Statins inhibit Akt/PKB signaling via P2X7 receptor in pancreatic cancer cells

Oras Mistafa, Ulla Stenius *

Institute of Environmental Medicine, Karolinska Institutet, S-171 77 Stockholm, Sweden

ARTICLE INFO

ABSTRACT

Cholesterol-lowering statins have been shown to inhibit growth of pancreatic cancer cells *in vitro* and *in vivo*. Epidemiological studies also indicate a chemopreventive effect of statins. We have investigated the effect of statins on Akt/protein kinase B signaling. We found that atorvastatin decreased constitutive- and insulin-induced pAkt in Panc-1 and MIA PaCa-2 cells. Statins also inhibited pAkt in combination with gemcitabine- and 5-fluorouracil, and sensitized cells to gemcitabine- and 5-fluorouracil-induced apoptosis and inhibition of cell proliferation. In line with our previous data, it was found that the P2X7-purinergic receptor mediated the effects of statins in Panc-1 and MIA PaCa-2 cells. Thus, experiments employing P2X7 siRNA and inhibitors supported an involvement of P2X7. In Capan-2 cells, which expressed P2X7 in low levels, statins did not reduce pAkt levels nor did statins sensitize them to cytostatic drugs. However, statin inhibited the growth of Capan-2 cells and this correlated to inhibition of NFκB and Raf/MEK pathways. As shown previously, these latter effects can be explained by an inhibited protein prenylation. Our data suggest that statins primarily target a functional P2X7-Akt signaling in pancreatic cancer cells. By targeting the P2X7-Akt axis, statins can sensitize pancreatic cancer cells to chemotherapeutic drugs. Our data are also in line with a role for P2X7 in the chemopreventive effect of statins on pancreatic cancer.

1. Introduction

Pancreatic cancer is resistant to conventional radio- and chemotherapy and is associated with poor prognosis. Improvement of therapies targeting specific signaling pathways in this tumor type is thus urgently needed. 3-Hydroxy-3-methyl-glutaryl-CoA reductase inhibitors, statins, have anticancer effects in different *in vitro* and *in vivo* models [1,2]. Several studies show that statins inhibit growth of pancreatic cancer cell lines *in vitro* and sensitize them for cytostatic drugs [3–6]. Gemcitabine, which is presently used for the treatment of pancreatic cancer, is one of these drugs. Thus, fluvastatin was shown to enhance the antiproliferatory effect of gemcitabine in MIA PaCa-2 pancreatic cancer cells [5]. Besides their *in vitro* effects, statins have been shown to inhibit pancreatic tumor growth *in vivo* [3,5,7]. In a recent study performed on nude mice it was shown that different statins decreased the tumor growth of transplanted Capan-2 cells [7]. This effect was explained by an inhibited cholesterol synthesis and an inhibited prenylation of signaling proteins such as Ras.

Statin-induced anticancer effects on pancreatic cancer progression are supported by epidemiological data [6,8–10]. A recent study indicate a dramatic (<50%) risk reduction in metastatic or fatal prostate cancer among statin users [9]. An even stronger effect was registered in a case-control study, involving about half a million veterans. In this study it was found that four years on statins reduced the risk of pancreatic cancer by 80% [10]. Together these data indicate that statins strongly affect pancreatic cancer cells and preneoplastic cells in pancreas. However, mechanisms for these anticancer effects are still not well characterized.

There are three major signaling pathways commonly altered in pancreatic cancers [11]. These include phosphatidylinositol 3-kinase- (PI3K/Akt), nuclear factor kappa-B- (NF-κB), and mitogen-activated protein kinase (MAPK) pathways [12–14]. We have previously shown that statins inhibit one of these pathways, PI3K/Akt signaling, in different cell lines [15,16]. Thus, incubation with statins decrease the levels of phosphorylated Akt in the cytoplasm and the nucleus and sensitized HepG2 and A549 cells to cytostatic drugs [16].

The Akt pathway is one of the major anti-apoptotic factors in cells. Akt is activated by growth factors and cellular stress and is

commonly overexpressed in pancreatic cancer, but is also often induced by cytostatic treatment [12,17,18]. In addition, PI3K/Akt has been implicated in the resistance of pancreatic cancer to gemcitabine [19]. Therefore, Akt is an attractive target for cancer therapy and in particular for pancreatic cancer.

Our recent data show that the statin-induced inhibition of nuclear pAkt is mediated via the P2X7-purinergic receptor in A549 lung cancer cells. Furthermore, we found evidence that P2X7 receptors, when activated by its natural ligand ATP, can regulate nuclear pAkt in epithelial cells [20]. P2X7 is expressed in pancreatic cells and is activated by extracellular purinergic nucleotides such as ATP. P2X7 receptor activation might stimulate pleiotropic cellular effects including the release of pro and inhibitory inflammatory cytokines [21,22]. Increased levels of P2X7 were recently detected in chronic pancreatitis and pancreas cancer indicating a possible involvement of P2X7 receptors in pancreatic cancer development [23].

In the present study, we have investigated the effects of atorvastatin on pancreatic cancer cell lines. We report that pharmacologically relevant concentrations of statins decrease the levels of pAkt in Panc-1 and MIA PaCa-2 cells and sensitize them to gemcitabine and 5-Fu. We also report that these effects are mediated by P2X7. On the other hand, in Capan-2 cells, with low expression of P2X7, statins did not affect pAkt levels and did not sensitize them to cytostatic drugs.

2. Materials and methods

2.1. Cell culture

Human pancreatic cell lines, Panc-1, Capan-2 and MIA PaCa-2, were obtained from the American Type Culture Collection, ATCC (Manassas, VA, USA). Capan-2 is well- to moderately well-differentiated ductal adenocarcinoma cell line expressing wt p53, while MIA PaCa-2 and Panc-1 are poorly differentiated ductal adenocarcinoma cell lines with mutant p53. Panc-1 and MIA PaCa-2 cells were maintained in Dulbecco's modified Eagle's medium (ATCC) while Capan-2 cells were maintained in McCoy's 5A Modified Medium (ATCC). Media was supplemented with 10% fetal calf serum and penicillin-streptomycine. Serum-starved cells were cultured in medium supplemented with 0.1% serum for 24 h. Cells were exposed for atorvastatin for 15 min–1 h to study the P2X7-mediated effects on Akt phosphorylation, while 24 or 48 h incubation time was used for detection of effects on cell proliferation or apoptosis. Human embryonic kidney (HEK) 293 cells stably expressing human P2X4 and P2X7 were kindly provided by A. Surprenant, Sheffield University, UK. HEK293 cells were grown in DMEM: F12 with 1 mM L-glutamine, 10% inactivated calf serum and 300 μg/ml G418. A549 cells were obtained from ATCC.

2.2. Reagents

Atorvastatin was provided by Pfizer (New York, NY). Pravastatin, LY294002, 5-fluorouracil (5-Fu), adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-triphosphate periodate oxidized sodium salt (o-ATP), 2′(3′)-O-(4-benzoyl–benzoyl) adenosine 5′-triphosphate triethylammonium salt, mixed isomer (BzATP) and KN-62 was purchased from Sigma–Aldrich (St. Louis, MO) and gemcitabine from Eli Lilly (Indianapolis, IN). The final concentration of DMSO (Sigma–Aldrich, St. Louis, MO) added to the cells was <0.2%. No effect of DMSO was observed.

2.3. Western blotting

Western blotting was performed as previously [17]. In brief the samples were subjected to SDS–PAGE and thereafter blotted onto a PVDF membrane (Bio-Rad, Hercules, CA). Some samples were subfractionated. The protein bands were probed using antibodies against Akt, Akt phosphorylated at residues Ser473 or Thr308, α-tubulin, ERK phosphorylated at residue Tyr208, survivin, PARP, P2X7 and Cdk2 from Santa Cruz (Santa Cruz, CA); glycogen synthase kinase 3 beta (GSK3β) phosphorylated at residue Ser9, pMEK1/2 Ser217/221, NFκB p65, pRaf Ser259 and p70 S6K Thr386 from Cell Signaling (Beverly, MA); cyclin D1 from Oncogene (Uniondale, NY). pAkt Ser473 antibody gave rise to a double band which consists of Akt1 and Akt2 and both have been used for evaluations. Proteins were visualized with ECL procedure (Amersham Biosciences, Uppsala, Sweden). The Western blot results were analyzed with NIH Image 1.62 software.

2.4. Quantification of apoptosis

Morphological evaluation of apoptotic cell death was performed by Hoechst staining. Cells were fixed in methanol for 1 h. After fixation the cells were stained with Hoechst 33342 (Sigma–Aldrich, St. Louis, MO) for 5 min. Nuclear morphology was then evaluated using fluorescent microscope. Cells whose nucleus exhibited brightly stained, condensed chromatin or nuclear fragments were designated apoptotic. The percentage of cells with apoptotic morphology was determined by counting at least 1000 cells per plate.

2.5. Cell viability and cell proliferation

Viability of cells was determined by the trypan blue exclusion assay. Cell proliferation was determined by 3-(4,5-dimethylthia-zol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay detecting the cellular mitochondrial capacity to convert MTT tetrazolium salt to formazan. Cells were incubated with the medium containing MTT (Sigma–Aldrich, St. Louis, MO) for 4 h. The cells were then lyzed in DMSO. The absorbance was measured at 570–620 nm.

2.6. Small interference RNA transfection

Cells were transfected with P2X7 small interference RNA (siRNA) (Cell signaling Technology, Beverly, MA) for 72 h according to the TranIT-TKO protocol from the manufacturer (Mirus, Madison, WI). Control siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, Santa Cruz, CA).

2.7. Immunocytological staining

Cells were fixed in 3.7% formaldehyde. After fixation the cells were stained with polyclonal antibodies against P2X7 and NFκB. After incubation with primary antibodies, secondary antibody conjugated with FITC (Dako, Glostrup, Danmark) was applied. No staining was detected when the primary antibodies were omitted. The staining intensity was analyzed with NIH Image 1.62 software.

2.8. Statistical analysis

Statistical analysis was conducted using student's t-test and two-way ANOVA. The data were presented as mean ± SD. Experiments were performed at least three times with different batches of cells. Results were considered to be statistically significant at $P \leq 0.05$.

3. Results

3.1. Statins decrease pAkt levels and inhibit cell proliferation in Panc-1 cells

The PI3K/Akt pathway has been implicated in the resistance of pancreatic cancer to cytostatic drugs [19] and we have recently
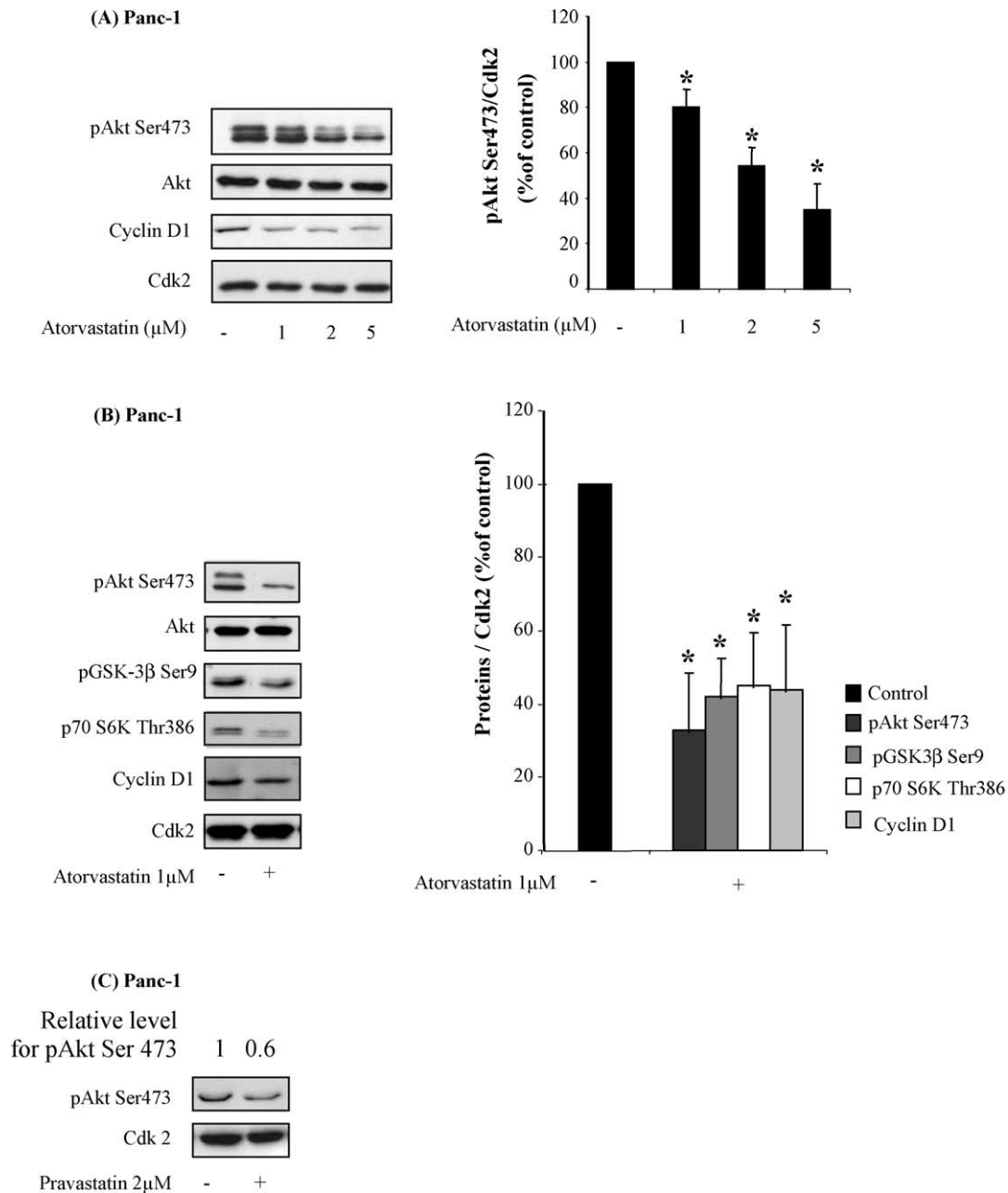
Fig. 1. Statins decrease pAkt levels, inhibit cell proliferation and induce apoptosis in Panc-1 cells. Panc-1 cells were treated with atorvastatin or pravastatin, at the concentrations indicated for 1 h (A), 24 h (B and C) or for times indicated (D). Samples were then analyzed by Western blotting, employing antibodies for Akt, pAkt Ser473, cyclin D1, pGSK-3β Ser9, p70 S6K Thr386, PARP and α-tubulin. Cdk2 was used as a loading control. Cell proliferation was estimated using MTT assay (D). Columns, mean ± SD from three independent experiments; *significantly different from corresponding samples without atorvastatin, $P \leq 0.05$. (E) Serum-starved cells were treated with insulin for 15 min and thereafter with atorvastatin for 15 min or 1 h in the concentrations indicated. (A, B, and E) Columns, mean ratio from densitometric analysis of pAkt Ser473/Cdk2, pGSK-3β Ser9/Cdk2, p70 S6K Thr386/Cdk2 and cyclin D1/Cdk2 from three independent experiments; mean ± SD from three independent experiments; *significantly different from corresponding control, $P \leq 0.05$.

shown that statins readily inhibit Akt in cancer cells [16]. Therefore, the effect of atorvastatin on the constitutive level of phosphorylated Akt (pAkt) was examined in Panc-1 cells. We found that incubation of cells with atorvastatin (1 μM for 1 or 24 h) decreased a high constitutive level of Akt phosphorylation at residue Ser473 (pAkt Ser473) (Fig. 1A and B). This reduction was associated with decreased levels of downstream targets, GSK3β phosphorylated at residue Ser9 (pGSK3β Ser9) and p70 S6K phosphorylated at residue Thr386 (p70 S6K Thr386) (Fig. 1A and B). Also cyclin D1 was reduced by atorvastatin (Fig. 1A and B). The reduction of pAkt Ser473 was also induced by pravastatin (Fig. 1C). Cyclin D1 has been implicated in cell cycle regulation. As shown by

densitometric analysis the level of pAkt correlated with pGSK3β Ser9, p70 S6K Thr386 and cyclin D1 levels (Fig. 1B). Total Akt levels were not affected (Fig. 1A and B). The effect of atorvastatin on cell proliferation was also studied. Incubation with 1–4 μM of atorvastatin for 48 h inhibited cell proliferation dose-dependently (Fig. 1D). PARP-assay was used to detect effects on apoptosis. As shown in Fig. 1D, a dose-dependent increase in the levels of PARP cleavage was detected when cells were incubated with atorvastatin for 24 h.

Growth factors induce the phosphorylation of Akt and the effects of statin on insulin-induced pAkt was studied. As shown in Fig. 1E preincubation of cells with atorvastatin for 1 h decreased
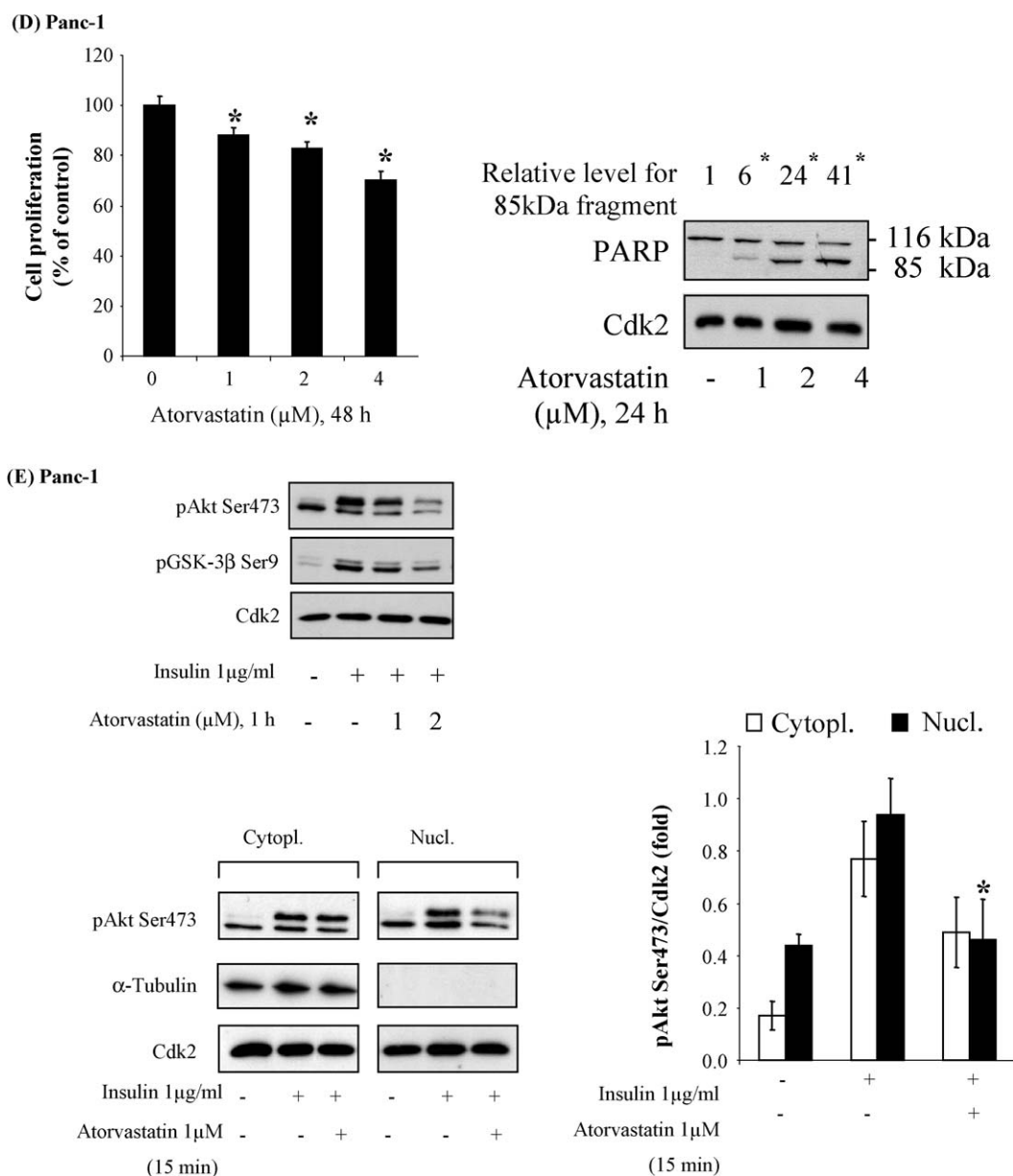
Fig. 1. (Continued).

the level of insulin-induced pAkt Ser473 and its downstream target pGSK3β Ser9. Nuclear translocation of Akt is crucial for its activity [24]. Several nuclear targets for Akt involved in the cell cycle regulation have been identified and exclusion from the nucleus may thus restrain the kinase activity on targets involved in regulation of the cell cycle [25]. To test whether statins could affect the nuclear localization of pAkt, Panc-1 cells were fractioned. We found that insulin-induced nuclear pAkt levels decreased within 15 min after addition of atorvastatin (1 μM) (Fig. 1E). The purity of the cytoplasmic extract was confirmed by analyzing α-tubulin. LY294002 (25 μM), a PI3K-inhibitor, was used as a control and prevented the insulin-induced nuclear pAkt accumulation (data not shown). Together, these data show that atorvastatin attenuate Akt signaling in Panc-1 cells.

3.2. Atorvastatin sensitizes Panc-1 cells to gemcitabine- and 5-fluorouracil

Gemcitabine is used to treat pancreatic cancer and we investigated whether statins affected pAkt or enhance the

efficiency of gemcitabine in Panc-1 cells. We found that gemcitabine significantly decreased phosphorylation of Akt at residue Ser473 and its downstream target p70 S6K Thr386. When cells were treated with atorvastatin pAkt was further reduced (Fig. 2A). Additional finding was that 5-Fu decreased pAkt and also this effect was potentiated by incubation with atorvastatin (Fig. 2B).

The effect of atorvastatin on the antiproliferative effect of gemcitabine and 5-Fu was studied. Cells were pretreated with atorvastatin for 1 h and thereafter exposed with gemcitabine or 5-Fu for 48 h. As expected, both drugs alone induced a significant inhibition of cell proliferation (Fig. 3A). As shown in Fig. 3A, 1 h pretreatment with atorvastatin strengthened the gemcitabine- and 5-Fu-induced inhibition of cell proliferation over a broad concentration range. In these experiments low concentrations of gemcitabine and 5-Fu were used and the cells were incubated for 48 h. The pretreatment of atorvastatin combined with 0.01 μM gemcitabine induced a similar inhibition of cell proliferation as 0.1 μM. This means a 10 times increased efficiency in this experiment (Fig. 3A).
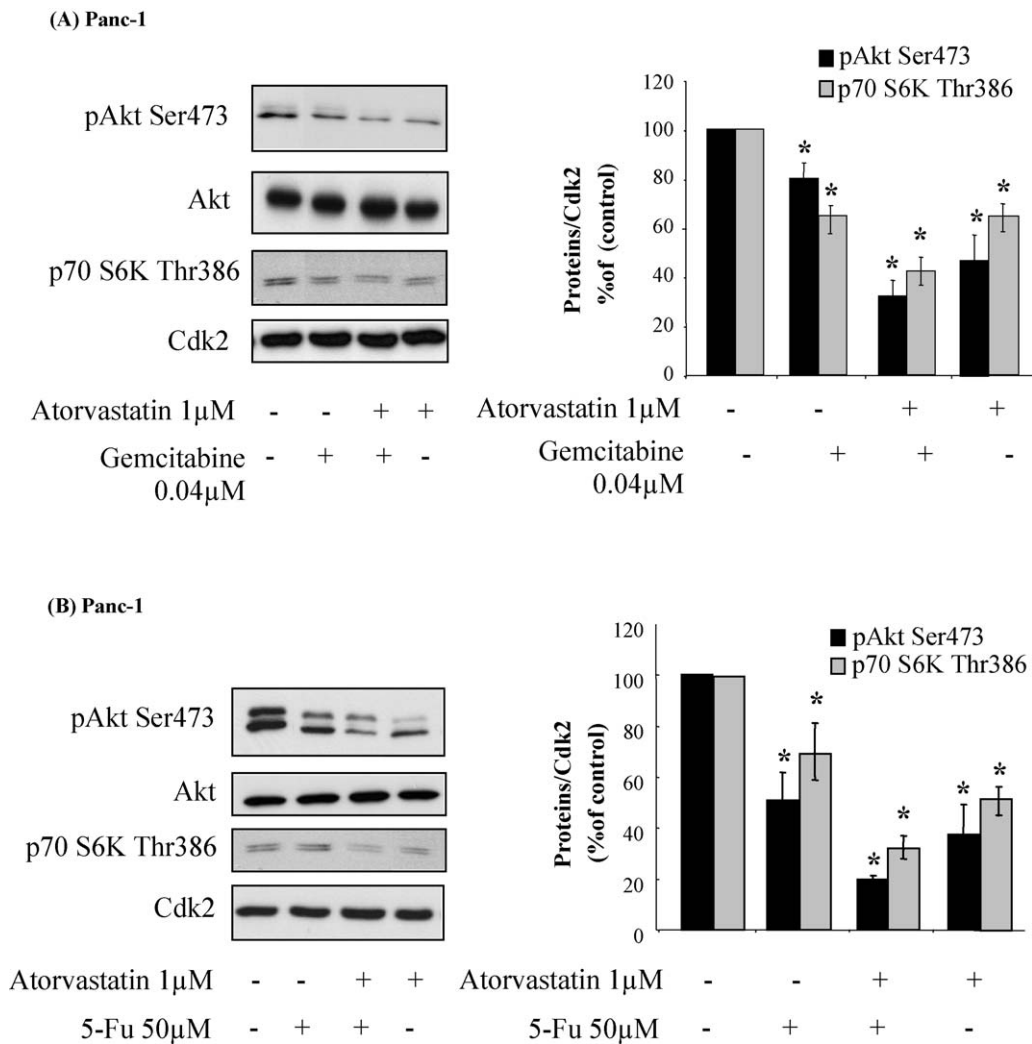
Fig. 2. Atorvastatin, gemcitabine and 5-fluorouracil induce increased effect on pAkt in Panc-1 cells. (A and B) Cells were treated with atorvastatin (1 μM) for 1 h and thereafter with gemcitabine (0.04 μM) or 5-Fu (50 μM) for 24 h. Samples were then analyzed by Western blotting, employing antibodies for Akt, pAkt Ser473 and p70 S6K Thr386. Cdk2 was used as a loading control. Columns, mean ratio from densitometric analysis of pAkt Ser473/Cdk2 and p70 S6K Thr386/Cdk2 from three independent experiments; mean ± SD from three independent experiments; *significantly different from corresponding control, $P \le 0.05$.

We next studied the effect of atorvastatin on gemcitabine- and 5-Fu-induced apoptosis. Apoptosis was assayed by Hoechst staining and by cleavage of PARP. As shown in Fig. 3B, the combination of atorvastatin (4 μM) and gemcitabine (0.04 μM) or 5-Fu (5 μM) significantly increased the level of apoptosis. 5 μM 5-Fu induced a minor effect on PARP (data not shown) and therefore higher concentrations were used to study the effect on PARP. As shown in Fig. 3C atorvastatin (4 μM), gemcitabine (0.04 μM) or 5-Fu (50 μM) resulted in cleavage of PARP and this effect was strengthened by combining atorvastatin with cytostatic drugs. Fig. 3C also shows the densitometric analysis of 85 kDa PARP fragment.

The effect of atorvastatin on the antiproliferative effect of LY294002 (PI3K-inhibitor) was also studied in Panc-1 cells (Supplemental data 1). In these experiments low concentrations of LY294002 (non-toxic concentration and without any detectable effect on pAkt) were used. Treatment of cells with the combination of atorvastatin (1 μM) and LY294002 (2.5 μM) significantly decreased the level of pAkt Ser473 and reduced growth of Panc-1 cells. Taken together, atorvastatin increased the effect 5-Fu or gemcitabine on cell proliferation and apoptosis in pancreatic cancer cells.

3.3. Statins decrease pAkt and increased the effect of gemcitabine in MIA PaCa-2, but not in Capan-2 cells

To study the cell specificity of the effect of statins we also studied two additional pancreatic cancer cell lines, Capan-2 and MIA PaCa-2. As shown in Fig. 4A atorvastatin and pravastatin decreased the constitutive level of pAkt Ser473 in MIA PaCa-2 cells. Furthermore, in this cell line increased inhibition of cell proliferation was induced by atorvastatin and gemcitabine (Fig. 4B). Atorvastatin also induced PARP cleavage (Fig. 4C). In Capan-2 cells incubation with atorvastatin or pravastatin did not decrease the level of pAkt and no increased inhibition of cell proliferation was induced by statins in combination with low doses of gemcitabine (Fig. 4A and B). However statins induced cleavage of PARP after 24 h (Fig. 4C).

3.4. Effects of atorvastatin in Panc-1 and MIA PaCa-2 are mediated by P2X7 receptors

We have previously shown that statins regulate nuclear pAkt via P2X7-purinergic receptor in lung cancer cells [20]. We also documented a statin-induced nuclear localization of the antigen in
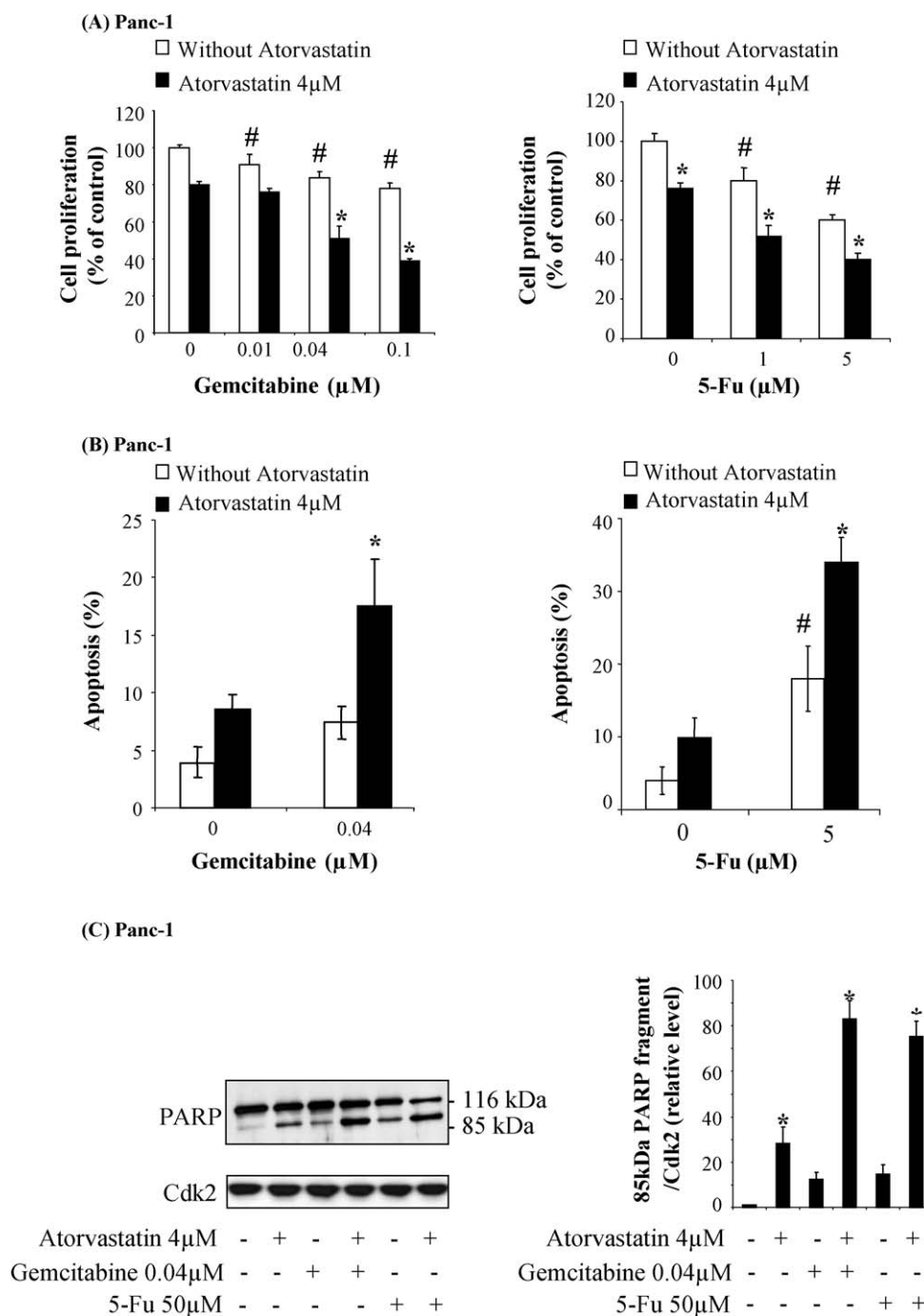
Fig. 3. Antiproliferative and apoptotic effects of combined treatment of gemcitabine or 5-fluorouracil and atorvastatin in Panc-1 cells. Cells were treated with atorvastatin (4 μM) for 1 h and thereafter with gemcitabine or 5-Fu for concentrations indicated for 48 h (A and B) or with gemcitabine (0.04 μM) or 5-Fu (50 μM) for 24 h (C). Cell proliferation was estimated using MTT assay (A). Columns, mean ± SD from three independent experiments; #significantly different from corresponding control, *significantly different from atorvastatin, $P \leq 0.05$. The percentage of apoptotic cells was quantified using Hoechst staining after 24 h (B). Samples were also analyzed by Western blotting, employing antibody for PARP (C). Cdk2 was used as a loading control. In panel (C) columns show densitometric analysis; mean ± SD from three independent experiments; *significantly different from corresponding control, $P \leq 0.05$.

rat hepatocytes *in situ*, indicating that statins may affect the P2X7 receptor *in vivo* [20]. We investigated the role of the P2X7 receptor in statin-induced effects in Panc-1 cells. The P2X antagonist, oxidized-ATP (o-ATP), and a more selective inhibitor of P2X7 receptor, KN-62, were tested [26]. As shown in Fig. 5A preincubation with both inhibitors abrogated the atorvastatin-induced effect on pAkt Ser473. The inhibitors alone did not have any effect on pAkt (data not shown). Next, the P2X7 agonists, ATP and a more selective agonist BzATP were tested. As shown in Supplemental

data 2 the levels of pAkt was decreased in Panc-1 and MIA PaCa-2, but not in Capan-2 cells. Further, BzATP increase the effect of gemcitabine in Panc-1, MIA PaCa-2, but not in Capan-2 cells (Supplemental data 2). Thus ATP and BzATP mimicked the effects of statins.

In another series of experiments Panc-1, Capan-2 and MIA PaCa-2 cells were transfected with siRNA against P2X7 for 72 h and thereafter incubated with atorvastatin for additional 48 h. As shown in Fig. 5B siRNA transfection decreased the level of P2X7
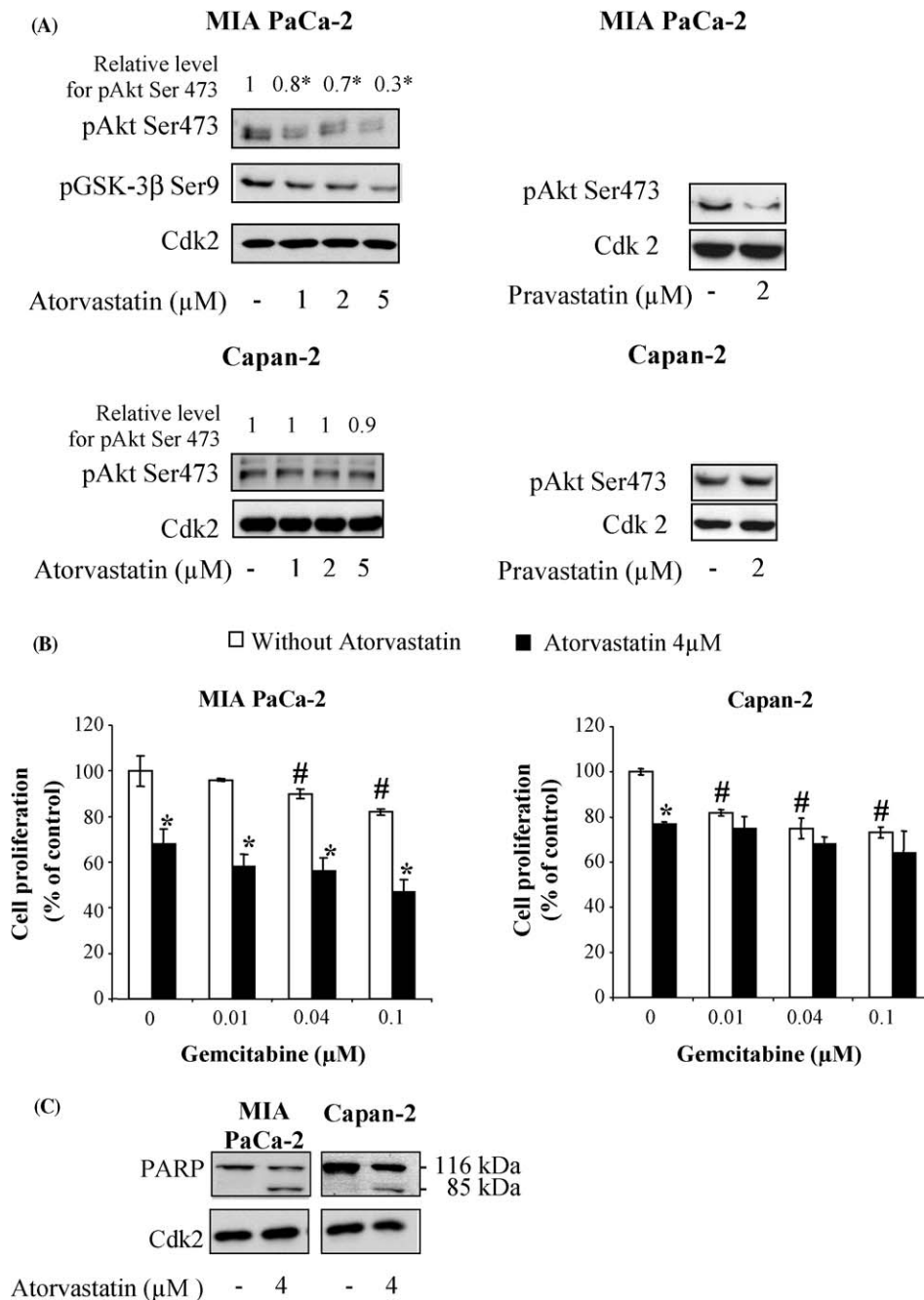
Fig. 4. Statins decrease pAkt and increase the effect of gemcitabine in MIA PaCa-2, but not in Capan-2 cells. In (A and C), cells were treated with atorvastatin or pravastatin for 1 or 24 h in concentrations indicated. Samples were analyzed by Western blotting, employing antibodies for pAkt Ser473, pGSK-3β Ser9 and PARP. Cdk2 was used as a loading control. In (B), cells were treated with atorvastatin for 1 h and thereafter with gemcitabine or 5-Fu for 48 h. Cell proliferation was estimated using MTT assay. Columns, mean ± SD from three independent experiments; #significantly different from corresponding control, *significantly different from atorvastatin, $P \leq 0.05$.

and prevented the effect induced by atorvastatin on cell proliferation in Panc-1 and MIA PaCa-2 but not in Capan-2 cells (Fig. 5B). Similar effects as induced by siRNA were induced by KN-62 (Fig. 5C). KN-62 did not affect the antiproliferative effect of gemcitabine (data not shown). To determine whether differences in response might be explained by differences in receptor levels, P2X7 was analyzed by Western blotting and immunocytology. 2-Fold, 4-fold and 10-fold dilutions of Panc-1 samples were ran alongside to compare band intensities between different cell lines. Capan-2 cells, in contrast to Panc-1 and MIA PaCa-2 cells, exhibited a low level of the P2X7 receptor (Fig. 5D). The specificity of the antibody was confirmed by using HEK293 cells heterologously expressing human P2X7 or P2X4 receptors (Fig. 5D). No binding

was detected in HEK293 cells heterologously expressing human P2X4 receptors. This was also supported by immunocytological staining. As shown in Fig. 5E staining for P2X7 was detected in Panc-1 and MIA PaCa-2, while a faint staining concentrated in droplets in the cytoplasm was seen in Capan-2 cells. Several studies have shown nuclear localization of the P2X7 [27,28]. A recent study showed an intense cytoplasmic and plasma membrane staining, using the same antibody as employed here [29]. These results support the notion that the effects of statins on pAkt and cell proliferation were mediated by P2X7 receptor.

To further confirm an involvement of P2X7 receptors in statin-induced inhibition of cell proliferation in P2X7 expressing Panc-1 and MIA PaCa-2 cells we used HEK293 expressing human P2X7 or
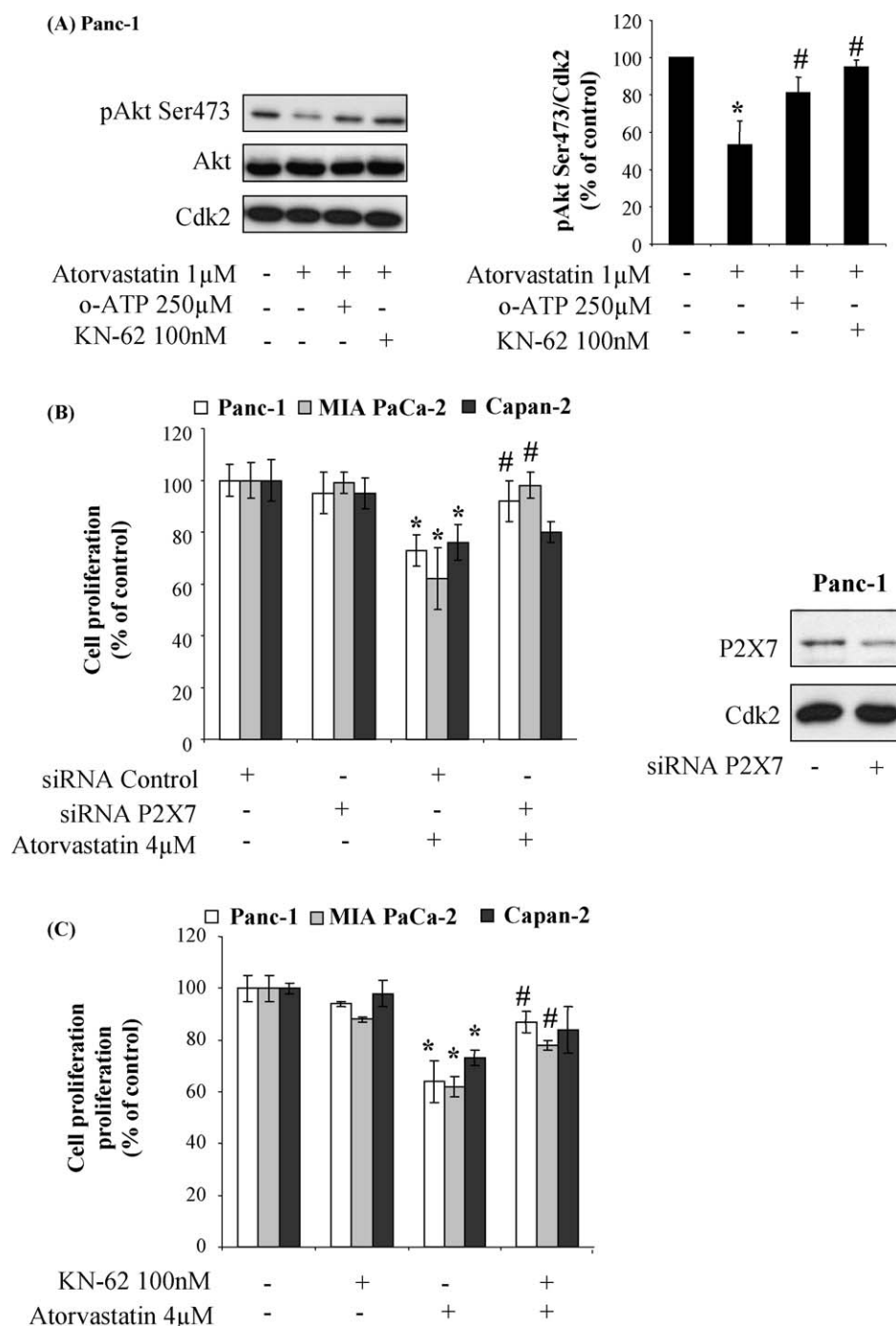
(A) Panc-1

pAkt Ser473

Akt

Cdk2

Atorvastatin 1μM	-	+	+	+
o-ATP 250μM	-	-	+	-
KN-62 100nM	-	-	-	+

(B)

☐ Panc-1 ☐ MIA PaCa-2 ■ Capan-2

siRNA Control	+	-	+	-
siRNA P2X7	-	+	-	+
Atorvastatin 4μM	-	-	+	+

Panc-1

P2X7

Cdk2

| siRNA P2X7 | - | + |

(C)

☐ Panc-1 ☐ MIA PaCa-2 ■ Capan-2

| KN-62 100nM | - | + | - | + |
| Atorvastatin 4μM | - | - | + | + |

Fig. 5. Effects of atorvastatin are mediated by P2X7 receptors. (A) Cells were treated with o-ATP (250 μM) or KN-62 (100 nM) for 10 min and thereafter with atorvastatin (1 μM) for 10 min. Samples were then analyzed by Western blotting, employing antibodies for Akt and pAkt Ser473. Cdk2 was used as a loading control. Panc-1 cells were transfected with siRNA against P2X7 (50 nM) for 72 h (B) or treated with KN-62 (100 nM) for 1 h (C) and thereafter with atorvastatin for 48 h. Cell proliferation was estimated using MTT assay and (B) shows mean ± SD from three independent experiments; significantly different from *siRNA control/siRNA P2X7 or #from siRNA control + atorvastatin, $P \leq 0.05$. (C) Column, mean ± SD from three independent experiments; significantly different from *control/KN-62 or #from atorvastatin, $P \leq 0.05$. In (B) Western blotting showing the level of P2X7 in siRNA P2X7 transfected Panc-1 cells. In (D) Western blotting (20, 10, 5 and 0.2 μg protein) of P2X7 expression in Panc-1, MIA PaCa-2, Capan-2 cells and HEK293 cells heterologously expressing human P2X7 or P2X4 receptors. Columns; mean ratio from densitometric analysis of P2X7/Cdk2 from three independent experiments. In (E and G), immunocytological staining of P2X7 expression in Panc-1, MIA PaCa-2, Capan-2 and A549 cells. In (F), HEK293 P2X7 and HEK293 P2X4 cells were treated with atorvastatin for 48 h in concentrations indicated. Cell proliferation was estimated by MTT assay. Column, mean ± SD from three independent experiments; significantly different from * corresponding control.

P2X4 receptors [30]. In our recent publication it was found that pAkt was induced by insulin in HEK295 P2X7 cells, and that atorvastatin decreased nuclear pAkt levels within 5 min. Furthermore, atorvastatin did not decrease pAkt in cells expressing P2X4 (20). Here we found that atorvastatin decreased cell proliferation in HEK293 P2X7 expressing cells, but had no effect on cells expressing P2X4 (Fig. 5F). Activation of P2X7 receptors has been shown to induce blebs [31]. As shown in Fig. 5G, incubation of A549 cells with statins induced formation of blebs, indicating an activation of P2X7 receptors. Taken together, these data indicate an involvement of P2X7 receptor in statin-induced inhibition of PI3K/Akt signaling in P2X7 expressing cells.
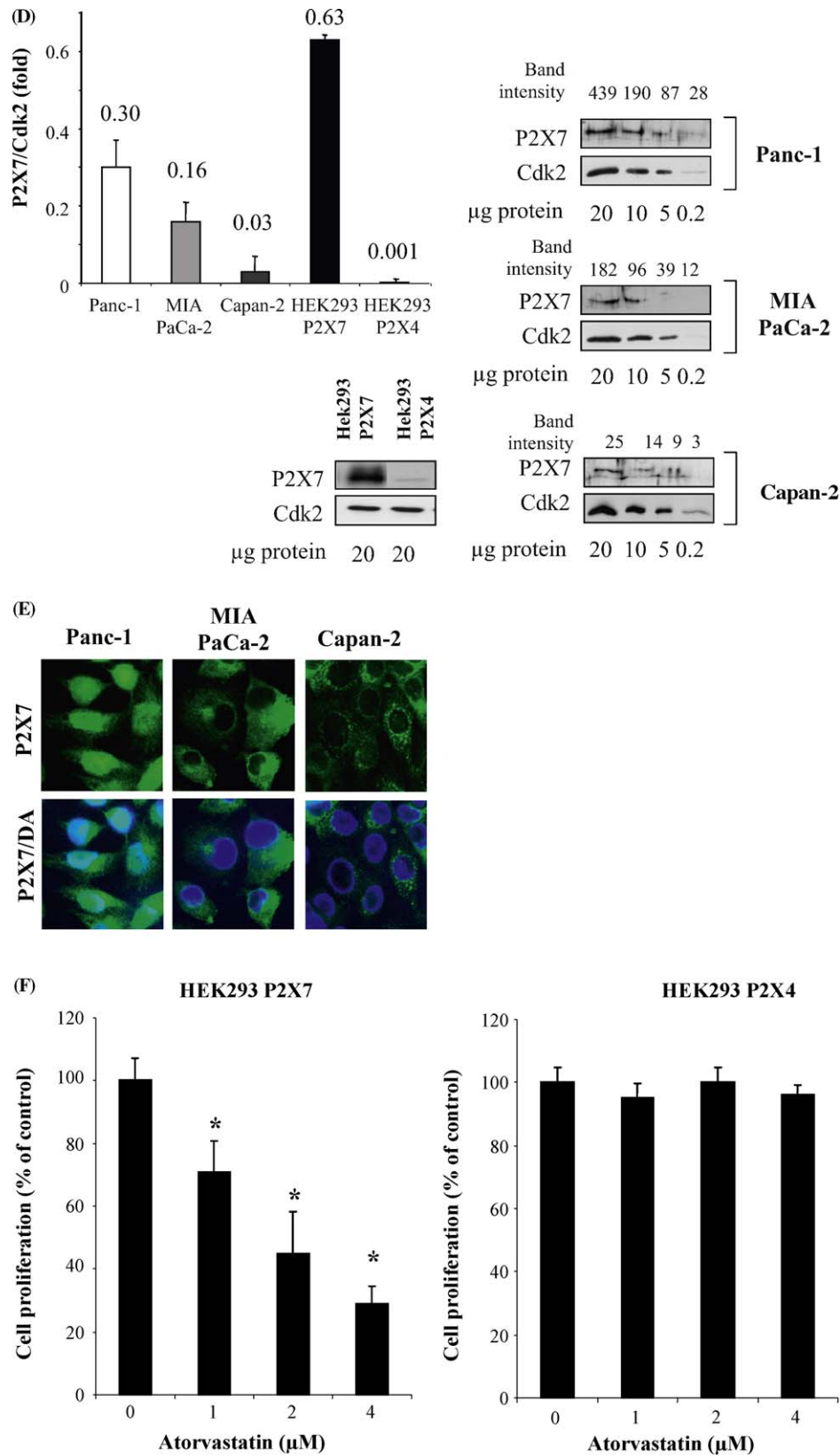
(D)

(E)

(F)

Fig. 5. (*Continued*).

3.5. Atorvastatin affects Raf/MEK and NFκB pathways in Capan-2 cells

Raf/MEK and NFκB are two additional pathways commonly involved in regulating apoptosis in pancreatic cancer cells [12,13]. Both have been shown to be down-regulated by statins via an inhibited prenylation [32]. Thus, we evaluated the effect of atorvastatin on Raf/MEK and NFκB in the three cell lines studied (Fig. 6A–C). As shown in Fig. 6A an inactivating phosphorylation of Raf at Ser259 was induced and this correlated to decreased levels of
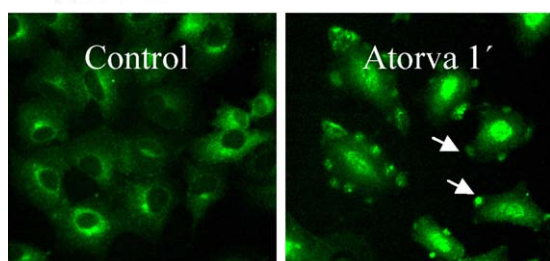
(G) A549-P2X7

Fig. 5. (*Continued*).

pMEK Ser217/221 in Capan-2 cells. Only small effects in this pathway were detected in MIA PaCa-2 and Panc-1 cells. Further, in Capan-2 cells, decreased levels of NFκB and its downstream target, survivin, were induced by atorvastatin. These data suggest that under conditions used here atorvastatin readily induced effects that can be explained by an inhibited prenylation in Capan-2 cells [7], but not in the Panc-1 and the MIA PaCa-2 cell lines.

4. Discussion

Our data show that statins in pharmacologically relevant concentrations inhibit phosphorylation of Akt in Panc-1 and MIA PaCa-2 pancreatic cancer cells. This effect was associated with
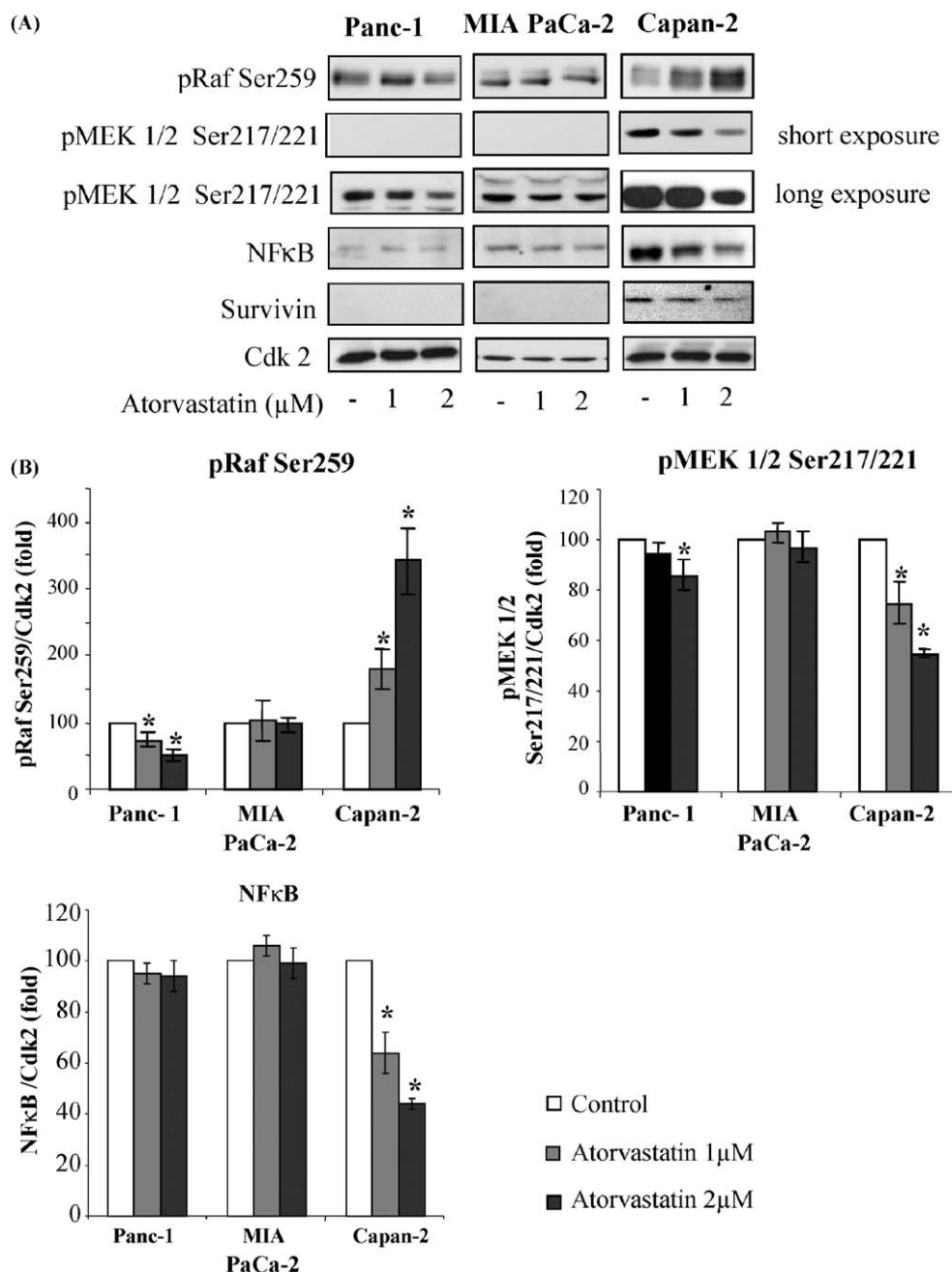

Fig. 6. Atorvastatin affects Raf/MEK- and NFκB pathway in Capan-2 cells. In (A) cells were treated with atorvastatin for 24 h in concentrations indicated. Samples were then analyzed by Western blotting, employing antibodies for pRaf Ser259, pMek 1/2 Ser217/221, survivin and NFκB p65. Cdk2 was used as a loading control and (B) shows mean ratio from densitometric analysis of pRaf Ser259/Cdk2, pMEK1/2 Ser217/221 or NFκB/Cdk2 from three independent experiments, mean ± SD; *significantly different from control, $P \leq 0.05$. In (C) Panc-1 and Capan-2 cells were treated with atorvastatin (4 μM) for 8 h and thereafter immunocytochemically stained for NFκB. Columns show relative staining intensity (mean ± SD from 100 cells). *Significantly different from corresponding control, $P \leq 0.05$.
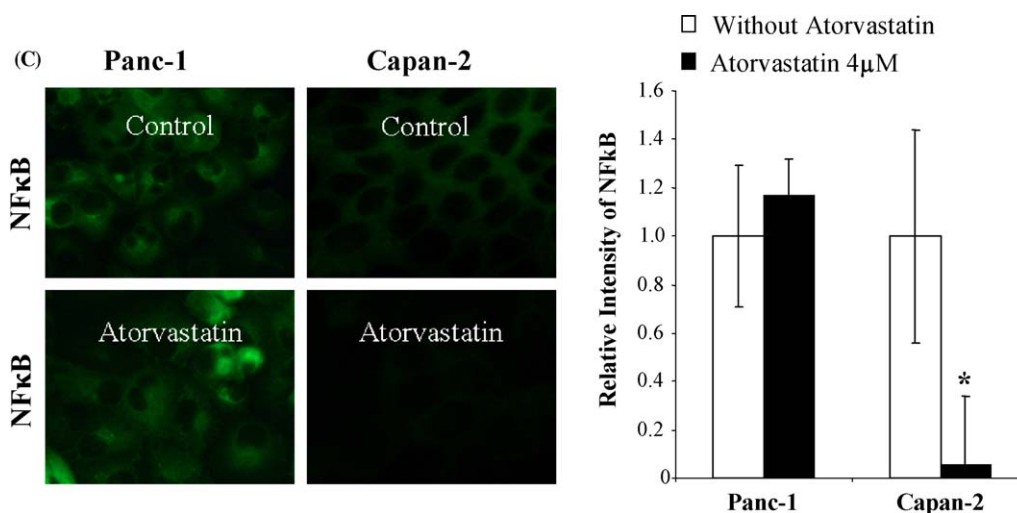
Fig. 6. (*Continued*).

inhibition of cell proliferation and induction of apoptosis. In addition, statin increased the effect of cytostatic drugs commonly used to treat pancreatic cancer. We also present data indicating that these effects are mediated by the P2X7 receptor.

The Akt pathway is commonly altered in pancreatic cancer [12]. We found that constitutive levels of Akt as well as insulin-induced activation of Akt were inhibited by statins and that this correlated with decreased phosphorylation of the downstream targets GSK3β Ser9 and 70 S6K Thr386. We also found that combined treatment of cells with atorvastatin and gemcitabine or 5-Fu induced increased inhibition of pAkt and that this correlated to inhibition of cell proliferation and increased the level of apoptosis in Panc-1 and MIA PaCa-2 cells. Inhibition of Akt has been shown to sensitize cancer cell lines to chemotherapeutic agents [33–35], and statins have been shown to sensitize pancreatic cancer cell lines to cytostatic treatment [1]. Our data is also in line with a recently published study showing that fluvastatin inhibits proliferation, induces apoptosis and potentiates the cytotoxic effect of gemcitabine in MIA PaCa-2 cells [5].

Gemcitabine is the current standard chemotherapeutic drug for treatment of pancreatic cancer but with minor benefit [36]. However, we found that in combination treatment, atorvastatin increased the effect of gemcitabine. Similar results were also induced by combining atorvastatin and 5-Fu. These effects correlated to decreased Akt phosphorylation. The same response was detected with combination of 5-Fu and atorvastatin. We also found that PI3K-inhibitor, LY294002, increased the effect of atorvastatin in Panc-1 cells. This effect suggests that statins act by inhibiting PI3K/Akt pathway.

Several lines of evidence indicate that the statin-induced growth inhibition in Panc-1 and MIA PaCa-2 cells was mediated by P2X7. Thus, P2X7 inhibitors, KN-62 and o-ATP, abrogated the atorvastatin-induced effect on pAkt and cell proliferation. Our data also show that silencing of P2X7 with siRNA prevented the effect of atorvastatin on cell proliferation. These data are in line with our recently published data showing that the statin-induced effect on nuclear pAkt is mediated via P2X7 [20]. In addition, we showed that the natural ligand for P2X7, extracellular ATP, inhibited insulin-induced Akt signaling in epithelial cells via P2X7. These effects were induced more rapidly than can be expected if an inhibited prenylation was involved [33]. Taken together data presented here indicate that atorvastatin inhibit the growth of pancreatic cancer cells via P2X7 signaling. However, a remaining question is how statins activate P2X7.

P2X7 is an ATP-gated cation channel leading to Ca^{2+} release and to pleiotropic effects [37]. Activation of the receptor is able to stimulate the release of proinflammatory cytokines and P2X7 activation can thus be involved in inflammation [22]. Chronic pancreatitis is a risk factor for development of pancreatic cancer and recently, increased levels of P2X7 were detected in chronic pancreatitis and pancreas cancer [23]. P2X7 might thus be involved in pancreatic cancer development and might also be an attractive target for chemoprevention.

In Capan-2 cells we found a low level of P2X7 concentrated in cytoplasmic droplets. The picture resembled that found in cells where P2X7 has been internalized in a trafficking process activated by ATP [38]. It thus seems that P2X7 is aberrantly regulated in a cell line in which atorvastatin did not affect pAkt levels nor sensitized them to cytostatic drugs. However, in these cells statins affected apoptosis. Statins have been shown to reduce NFκB activation by inhibiting prenylation [1,36] and our data show that in Capan-2 cells NFκB was reduced. In the same time phosphorylation of Raf and MEK were affected, which can be explained by an inhibited prenylation of Ras [32–35,1,36–40]. The reason why Panc-1 and MIA PaCa-2 cells were less sensitive to these effects remains to be studied. One possibility is that abundantly expressed P2X7 in Panc-1 and MIA PaCa-2 cells in some way prevents these effects.

Pancreatic ductal adenocarcinoma is a highly malignant neoplasm that still carries an extremely poor prognosis. The use of adjuvant chemotherapy has not improved much on survival. Our data suggests that in cells with functional P2X7, P2X7-Akt signaling is a useful target that can sensitize many pancreatic cancers to chemotherapy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.06.016.

References

[1] Chan KK, Oza AM, Siu LL. The statins as anticancer agents. Clin Cancer Res 2003;9:10–9.
[2] Demierre MF, Higgins PD, Gruber SB, Hawk E, Lippman SM. Statins and cancer prevention. Nat Rev Cancer 2005;5:930–42.
[3] Issat T, Nowis D, Legat M, Makowski M, Klejman MP, Urbanski J, et al. Potentiated antitumor effects of the combination treatment with statins and pamidronate in vitro and in vivo. Int J Oncol 2007;30:1413–25.

[4] Yao CJ, Lai GM, Chan CF, Cheng AL, Yang YY, Chuang SE. Dramatic synergistic anticancer effect of clinically achievable doses of lovastatin and troglitazone. Int J Cancer 2006;118:773–9.

[5] Bocci G, Fioravanti A, Orlandi P, Bernardini N, Collecchi P, Del Tacca M, et al. Fluvastatin synergistically enhances the antiproliferative effect of gemcitabine in human pancreatic cancer MIAPaCa-2 cells. Br J Cancer 2005;93:319–30.

[6] Kusama T, Mukai M, Iwasaki T, Tatsuta M, Matsumoto Y, Akedo H, et al. 3-Hydroxy-3-methylglutaryl-coenzyme a reductase inhibitors reduce human pancreatic cancer cell invasion and metastasis. Gastroenterology 2002;122:308–17.

[7] Gbelcová H, Lenícek M, Zelenka J, Knejzlík Z, Dvoráková G, Zadinová M, et al. Differences in antitumor effects of various statins on human pancreatic cancer. Int J Cancer 2008;122:1214–21.

[8] Kaminski R, Kozar K, Kopec M, Basak G, Skierski JS, Koronkiewicz M, et al. Discussion on 3-hydroxy-3-methylglutaryl-coenzyme a reductase inhibitors reduce human pancreatic cancer cell invasion and metastasis. Gastroenterology 2002;123:1747–8.

[9] Platz EA, Leitzmann MF, Visvanathan K, Rimm EB, Stampfer MJ, Willett WC, et al. Statin drugs and risk of advanced prostate cancer. J Natl Cancer Inst 2006;98:1819–25.

[10] Khurana V, Sheth A, Caldito G, Barkin JS. Statins reduce the risk of pancreatic cancer in humans: a case-control study of half a million veterans. Pancreas 2007;34:260–5.

[11] Holcomb B, Yip-Schneider MT, Matos JM, Dixon J, Kennard J, Mahomed J, et al. Pancreatic cancer cell genetics and signaling response to treatment correlate with efficacy of gemcitabine-based molecular targeting strategies. J Gastrointest Surg 2008;12:288–96.

[12] Altomare DA, Tanno S, De Rienzo A, Klein-Szanto AJ, Tanno S, Skele KL, et al. Frequent activation of AKT2 kinase in human pancreatic carcinomas. J Cell Biochem 2003;88:470–6.

[13] Holcomb B, Yip-Schneider M, Schmidt CM. The role of nuclear factor kappaB in pancreatic cancer and the clinical applications of targeted therapy. Pancreas 2008;36:225–35.

[14] Zhao Y, Shen S, Guo J, Chen H, Greenblatt DY, Kleeff J, et al. Mitogen-activated protein kinases and chemoresistance in pancreatic cancer cells. J Surg Res 2006;136:325–35.

[15] Pääjärvi G, Roudier E, Crisby M, Högberg J, Stenius U. HMG-CoA reductase inhibitors, statins, induce phosphorylation of Mdm2 and attenuate the p53 response to DNA damage. FASEB J 2005;19:476–8.

[16] Roudier E, Mistafa O, Stenius U. Statins induce mammalian target of rapamycin (mTOR)-mediated inhibition of Akt signaling and sensitize p53-deficient cells to cytostatic drugs. Mol Cancer Ther 2006;5:2706–15.

[17] Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. Nat Rev Cancer 2002;2:897–909.

[18] Heinemann V. Present and future treatment of pancreatic cancer. Semin Oncol 2002;29:23–31.

[19] Ng SSW, Tsao MS, Chow S, Hedley DW. Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. Cancer Res 2000;60:5451–5.

[20] Mistafa O, Högberg J, Stenius U. Statins and ATP regulate nuclear pAkt via the P2X7 purinergic receptor in epithelial cells. BBRC 2008;365:131–6.

[21] Lister MF, Sharkey J, Sawatzky DA, Hodgkiss JP, Davidson DJ, Rossi AG, et al. The role of the purinergic P2X7 receptor in inflammation. J Inflamm 2007;16:4–5.

[22] Hughes JP, Hatcher JP, Chessell IP. The role of P2X(7) in pain and inflammation. Purinergic Signal 2007;3:163–9.

[23] Künzli BM, Berberat PO, Giese T, Csizmadia E, Kaczmarek E, Baker C, et al. Upregulation of CD39/NTPDases and P2 receptors in human pancreatic disease. Am J Gastrointest Liver Physiol 2007;292:223–30.

[24] Meier R, Alessi DR, Cron P, Andjelkovic M, Hemmings BA. Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase. Bh J Biol Chem 1997;272(30):491–7.

[25] Brazil DP, Park J, Hemmings BA. PKB binding proteins. Getting in on the Akt. Cell 2002;111:293–303.

[26] Humphreys BD, Virginio C, Surprenant A, Rice J, Dubyak GR. Isoquinolines as antagonists of the P2X7 nucleotide receptor: high selectivity for the human versus rat receptor homologues. Mol Pharmacol 1998;54:22–32.

[27] Franke H, Gunther A, Grosche J, Schmidt R, Rossner S, Reinhardt R. P2X7 receptor expression after ischemia in the cerebral cortex of rats. J Neuropathol Exp Neurol 2004;63:686–99.

[28] Atkinson L, Milligan CJ, Buckley NJ, Deuchars J. An ATP-gated ion channel at the cell nucleus. Nature 2002;420:42.

[29] Hansen MR, Krabbe S, Novak I. Purinergic receptors and calcium signalling in human pancreatic duct cell lines. Cell Physiol Biochem 2008;22:157–68.

[30] Jiang LH, Mackenzie AB, North RA, Surprenant A. Brilliantblue G selectively blocks ATP-gated rat P2X(7) receptors. Mol Pharmacol 2000;58:82–8.

[31] Mackenzie AB, Young MT, Adinolfi E, Surprenant A. Pseudoapoptosis induced by brief activation of ATP-gated P2X7 receptors. J Biol Chem 2005;40:33968–76.

[32] Yang BC, Wang YS, Liu HS, Lin SJ. Ras signaling is involved in the expression of Fas-L in glioma. Lab Invest 2000;80:529–37.

[33] Krystal GW, Sulanke G, Litz J. Inhibition of phosphatidylinositol 3-kinase-Akt signaling blocks growth, promotes apoptosis, and enhances sensitivity of small cell lung cancer cells to chemotherapy. Mol Cancer Ther 2002;1:913–22.

[34] Warshamana-Greene GS, Litz J, Buchdunger E, Garcia-Echeverria C, Hofmann F, Krystal GW. The insulin-like growth factor-I receptor kinase inhibitor, NVP-ADW742, sensitizes small cell lung cancer cell lines to the effects of chemotherapy. Clin Cancer Res 2005;11:1563–71.

[35] Tsurutani J, West KA, Sayyah J, Gills JJ, Dennis PA. Inhibition of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway but not the MEK/ERK pathway attenuates laminin-mediated small cell lung cancer cellular survival and resistance to imatinib mesylate or chemotherapy. Cancer Res 2005;65:8423–32.

[36] Morgan MA, Parsels LA, Maybaum J, Lawrence TS. Improving gemcitabine-mediated radiosensitization using molecularly targeted therapy: a review. Clin Cancer Res 2008;14:6744–50.

[37] Ortego M, Bustos C, Hernández-Presa MA, Tuñón J, Díaz C, Hernández G, et al. Atorvastatin reduces NF-kappaB activation and chemokine expression in vascular smooth muscle cells and mononuclear cells. Atherosclerosis 1999;147:253–61.

[38] Feng Y-H, Wang L, Wang Q, Li X, Zeng R, Gorodeski G.I.. ATP stimulates GRK-3 phosphorylation and β-arrestin-2-dependent internalization of P2X7 receptor. Am J Physiol Cell Physiol 2005;288:1342–56.

[39] Denoyelle C, Vasse M, Körner M, Mishal Z, Ganné F, Vannier JP, et al. Cerivastatin, an inhibitor of HMG-CoA reductase, inhibits the signaling pathways involved in the invasiveness and metastatic properties of highly invasive breast cancer cell lines: an in vitro study. Carcinogenesis 2001;22:1139–48.

[40] Dai Y, Khanna P, Chen S, Pei XY, Dent P, Grant S. Statins synergistically potentiate 7-hydroxystaurosporine (UCN-01) lethality in human leukemia and myeloma cells by disrupting Ras farnesylation and activation. Blood 2007;15:4415–23.