Is a Target for the Anticancer and Radiosensitizing Agent PKC412, an Inhibitor of Protein Kinase C¹

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ABSTRACT

Activation of the phosphatidylinositol 3'-kinase (PI3K)/Akt survival pathway protects against apoptotic stress stimuli. Therefore, compounds that down-regulate this pathway are of clinical interest for single and combined anticancer treatment modalities. Here we demonstrate that the cytotoxic effect of the protein kinase C (PKC)-inhibitor *N*-benzoylated staurosporine (PKC412) is mediated via the PI3K/Akt pathway. Dose-dependent down-regulation of the proliferative activity, activation of the apoptotic machinery, and cell killing by PKC412 (0–1 μ M) in Rat1a-fibroblasts and *H-ras*-oncogene-transformed fibroblasts correlated with a decrease of Akt phosphorylation and a reduced phosphorylation of the endogenous Akt-substrate GSK3- α . Expression of the dominant-active myristoylated form of Akt abrogated this cytotoxic effect of PKC412. Experiments with Apaf-1-deficient cells revealed that PKC412-induced cytotoxicity depends on an intact apoptosome but that the decrease of Akt phosphorylation is not attributable to apoptosis execution. Comparative experiments indicate that PKC412 and the parent-compound staurosporine down-regulate this survival pathway upstream or at the level of Akt but by a different mechanism than the PI3K-inhibitor LY294002. Furthermore, inhibition of this pathway by PKC412 is relevant for sensitization to ionizing radiation. These results demonstrate the specific role of this signaling pathway for the PKC412-mediated down-regulation of an apoptotic threshold and its cytotoxicity.

INTRODUCTION

The PI3K³/Akt pathway is activated in response to as a mediator of growth factor-related survival signals and plays an important role in diverse cellular processes (1, 2). On growth factor stimulation, PI3K phosphorylates membrane phosphoinositides at the 3D position that acts as second messengers to mediate the downstream effects of PI3K. One of the downstream targets is the serine-threonine protein kinase Akt, also referred to as PKB (3, 4). Activation of Akt involves the binding of PI3K-phosphorylated phosphoinositides to the Akt-pleckstrin domain and translocation to the plasma membrane, where Akt is phosphorylated by the PI-dependent kinase PDK1 and an unidentified kinase referred to as PDK2. Phosphorylation of the two phosphorylation sites Thr308 by PDK1 and Ser473 is required for its activation (5–8). Several downstream targets of PKB/Akt have been identified,

e.g., the *bcl-2* family member Bad, caspase-9, and the transcription factor forkhead, all involved in apoptosis regulation, or GSK3 regulating glucose transport and glycogen synthesis (9–11). Akt is also involved in such cell- and stress-specific processes like hypoxia-induced expression of vascular endothelial growth factor. Thus, PKB/Akt is involved in multiple cellular processes, and different cross-talks exist between other growth-promoting and cell survival signaling pathways, but the exact mechanism of their regulation is far from clear (12–18).

Constitutively active PI3K or Akt results in an enhanced protection against apoptotic cellular insults, such as growth factor deprivation, UV irradiation, or loss of matrix attachment (19, 20). Both elevated PI3K and Akt activities have been identified in various tumor types because of amplified gene copy numbers, and this pathway has been linked not only to apoptosis suppression but also to oncogenesis (21–23). Furthermore, inactivating mutations of the lipid-phosphatase and tumor suppressor PTEN that acts as a PI3K signaling antagonist also often correlate with an apoptosis-resistant tumor phenotype (24).

Different inhibitors of the PI3K/Akt pathway are known. Growth factor-induced activation is almost completely prevented by pretreatment of the cells with the specific PI3K inhibitors LY294002 and Wortmannin or by overexpression of a dominant-negative form of PI3K (25, 26). The phosphorylation status of Akt is also modulated in a PI3K-independent way by the phosphatase inhibitors pervanadate and okadaic acid, also suggesting that Akt is a target of protein phosphatase 2A (27–29). Although, these inhibitors are exceedingly toxic for clinical application. Nevertheless, inhibition of PI3K activity by LY294002 abrogates a high apoptotic threshold in otherwise treatment-resistant cancer cells and sensitizes to chemotherapy and radiotherapy (30, 31).

Recently, a cross-talk between the PI3K/Akt pathway and PKC activity has been observed. Overexpression of PKC stimulated Akt activity and suppressed cytokine-dependent apoptosis. On the other hand, the phorbol ester phorbol 12-myristate 13-acetate, an activator of PKC, down-regulates growth factor-induced Akt activation, and specific isoforms of PKC directly interact as negative regulators of Akt (32–34). Thus, PKC inhibitors might be potential modulators of this survival pathway.

A key step for genotoxic- and chemotherapeutic-induced apoptosis is mitochondrial cytochrome *c* release that was originally identified by the PKC-inhibitor STP. PKC inhibitors can induce apoptosis as single agent but also sensitize cells for stress-induced apoptosis when used in combination with other cytotoxic agents (35, 36). Although, it is not clear which cytosolic signaling pathway upstream of cytochrome *c* release is responsible for STP-induced apoptosis. STP displays a potent inhibitory effect on PKC activity; however, STP also reveals unspecific inhibition of other protein kinases. Chemical modification of STP resulted in clinically relevant antineoplastic derivatives, such as PKC412 that is very selective against the conventional α , β , γ -PKC-subtypes and that displays much less unspecific side effects (37, 38). Although attributable to the multitude of PKC

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³ The abbreviations used are: PI3K, phosphatidylinositol 3'-kinase; IR, ionizing radiation; MEF, mouse embryo fibroblast; myrAkt, myristoylated Akt; PKC, protein kinase C; STP, staurosporine; PKC412, *N*-benzoylated staurosporine; PKB, protein kinase B; GSK, glycogen synthase kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; pNA, p-nitroanilide; Apaf-1, apoptosis-associated factor-1; PDK-1, phosphoinositide-dependent kinase-1.

substrates, it is less clear which downstream signaling pathways are responsible for the effect of PKC and its corresponding inhibitors. In this report, we investigated the response of the PI3K/Akt-survival pathway to treatment with the PKC-inhibitor PKC412, especially because down-regulation of the PI3K/Akt-survival pathway might be a valid target to overcome an apoptotic threshold.

MATERIALS AND METHODS

Cell Cultures, Irradiation, and Treatment with PKC412 and LY294002. Rat1a control and myrAkt-transformed Rat 1a cells and *EIA/ras*-wild-type Apaf-1+/+ and Apaf-1-/- MEFs were cultured at 37°C and 5% CO₂ atmosphere in DMEM containing 10% FCS (HyClone Laboratories), supplemented with penicillin and streptomycin. *EIA/ras*-transformed MEFs (39) were cultured in DMEM, 10% FCS, and 10% bovine calf serum (HyClone Laboratories). LNCaP cells were cultured in RPMI 1640 and 10% FCS. Irradiation was carried out at room temperature using a Pantak Therapax 300kV X-ray unit at 0.7 Gy/min. PKC412 (Novartis Pharma, Inc.), STP (Sigma Chemical Co.), and PI3K inhibitor (LY294002; Sigma Chemical Co.) were dissolved in DMSO and diluted in DMEM containing 10% FCS. For combined treatments, cells were preincubated with PKC412 and LY294002, respectively, 1 h before irradiation.

Proliferation and Cell Viability Assay. Tumor cell proliferation was assessed 48 h after treatment with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-like colorimetric alamarBlue assay that is based on the detection of metabolic activity (Biosource International, Camarillo, CA). Absorption was measured at 570 and 600 nm using a Dynatech MR5000 spectrophotometer. For cell viability, floating and adherent cells were collected at the indicated time points, spun down, and resuspended in the corresponding cell media. The cells were then diluted 1:1 with 0.4% trypan blue solution (Sigma Chemical Co.) and scored under a light microscope. The results represent the mean ±SD of two independent experiments, with a minimum of 100 cells scored/treatment.

Akt Stimulation and Western Blotting. Rat1a cells (2×10^5) or *EIA/ras*-transformed MEFs and LNCaP cells (5×10^5) were plated in a 30-mm dish. For Akt stimulation, Rat1a cells were incubated in serum-free DMEM, and after 2 h serum starvation, the medium was replaced by DMEM containing 10% FCS. No serum starvation was necessary for Akt stimulation in *EIA/ras*-transformed MEFs or LNCaP cells. PKC412 or LY294002 was added 5 min after serum stimulation. Cells were harvested at different time points by scraping off the cells in 100 μ l of SDS sample buffer and stored at -80°C. Samples were analyzed by SDS-PAGE followed by blotting onto polyvinylidene difluoride-membranes. Membranes were probed with either rabbit polyclonal anti-Akt, anti-Akt-phosphoThr-308, or Ser-473 (New England Biolabs) sheep polyclonal anti-GSK3- α -phosphoSer-21-antibodies (Upstate Biotechnology). Antibody detection was achieved by enhanced chemiluminescence (Amersham), according to the manufacturer's protocol. Quantification of the blots was performed with Scion Image computer software. All experiments were carried out independently at least three times.

Cell Fractionation. Cells were harvested by centrifugation and washed with ice-cold PBS. The cell pellet was suspended in five volumes of ice-cold buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, 250 mM Sucrose, and 0.1 mM phenylmethylsulfonyl fluoride, supplemented with protease inhibitors (5 μ g/ml pepstatin A, 10 μ g/ml leupeptin, and 2 μ g/ml aprotinin)]. After sitting on ice for 15 min, the cells were disrupted by douncing 15 times in a dounce homogenizer. Cell lysates were centrifuged at 1000 $\times g$ for 10 min at 4°C, and the supernatant was additionally centrifuged at 100,000 $\times g$ for 1 h. The resulting supernatant (S-100 fraction) was stored at -80°C.

In Vitro Caspase Assay. To determine caspase 3-like activity, 50–80 μ g of protein (S-100 fraction) were incubated at 37°C with the colorimetric caspase-3 substrate Ac-DEVD-pNA (100 μ M; Calbiochem) and 1 mM dATP in a final volume of 120 μ l. Control measurements were performed in the presence of the caspase-specific inhibitors Ac-DEVD-aldehyde (20 μ M) to correct for unspecific background activity. The cleavage of the caspase substrates was monitored at 405 nm using a Dynatech MR5000 spectrophotometer.

MyrAkt-overexpressing Cell Lines. ϕ NX-packaging cells were transfected with a total of 8 μ g of pBabe(puro) plasmid DNA or its derivative

(containing the myristoylated/palmitoylated-HA-PKB α DNA construct; Ref. 40) by calcium phosphate coprecipitation as described (41). The medium containing the retrovirus (supernatant) was harvested 48 h post-transfection after the removal of the precipitate and was used to infect the target cells. The *EIA/ras*-transformed MEF target cells were infected at 40% confluence in the presence of Polybrene (4 μ g/ml; Sigma Chemical Co.). Puromycin selection (2 μ g/ml) to enrich a transfected cell population was initiated 30 h after infection and was performed over ≥ 14 days.

RESULTS

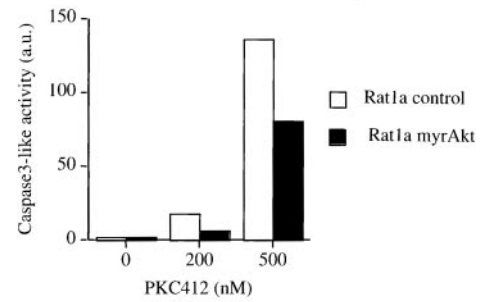
The Akt Pathway Is a Target for the Antiproliferative PKC-Inhibitor PKC412. Activation of the PI3K/Akt survival pathway protects against cellular stress induced by external apoptotic stimuli (e.g., chemo therapeutic agents; References 19 and 32). We investigated the role of this pathway for the antiproliferative and apoptotic effect of the PKC-inhibitor PKC412. PKC412 is a STP-derivative that is very selective against the conventional α, β, γ -PKC isoforms (37). The proliferative activity of Rat1a cells and cells expressing dominant-active myrAkt was tested with the alamarBlue assay in the absence and after treatment with increasing concentrations of PKC412. The alamarBlue assay is a proliferation assay that assesses the metabolic activity comparable with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-tetrazolium-based quantification of cell metabolism. The proliferative index of the two cell populations as determined by cell counting over 48 h were similar in the absence of treatment. After 48 h, the proliferative activity in the Rat1a cell population was decreased in a dose-dependent way (0–1 μ M PKC412), reaching 20% of the activity compared with untreated cells. Interestingly, Rat1a-myrAkt cells were less sensitive to treatment with PKC412. High doses of PKC412 (at ≥ 500 nM) reduced the proliferative activity only to 65% (Fig. 1A). A cytotoxic effect of treatment with low and high dose of PKC412 (200 and 500 nM, respectively) was determined and quantified with the trypan blue exclusion assay. Treatment with 200 nM PKC412 induced minimal cell death in both Rat1a control and myrAkt-expressing cells similar to the minimal antiproliferative effect observed in both cell lines at this concentration. On the other hand, treatment with increased PKC412 concentration (500 nM) that induced a large antimetabolic effect in the Rat1a control cell population but not in myrAkt-expressing cells resulted in massive cell death in the control cells but only low amounts of myrAkt-expressing Rat1a cells were trypan blue positive (Fig. 1B).

To assess whether PKC412 induces apoptosis, the effector protease activity (caspase-3-like/DEVDase activity) was measured in cytosolic S-100 fraction 18 h after treatment. Using Ac-DEVD-pNA as colorimetric caspase-3-substrate, a dose-dependent increase in DEVDase activity was observed both in control and myrAkt-expressing cells. However, PKC412 treatment activated caspase-3 to a much higher level in the Rat1a cells than in the myrAkt-expressing cells (Fig. 1C). Immunoblot experiments with an anticaspase-3 antibody that specifically recognizes the active form of caspase-3 also resulted in enhanced processing of caspase-3 in the Rat1a control cells (data not shown).

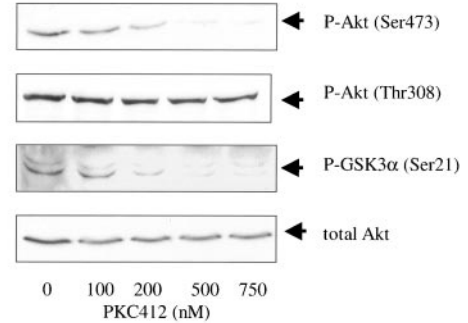
Thus, the strongly reduced proliferative activity in the Rat1a cell population by submicromolar concentration of PKC412 is most probably attributable to apoptosis induction and cell killing by PKC412. Furthermore, these results indicate that expression of a dominant-active form of Akt abrogated, to a large extent, this cytotoxic effect of PKC412.

PKC412 Down-Regulates Phosphorylation of Akt Ser-473. Akt activation is a multistep process and requires phosphorylation of Akt Ser-473. To investigate the effect of PKC412 on the molecular level, the phosphorylation status of Akt Ser-473 and the activity of Akt were determined after treatment with increasing concentrations of PKC412.

C



D



E

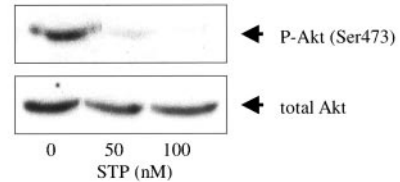
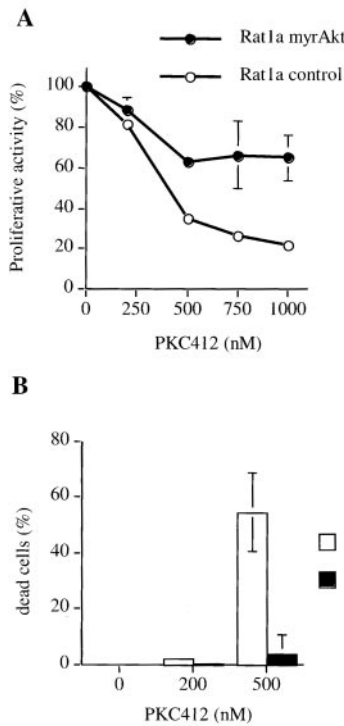


Fig. 1. The antiproliferative PKC-inhibitor PKC412 down-regulates phosphorylation of Akt and GSK3- α . Rat1a cells or Rat1a myrAkt cells were treated with increasing concentrations of PKC412 (A, 0–1 μ M; B–D, 0–0.5 μ M). Proliferative activity (A) and cell viability (B) were determined 48 h after treatment. Caspase-3-like activity was determined 18 h after treatment in cytosolic extracts using Ac-DEVD-pNA as a colorimetric substrate-3 substrate. Caspase-3 activity measurements show results from a representative experiment (C). In D, after serum stimulation, Rat1a cells were treated with increasing concentrations of PKC412 for 5 h. The phosphorylation level of Akt and GSK3- α was determined in whole cell extracts using an anti-Akt phosphoSer-473, phosphoThr-308, or an anti-GSK3- α phosphoSer-21-specific antibody. In E, down-regulation of Akt phosphorylation Ser-473 was also probed after treatment with increasing concentrations of STP (0–100 nM). Samples were reanalyzed with an antibody against total Akt to ensure the loading of equal amounts of protein. All experiments were repeated at least three times.



Rat1a cells were stimulated with serum to activate the growth factor-stimulated PI3K/Akt pathway followed by treatment with PKC412 for 5 h. Whole cell extracts were probed by Western blotting with a Ser-473 site-specific antiphospho-Akt antibody (Fig. 1D). A 50% reduced level of Ser-473 phosphorylation was observed after treatment with PKC412 above 200 nM. Further, the phosphorylation status of Akt Thr-308, which is also critical for Akt activity, was tested with a site-specific antiphosphoThr-308 antibody. Although the effect was not as strong as detected at the Akt-Ser-473 site, PKC412 treatment also induced a dose-dependent down-regulation of Akt Thr-308 phosphorylation at this time point. The same samples were analyzed with a site-specific antiphospho-GSK3- α antibody to detect the Ser-21-phosphorylation status of GSK3- α , an endogenous substrate of Akt. A dose-dependent decrease of GSK3- α phosphorylation was observed on treatment with increasing concentrations of PKC412 (Fig. 1D). The same samples were also analyzed with an anti-Akt antibody to ensure equal protein loading (Fig. 1D). Thus, these results suggest that the cytotoxic effect of the PKC-inhibitor PKC412 is mediated via down-regulation of Akt Ser-473 phosphorylation and concomitant down-regulation of Akt activity.

In parallel, the parent compound and broad-range PKC-inhibitor STP was tested for its effect on Akt phosphorylation. STP also induced a dose-dependent decrease of Akt phosphorylation 5 h after treatment in serum-stimulated Rat1a cells (Fig. 1E), indicative for a general effect of this class of PKC inhibitors. The difference in the potency between STP and PKC412 to down-regulate Akt phosphorylation (50 versus 500 nM, respectively) is in a similar range as also observed in various other PKC-inhibitory assays (42). Additional

experiments were performed with the clinically relevant PKC-inhibitor PKC412.

PKC412 Also Targets the Akt Pathway in H-ras-Oncogene-transformed Cells. Up-regulation of the PI3K/Akt survival pathway often correlates with a cellular stress-resistant phenotype. To detect an antiproliferative effect of PKC412 in cells with an endogenous high Akt phosphorylation and activity level, *E1A/ras*-transformed MEFs were treated with increasing concentrations of PKC412, and the metabolic activity was determined 48 h after treatment with the alamarBlue assay. In *E1A/ras*-transformed MEFs, the equilibrium between unphosphorylated and phosphorylated Akt is shifted toward the active form attributable to a continuous stimulation of PI3K activity by mutated T24 H-ras (43).

The *E1A/ras*-transformed MEFs were also transfected with a high-titer control retrovirus and a retrovirus encoding the dominant-active myrAkt construct. Similar to the Rat1a cells expressing myrAkt, the *ras*-transformed myrAkt-MEFs were more resistant to treatment with PKC412 than cells infected with a control retrovirus. A dose-dependent decrease of the proliferative activity was observed in both control and myrAkt-infected MEF cell populations below treatment with 500 nM of PKC412 that was only slightly more reduced on treatment with higher PKC412 concentrations. Although, the proliferative activity in the myrAkt-expressing cell population remained 50% versus 18% in control-infected cells after treatment with PKC412 (500 nM) in comparison with untreated cells (Fig. 2A). To determine the cytotoxic effect of PKC412 (200 and 500 nM, respectively) on the *E1A/ras*-transformed MEFs, quantitative analysis of the amount of dead cells after treatment was assessed by trypan blue exclusion. Treatment with

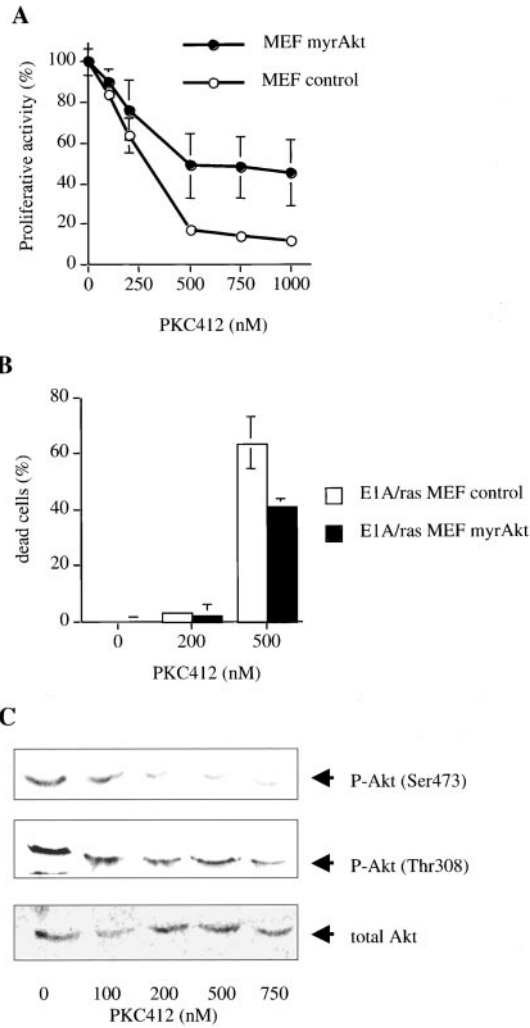


Fig. 2. PKC412 decreases Akt phosphorylation and cell survival in *ras*-oncogene-transformed cells. *E1A/ras*-transformed MEFs carrying a control vector or *E1A/ras*-transformed MEFs expressing dominant-active myrAkt were treated with increasing concentrations of PKC412 (A, 0–1 μ M; B, 0–0.5 μ M). Proliferative activity (A) and cell viability (B) were determined 48 h after treatment. In C, the phosphorylation level of Akt was determined in whole cell extracts by Western blotting using anti-Akt phosphoSer-473 and phosphoThr-308-specific antibodies after cellular treatment with increasing concentrations of PKC412 for 5 h. Samples were reanalyzed with an antibody against total Akt to ensure the loading of equal amounts of protein.

200 nM PKC412 induced minimal cell death in both control and myrAkt-expressing cells similar to the low antiproliferative effect observed in both cell lines at this concentration. On the other hand, treatment with a high PKC412 concentration (500 nM) resulted in massive cell death in the control cells, and expression of dominant-active Akt partially abrogated the cytotoxic effect of this PKC inhibitor, similar to the response observed on the level of proliferation (Fig. 2B).

These results obtained with the Rat1a cells and *E1A/ras*-transformed MEFs and the corresponding myrAkt-overexpressing cells demonstrate that the antiproliferative effect of PKC412 in these cell populations correlates with PKC412-induced cytotoxicity.

To detect whether PKC412 decreases the elevated Akt-phosphorylation status in the *ras*-transformed MEFs, cells were incubated with increasing concentrations of PKC412, and whole cell extracts were analyzed for site-specific Akt Ser-473 phosphorylation 5 h after treatment. A dose-dependent decrease of the phosphorylation of Akt Ser-473 was detected (Fig. 2C) at this time point. In parallel, the phosphorylation status of Akt Thr-308 was also tested with a site-

specific antiphosphoThr-308 antibody. Similar to the Rat1a cell line, down-regulation of the phosphoThr-308 status was also not as strong in these cells as detected at the Akt-Ser-473 site at this time point (Fig. 2C). As a control, the membranes were probed with an anti-Akt antibody to ensure equal protein loading. Likewise, down-regulation of Akt Ser-473 by PKC412 was also induced in isogenic *ras*-transformed but p53-deficient MEFs, indicating that PKC412-mediated phospho-Akt down-regulation is independent of the p53 status (data not shown).

To determine the role of apoptosis as a mode of cell death, *E1A/ras*-transformed wild-type MEFs and *E1A/ras*-transformed MEFs derived from Apaf-1-deficient mice were treated with PKC412. These cells were derived from uncloned mass culture transfection with the two oncogenes *E1A* and T24 H-*ras*. STP-related compounds induce apoptosis via the cytochrome *c*-mediated apoptotic pathway, and Apaf-1 is essential for the integrity of this stress-induced apoptotic signal transduction pathway (44, 45). A dose-dependent increase of cell death was induced by PKC412 in the wild-type cells, whereas the Apaf-1-deficient cells were highly resistant against PKC412 as assessed by trypan blue staining (Fig. 3A). To complement this genetic cell-based approach, caspase-3-like activity was determined in cytosolic S-100 fractions with Ac-DEVD-pNA as colorimetric caspase-3 substrate. Dose-dependent DEVDase activity was induced by

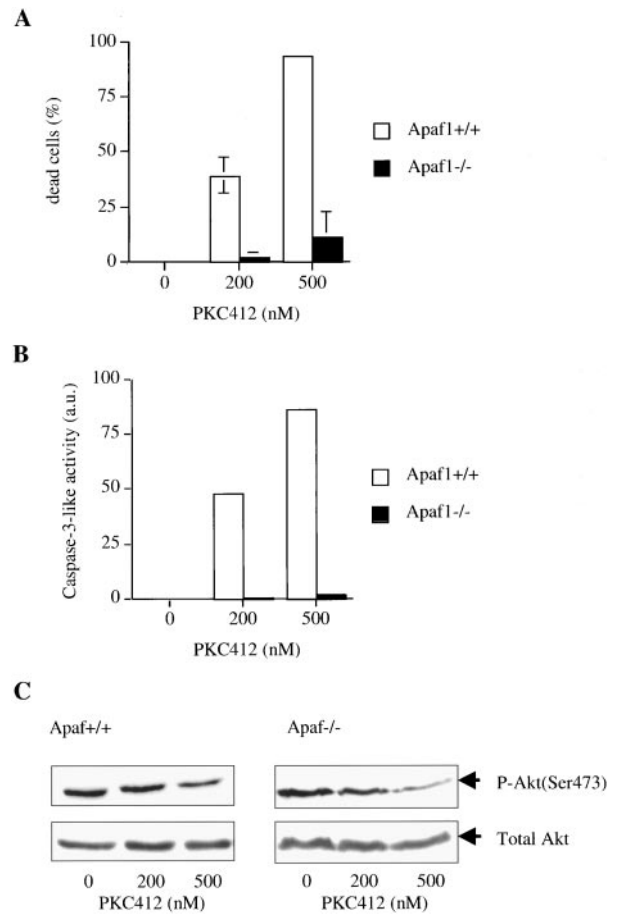
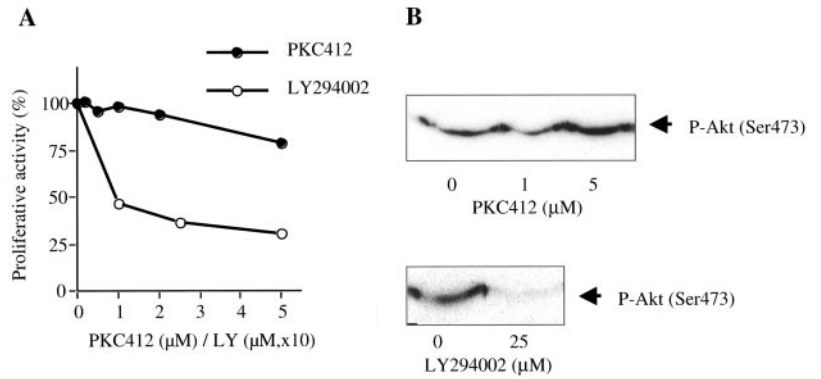


Fig. 3. Phospho-Akt regulation by PKC412 occurs upstream of apoptosis execution. *E1A/ras*-transformed Apaf-1 wild-type and Apaf-1-deficient MEFs were treated with increasing concentrations of PKC412 (0–500 nM). Cell viability was determined by trypan blue exclusion 48 h after treatment (A). Caspase-3-like activity was determined 18 h after treatment in cytosolic extracts using Ac-DEVD-pNA as a colorimetric substrate-3 substrate. Caspase-3 activity measurements show results from a representative experiment (B). The phosphorylation of Akt was determined in whole cell extracts 5 h after treatment using an anti-Akt phosphoSer-473-specific antibody (C). All experiments were repeated at least three times.

Fig. 4. The PI3K-inhibitor LY294002 but not PKC412 compromises proliferation and Akt phosphorylation in PTEN-deficient cells. In A, PTEN-deficient LNCaP prostate carcinoma cells were treated with increasing concentrations of PKC412 (0–5 μM) or LY294002 (0–50 μM), and the proliferative activity was measured 48 h after treatment. Absence of error bars is attributable to minimal SD. In B, cells were treated for 5 h with increasing concentrations of PKC412 (0–5 μM) and for 0.5 h with LY294002 (25 μM). The phosphorylation level of Akt in whole cell extracts was determined by Western blotting using an anti-Akt phosphoSer-473-specific antibody.



PKC412 in Apaf-1 wild type but absent in Apaf-1-deficient cells (Fig. 3B). These results demonstrate that PKC412-induced cytotoxicity in *E1A/ras*-transformed MEFs is mediated via the Apaf-1-associated apoptotic pathway. Down-regulation of the cellular Akt protein level has been observed in different cells as part of a catabolic process attributable to caspase-3 cleavage of Akt (46). To investigate whether PKC412-dependent down-regulation of Akt phosphorylation is attributable to the execution of apoptosis, the phospho Akt-Ser-473 level was determined in the wild-type *E1A/ras*-transformed cells and the Apaf-1-deficient cells that do not undergo apoptosis after treatment with PKC412. PKC412-mediated down-regulation of the phospho Akt-Ser-473 was observed to a similar extent in both apoptosis-prone wild type and apoptosis-resistant cells (Fig. 3C), and the total Akt level was not affected by PKC412 treatment either. These results indicate that phospho-Akt down-regulation by PKC412 does occur upstream of apoptosis induction and does not result as part of apoptosis execution.

An endogenous high Akt phosphorylation and activity level is found in cells with mutated *ras* but can also be attributable to a lack of intact PTEN. PTEN is a phosphatase that acts on the D3 position of phosphatidylinositol (3–5) trisphosphate, a direct product of PI3K activity, and thus, PTEN-mutant cells have elevated 3'-phosphorylated phosphatidylinositol levels. LNCaP prostatic cancer cells are characterized by having a PTEN mutation and a high Akt-phosphorylation status (47). To determine the effect of PKC412 in these cells, LNCaP cells were treated with increasing concentrations of PKC412. Interestingly, the proliferative activity in this cell line was only minimally affected by PKC412 (80% proliferative activity after treatment with 5 μM PKC412), even at concentrations 10 times higher than applied in the Rat1a or *E1A/ras*-transformed cells. On the other hand, the PI3K-specific inhibitor LY294002 reduced the proliferative activity of this PTEN-mutated cell population in a dose-dependent way (Fig. 4A).

In parallel, the Akt-phosphorylation status was also determined in the PTEN-mutated LNCaP cells after treatment with PKC412. Treatment with even high concentrations of PKC412 (1–5 μM) did not decrease the Akt-phosphorylation level (Fig. 4B). On the other hand, treatment of LNCaP cells with the specific PI3K-inhibitor LY294002 resulted in a rapidly (after 0.5 h) reduced level of Akt phosphorylation in these PTEN-inactive cells most probably attributable to the lack of newly generated 3'-phosphorylated phosphatidylinositols (Fig. 4B). The results on the phospho-Akt level correlate with the response to PKC412 on the level of proliferation in these cells.

Down-Regulation of Phospho-Akt by PKC412 Is Different from the Specific PI3K-Inhibitor LY294002. The different response of Akt Ser-473 phosphorylation in LNCaP cells after treatment with LY294002 and PKC412 suggested that the two agents do not act in a similar way. Furthermore, down-regulation of phosphorylated Akt

Ser-473 by PKC412 in Rat1a cells and MEFs was always only observed 4–8 h after treatment. To investigate the time course of this PKC412 effect in more detail, a time course of phospho-Akt-level reduction in *ras*-transformed MEFs was determined by Western blotting after treatment with PKC412 (500 nM) and compared with the effect of the specific PI3K-inhibitor LY294002 (25 μM). Interestingly, treatment with the PI3K-inhibitor LY294002 completely reduced the phospho-Akt level already 0.5 h after treatment, and the phospho-Akt level again increased at later time points in these *ras*-transformed cells (Fig. 5A). On the other hand, down-regulation of the phospho-Akt level by PKC412 was only observed at later time points (Fig. 5B), and the phospho-Akt level remained reduced for several h (data not shown). Likewise, the serum-stimulated phospho-Akt level in Rat1a cells was immediately reduced on treatment with LY294002, and PKC412-mediated down-regulation was only observed at later time points (data not shown). However, treatment with both LY294002 and PKC412 did not affect the elevated phospho-Akt level in *myrAkt*-transformed cells (data not shown).

To determine the effect of PKC412 in *E1A/ras*-transformed cells on the activity of Akt, dose-response and time-course experiments were performed detecting the phosphorylation of the endogenous Akt-substrate GSK3- α . Ser21 site-specific phosphorylation of GSK3- α was reduced in a similar dose response with increasing concentrations of PKC412 as the phosphorylation status of Akt Ser-473 was affected by treatment with PKC412 (Fig. 6 and see Fig. 1D). Likewise, decreased phosphorylation of GSK3- α became prominent only 4 h

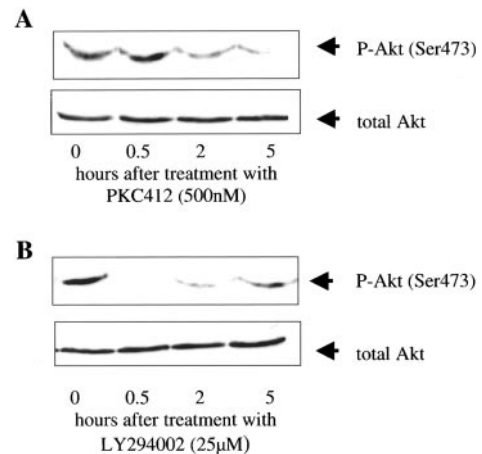


Fig. 5. Down-regulation of phospho-Akt by PKC412 is different from the specific PI3K-inhibitor LY294002. *E1A/ras*-transformed MEF was treated with PKC412 (500 nM; A) or LY294002 (25 μM ; B), and the phosphorylation level of Akt was determined in whole cell extracts after treatment for 0, 0.5, 2, and 5 h by Western blotting using anti-Akt phosphoSer-473-specific antibody. Samples were reblotted with an antibody against total Akt to ensure the loading of equal amounts of protein.

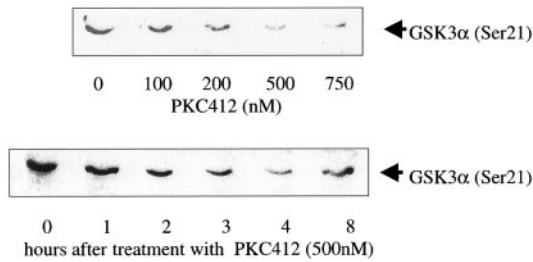


Fig. 6. Down-regulation of the phospho-Akt level by PKC412 correlates with the phosphorylation of the endogenous Akt-kinase substrate GSK3- α . *E1A/ras*-transformed cells were treated with increasing concentrations of PKC412 (0–0.75 μ M) for different time periods (0–8 h), and the phosphorylation level of GSK3- α was determined in whole cell extracts by Western blotting using an anti-GSK3- α phosphoSer-21-specific antibody.

after treatment, and the effect of PKC412 remained for up to 8 h after treatment start (Fig. 6).

Down-Regulation of the PI3K/Akt Pathway Sensitizes for IR. We reported previously that PKC inhibitors sensitize for IR-induced apoptosis in the *E1A/ras*-transformed MEFs, and recently, several reports have described that inhibition of the PI3K/Akt-survival pathway by LY294002 sensitizes for IR-induced cell death (30, 31, 35). Therefore, down-regulation of the phospho-Akt level by PKC412 (200 nM), IR (2 Gy), and after combined treatment was tested in *E1A/ras*-transformed cells. Whereas PKC412 treatment of the cells lowered the endogenous phospho-Akt level, irradiation rather enhanced the phospho-Akt level in these cells (Fig. 7A). Treatment with higher doses of IR did not additionally increase this effect (data not shown). Combined treatment with PKC412 and IR shifted the phospho-Akt level below control levels, suggesting that IR-induced phospho-Akt up-regulation is antagonized by PKC412.

Furthermore, the antiproliferative effect of PKC412, IR, and in combination was tested in *E1A/ras*-transformed MEFs and cells expressing dominant-active Akt. The partial antiproliferative effect of IR and PKC412 treatment alone was similar in both cell populations as also observed at this concentration (200 nM) when testing a dose response against increasing concentrations of PKC412 (see Fig. 2A). Combined treatment with PKC412 and irradiation resulted in at least an additive effect in the *E1A/ras*-transformed cells, but expression of dominant-active Akt abrogated this cooperative effect to a large extent (Fig. 7B). These results suggest that the radiosensitizing effect of PKC412 is at least in part attributable to the down-regulation of the PI3K/Akt-survival pathway.

DISCUSSION

Our studies with the STP-derivative PKC412 show that this protein kinase inhibitor mediates its cytotoxic effect at least in part via down-regulation of the Akt-survival pathway. The cytotoxic effect of PKC412 in nontransformed and oncogene-transformed fibroblasts was rescued in these cells when expressing dominant-active forms of Akt. PKC412 and its parent compound and broad-range PKC-inhibitor STP decreased in a dose-dependent way the site-specific phosphorylation status of Akt, required for its activity, and the subsequent phosphorylation of GSK3- α that serves as an endogenous Akt-kinase substrate. Comparative experiments with the known PI3K-inhibitor LY294002 indicate that PKC412 regulates the phosphorylation status of Akt in a different way than LY294002. Down-regulation of this survival pathway by PKC412 may cooperate with other stress factors to overcome an apoptotic threshold.

Only limited studies investigated the effect of STP or PKC412 on the PI3K/Akt pathway, and mostly elevated concentrations of STP were applied (7, 48–52). PKB/Akt is structurally related to protein

kinase A and C. However, based on the IC₅₀ concentrations required for STP and PKC412 to inhibit purified Akt kinase *in vitro* (>10 μ M), it is unlikely that Akt is directly inhibited by PKC412 *in vivo* as reported here (53). A direct inhibition of the upstream PDK-1 by staurosporine has recently been demonstrated *in vitro* and *in vivo*, but interestingly, inhibition of PDK-1 only affected Thr-308-Akt phosphorylation but not Ser-473 and additionally indicates that Ser-473 is phosphorylated by a thus far unidentified kinase (51). With STP and PKC412, we also observed a decrease of Thr-308 phosphorylation on treatment with PKC412, and the major effect of decreased phosphorylation status was observed on Ser-473. These conflicting results might be mainly attributable to the low PKC-inhibitor concentration range used in this report and time points at which the phosphorylation pattern has been analyzed. On treatment with chemical agents, usually the immediate short-term response on Akt phosphorylation is investigated. In this report, PKC412-mediated decrease of Akt phosphorylation has been shown on the level of endogenous Akt. In contrast to the rapid dephosphorylation of Akt when cells are treated with, e.g., LY294002, the PKC412-mediated effect is delayed and only observed after 4–8 h. This difference between LY294002 and PKC412 is not attributable to minimal inhibitory concentrations of PKC412 used because treatment with also 10 \times higher concentrations of PKC412 did not shorten this kinetic difference (data not shown). It is noteworthy that down-regulation of GSK3- α phosphorylation used as a marker for Akt-kinase activity followed the same time course as PKC412-induced decrease of Akt phosphorylation. Furthermore, the enhanced antiproliferative and cytotoxic effect on treatment with increasing PKC412 concentrations well correlated with the level of phospho-Akt down-regulation. The PKC412-mediated effect on GSK3- α was rescued after an additional 4 h, indicating that PKC412-induced Akt-kinase inhibition is transient. Interestingly, LY294002 also induced only a transient effect on Akt phosphorylation (compare Figs. 4B and 5). Thus, a short inhibition of this pathway might be sufficient for its downstream mediated cytotoxic effect.

Although no thorough analysis against the various PI3K isoforms

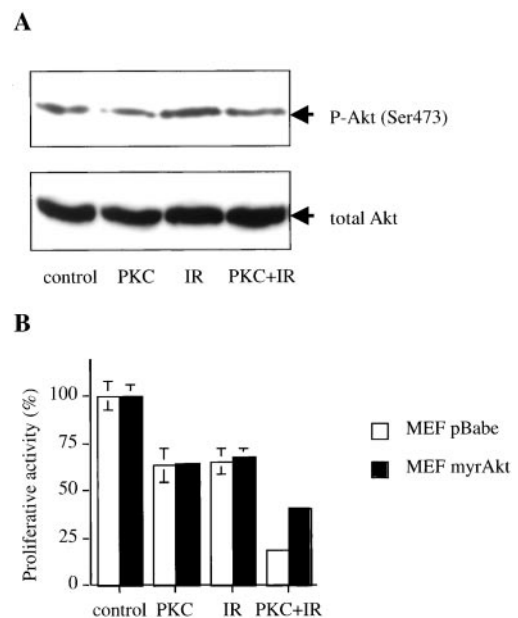


Fig. 7. PKC412 sensitizes for treatment with IR. In A, *E1A/ras*-transformed MEF cells were treated with 200 nM PKC412, 2 Gy of IR, or in combination, and the phosphorylation status of Akt was determined 5 h after treatment in whole cell extracts by Western blotting using an anti-Akt phosphoSer-473-specific antibody. In B, *E1A/ras*-transformed MEFs carrying a control vector and *E1A/ras*-transformed MEFs expressing dominant-active myrAkt were treated with 200 nM PKC412, 2 Gy of IR, or in combination, and the proliferative activity was determined 48 h after treatment.

has been performed, it is unlikely that PKC412 directly inhibits PI3K. Only micromolar concentrations of STP derivatives are reported to inhibit PI3K, and likewise, the PI3K-related ataxia telangiectasia mutated kinase is not inhibited *in vitro* and *in vivo* by PKC412 (54).⁴ LY294002, the specific PI3K inhibitor, and PKC412 have a different kinetic inhibitory pattern to down-regulate Akt phosphorylation *in vivo*. Furthermore, the PI3K-inhibitor LY294002 completely blocked Akt phosphorylation in PTEN-deficient cells most probably attributable to the direct inhibition of 3'-phospholipid generation. However, PKC412 did not decrease Akt phosphorylation and displayed no antiproliferative effect anymore in these cells. These results indicate that LY294002 and PKC412 do not down-regulate the Akt Ser-473 phosphorylation level by the same mechanism and that PKC412 does not directly inhibit PI3K. Interestingly, whereas PKC412 failed to affect the elevated phospho-Akt level in PTEN-mutated cells, PKC412 down-regulated the high phospho-Akt level and Akt activity attributable to H-*ras* mutation. This result points to the use of PKC412 as a therapeutic agent in *ras*-oncogene-transformed cancer cells (31, 55).

Different PKC isoforms interact *in vitro* with the PI3K/PDK1/2/Akt-lipid-mediated multienzyme complex (56) and might affect the phosphorylation status and activity of Akt. Interestingly, the specific PKC ζ isoform can also directly be coimmunoprecipitated with Akt from cell extracts, though this isoform acts as a negative regulator of Akt (34). The affinity of PKC412 toward PKC ζ is in the micromolar range, and this low affinity additionally supports that this isoform is not the responsible target for the observed effect of PKC412. Thus, PKC and its inhibitor modulate this multienzyme complex in a thus far unknown way, and we cannot rule out that the relevant target of PKC412 is not a PKC isoform.

PKC412 induced a dose-dependent cytotoxic effect in Rat1 fibroblasts, and oncogene-H-*ras*-transformed MEFs and PKC412 down-regulated the phosphorylation status of Akt critical for its activity. But PKC412 did not affect the myrAkt-phosphorylation status (data not shown) and displayed only a limited cytotoxicity in these cells expressing a dominant-active form of Akt. Likewise, PKC412 did not affect the phosphorylation status and proliferation of LNCaP cells that are characterized by high Akt-phosphorylation status attributable to a lack of functional PTEN. Thus, a detailed analysis will be required to investigate which factors influence the response to this class of PKC inhibitors and which protein is the ultimate target of PKC412. However, our results demonstrate that the PI3K/Akt-survival pathway is down-regulated by PKC412, and down-regulation contributes to the cytotoxic effect of PKC412. STP- and PKC412-induced cytotoxicity is mediated through the induction of apoptosis in these cells as shown previously and in this report (35). Thus, these results suggest that down-regulation of the PI3K/Akt pathway overcomes an intrinsic apoptotic threshold, and this might be sufficient for itself or support additional PKC412-mediated processes to induce apoptosis. Several downstream targets of Akt have recently been identified that balance cellular homeostasis (reviewed in Ref. 57). These substrates include pro and antiapoptotic components of the apoptotic machinery, such as caspase-9 or the Bcl-2-family member Bad, respectively; GSK3- α ; I κ B kinase, which regulates the nuclear factor κ B; and transcription factors of the forkhead family. We do not know which of these Akt targets is responsible for PKC412-mediated apoptosis induction, though Akt-dependent inhibition of caspase-9 does not seem plausible, because the Akt-specific phosphorylation site is not present in the murine isoform of caspase-9 (58).

Cleavage and degradation of Akt can occur as part of the apoptotic process by activated caspase-3. Apoptosis induction and cell killing

by PKC412 was completely abrogated in Apaf-1-deficient cells that are devoid of an intact apoptosome. These results confirm previous experiments that PKC412-dependent apoptosis is mediated through the cytochrome *c*/Apaf-1/caspase-9/-3 apoptotic pathway, and PKC412-related cytotoxicity occurs via apoptosis in these cells. More important treatment with PKC412 also decreased the phospho-Akt level in the Apaf-1-deficient cells. In wild-type cells, also no DEV-Dase activity could be detected at the 4-h time point at which PKC412 decreased Akt phosphorylation. In addition, the total Akt level was not affected at the different time points of phospho-Akt determination in all cell lines tested. These results demonstrate that PKC412-mediated Akt-dephosphorylation occurs upstream of apoptosis-induction and is not attributable to the activation of the caspase-dependent apoptotic machinery.

Several studies have demonstrated the importance of Akt in cell survival and in response to different apoptotic stimuli, such as growth factor withdrawal, UV irradiation, matrix detachment, and in various differentiated cell types, including cancer cells, *e.g.*, only recently constitutive activation of Akt was demonstrated to promote cellular survival and resistance to combined treatment modalities in non-small cell lung cancer cells. We reported previously that the clinically relevant PKC-inhibitor PKC412 and its parent compound STP sensitize tumor cells *in vitro* and *in vivo* to irradiation and demonstrated that this combined treatment results in the activation of the apoptotic machinery or cell cycle arrest, depending on the genetic background (35, 36). Here we could show that also this sensitizing effect of PKC412 is mediated at least in part via the Akt pathway, suggesting that treatment of PKC412 cells decreases an Akt-regulated stress-response threshold.

STP and PKC412 also induce apoptosis when applied as single agent and also in cells lacking active p53. But such a p53-independent effect requires low micromolar concentrations of these kinase inhibitors (59, 60). Small pharmacological compounds might interfere with multiple cellular pathways, and here we show that the cytotoxic effect of PKC412 is mediated at least in part via inhibition of this specific survival pathway. PKC412 is used for its anticancer activity *in vitro*, and *in vivo* is currently under clinical investigations. It will be important to investigate its activity profile on the molecular level in healthy and malignant cells to understand its enhanced effect in the malignant environment. In addition to Akt inhibition, other growth-promoting and p53-independent, antiapoptotic pathways signaling mechanism might be affected by PKC412 to cooperate to its full effect. Identification of these additional signaling pathways will increase our view of the apoptotic network and its cross-talks with the PI3K/Akt pathway.

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⁴ M. Lavin, personal communication.

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