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3 **Statins inhibit Akt/PKB signaling via P2X7 receptor in**
4 **pancreatic cancer cells**
5

6
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22 **Running title:** Statins inhibit Akt via P2X7
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24

25 **Abbreviations:** 3-Hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors,
26 5-fluorouracil (5-Fu), phosphatidylinositol 3-kinase (PI3K), glycogen synthase kinase 3
27 beta (GSK3 β), small interference RNA (siRNA), phosphorylation of Akt on residue
28 Ser473 (pAkt Ser473), phosphorylation of Akt on residue Thr308 (pAkt Thr308), GSK3 β
29 on residue Ser9 (pGSK3 β Ser9), nuclear factor kappa-B- (NF- κ B), mitogen-activated
30 protein kinas (MAPK), Purinergic receptor (P2X), ligand-gated ion channel 7 (P2X7),
31 Human embryonic kidney (HEK), adenosine 5'-triphosphate periodate oxidized sodium
32 salt (o-ATP), Poly (ADP-ribose) polymerase (PARP).
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36 **Key words:** atorvastatin, P2X7, Akt, chemotherapy, pancreatic cancer
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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 NF κ B 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-3methyl-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.

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5 There are three major signaling pathways commonly altered in pancreatic cancers [11].
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7 These include phosphatidylinositol 3-kinase- (PI3K/Akt), nuclear factor kappa-B- (NF-
8 κ B), and mitogen-activated protein kinas (MAPK) pathways [12-14]. We have previously
9
10 shown that statins inhibit one of these pathways, PI3K/Akt signaling, in different cell
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12 lines [15,16]. Thus, incubation with statins decrease the levels of phosphorylated Akt in
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14 the cytoplasm and the nucleus and sensitized HepG2 and A549 cells to cytostatic drugs
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19 [16].
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24 The Akt pathway is one of the major anti-apoptotic factors in cells. Akt is activated by
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26 growth factors and cellular stress and is commonly overexpressed in pancreatic cancer,
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28 but is also often induced by cytostatic treatment [12, 17, 18]. In addition, PI3K/Akt has
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30 been implicated in the resistance of pancreatic cancer to gemcitabine [19]. Therefore, Akt
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32 is an attractive target for cancer therapy and in particular for pancreatic cancer.
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38 Our recent data show that the statin-induced inhibition of nuclear pAkt is mediated via
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40 the P2X7 purinergic receptor in A549 lung cancer cells. Furthermore, we found evidence
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42 that P2X7 receptors, when activated by its natural ligand ATP, can regulate nuclear pAkt
43
44 in epithelial cells [20]. P2X7 is expressed in pancreatic cells and is activated by
45
46 extracellular purinergic nucleotides such as ATP. P2X7 receptor activation might
47
48 stimulate pleiotropic cellular effects including the release of pro and inhibitory
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50 inflammatory cytokines [21, 22]. Increased levels of P2X7 were recently detected in
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52 chronic pancreatitis and pancreas cancer indicating a possible involvement of P2X7
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54 receptors in pancreatic cancer development [23].
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5 In the present study, we have investigated the effects of atorvastatin on pancreatic cancer
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7 cell lines. We report that pharmacologically relevant concentrations of statins decrease
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9 the levels of pAkt in Panc-1 and MIA PaCa-2 cells and sensitize them to gemcitabine and
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11 5-Fu. We also report that these effects are mediated by P2X7. On the other hand, in
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13 Capan-2 cells, with low expression of P2X7, statins did not affect pAkt levels and did not
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15 sensitize them to cytostatic drugs.
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20 21 **2. Materials and methods**

22 23 24 **2.1 Cell culture**

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26 Human pancreatic cell lines, Panc-1, Capan-2 and MIA PaCa-2, were obtained from the
27
28 American Type Culture Collection, ATCC (Manassas, VA, USA). Capan-2 is well- to
29
30 moderately well-differentiated ductal adenocarcinoma cell line expressing wt p53, while
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32 MIA PaCa-2 and Panc-1 are poorly differentiated ductal adenocarcinoma cell lines with
33
34 mutant p53. Panc-1 and MIA PaCa-2 cells were maintained in Dulbecco's modified
35
36 Eagle's medium (ATCC) while Capan-2 cells were maintained in McCoy's 5A Modified
37
38 Medium (ATCC). Media was supplemented with 10% fetal calf serum and penicillin-
39
40 streptomycin. Serum-starved cells were cultured in medium supplemented with 0.1%
41
42 serum for 24 h. Cells were exposed for atorvastatin for 15 minutes – 1 h to study the
43
44 P2X7-mediated effects on Akt phosphorylation, while 24 or 48 h incubation time was
45
46 used for detection of effects on cell proliferation or apoptosis. Human embryonic kidney
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48 (HEK) 293 cells stably expressing human P2X4 and P2X7 were kindly provided by A.
49
50 Surprenant, Sheffield University, UK. HEK293 cells were grown in DMEM: F12 with 1
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3 mM l-glutamine, 10% inactivated calf serum and 300 µg/ml G418. A549 cells were
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5 obtained from ATCC.
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7 8 9 **2.2 Reagents**

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11 Atorvastatin was provided by Pfizer (New York, NY). Pravastatin, LY294002, 5-
12
13 fluorouracil (5-Fu), Adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'-
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15 triphosphate periodate oxidized sodium salt (o-ATP), 2'(3')-O-(4-benzoyl-benzoyl)
16
17 adenosine 5'-triphosphate triethylammonium salt, mixed isomer (BzATP) and KN-62 was
18
19 purchased from Sigma-Aldrich (St. Louis, MO) and gemcitabine from Eli Lilly
20
21 (Indianapolis, IN). The final concentration of DMSO (Sigma-Aldrich, St. Louis, MO)
22
23 added to the cells was <0.2%. No effect of DMSO was observed.
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31 32 **2.3 Western blotting**

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34 Western blotting was performed as previously [17]. In brief the samples were subjected
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36 to SDS-PAGE and thereafter blotted onto a PVDF membrane (Bio-Rad, Hercules, CA).
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38 Some samples were subfractionated. The protein bands were probed using antibodies
39
40 against Akt, Akt phosphorylated at residues Ser473 or Thr308, α -tubulin, ERK
41
42 phosphorylated at residue Tyr208, Survivin, PARP, P2X7 and Cdk2 from Santa Cruz
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44 (Santa Cruz, CA); glycogen synthase kinase 3 beta (GSK3 β) phosphorylated at residue
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46 Ser9, pMEK1/2 Ser217/221, NF κ B p65, pRaf Ser259 and p70 S6K Thr386 from Cell
47
48 Signaling (Beverly, MA); Cyclin D1 from Oncogene (Uniondale, NY). pAkt Ser473
49
50 antibody gave rise to a double band which consists of Akt1 and Akt2 and both have been
51
52 used for evaluations. Proteins were visualized with ECL procedure (Amersham
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54 Biosciences, Uppsala, Sweden). The Western blot results were analyzed with NIH Image
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3 1.62 software.
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7 ***2.4 Quantification of apoptosis*** 8 9

10 Morphological evaluation of apoptotic cell death was performed by Hoechst staining.
11 Cells were fixed in methanol for one hour. After fixation the cells were stained with
12 Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) for 5 minutes. Nuclear morphology was
13 then evaluated using fluorescent microscope. Cells whose nucleus exhibited brightly
14 stained, condensed chromatin or nuclear fragments were designated apoptotic. The
15 percentage of cells with apoptotic morphology was determined by counting at least 1000
16 cells per plate.
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28 ***2.5 Cell viability and cell proliferation*** 29 30

31 Viability of cells was determined by the trypan blue exclusion assay. Cell proliferation
32 was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
33 assay detecting the cellular mitochondrial capacity to convert MTT tetrazolium salt to
34 formazan. Cells were incubated with the medium containing MTT (Sigma-Aldrich, St.
35 Louis, MO) for 4 hours. The cells were then lyzed in DMSO. The absorbance was
36 measured at 570-620nm.
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48 ***2.6 Small Interference RNA transfection*** 49

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

Cells were fixed in 3.7% formaldehyde. After fixation the cells were stained with polyclonal antibodies against P2X7 and NF κ B. 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 \pm 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 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 μ M for 1 or 24 hours) decreased a high constitutive level of Akt phosphorylation at residue Ser473 (pAkt Ser473) (Fig. 1A and B). This reduction was

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3 associated with decreased levels of downstream targets, GSK3 β phosphorylated at
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5 residue Ser9 (pGSK3 β Ser9) and p70 S6K phosphorylated at residue Thr386 (p70 S6K
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7 Thr386) (Fig 1 A and B). Also cyclin D1 was reduced by atorvastatin (Fig 1 A and B).
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9 The reduction of pAkt Ser473 was also induced by pravastatin (Fig 1C). Cyclin D1 has
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11 been implicated in cell cycle regulation. As shown by densitometric analysis the level of
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13 pAkt correlated with pGSK3 β Ser9, p70 S6K Thr386 and cyclin D1 levels (Fig. 1B).
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15 Total Akt levels were not affected (Fig. 1A and B). The effect of atorvastatin on cell
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17 proliferation was also studied. Incubation with 1 to 4 μ M of atorvastatin for 48 hours
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19 inhibited cell proliferation dose-dependently (Fig. 1D). PARP-assay was used to detect
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21 effects on apoptosis. As shown in figure 1D, a dose-dependent increase in the levels of
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23 PARP cleavage was detected when cells were incubated with atorvastatin for 24 hours.
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32 Growth factors induce the phosphorylation of Akt and the effects of statin on insulin-
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34 induced pAkt was studied. As shown in figure 1E preincubation of cells with atorvastatin
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36 for 1 hour decreased the level of insulin-induced pAkt Ser473 and its downstream target
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38 pGSK3 β Ser9. Nuclear translocation of Akt is crucial for its activity [24]. Several nuclear
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40 targets for Akt involved in the cell cycle regulation have been identified and exclusion
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42 from the nucleus may thus restrain the kinase activity on targets involved in regulation of
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44 the cell cycle [25]. To test whether statins could affect the nuclear localization of pAkt,
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46 Panc-1 cells were fractionated. We found that insulin-induced nuclear pAkt levels
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48 decreased within 15 minutes after addition of atorvastatin (1 μ M) (Fig. 1E). The purity of
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50 the cytoplasmic extract was confirmed by analyzing α -tubulin. LY294002 (25 μ M), a
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52 PI3K-inhibitor, was used as a control and prevented the insulin-induced nuclear pAkt
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3 accumulation (data not shown). Together, these data show that atorvastatin attenuate Akt
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5 signaling in Panc-1 cells.
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10 **3.2 Atorvastatin sensitizes Panc-1 cells to gemcitabine- and 5-Fluorouracil**

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12 Gemcitabine is used to treat pancreatic cancer and we investigated whether statins
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14 affected pAkt or enhance the efficiency of gemcitabine in Panc-1 cells. We found that
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16 gemcitabine significantly decreased phosphorylation of Akt at residue Ser473 and its
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18 downstream target p70 S6K Thr386. When cells were treated with atorvastatin pAkt was
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20 further reduced (Fig. 2A). Additional finding was that 5-Fu decreased pAkt and also this
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22 effect was potentiated by incubation with atorvastatin (Fig. 2B).
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29 The effect of atorvastatin on the antiproliferative effect of gemcitabine and 5-Fu was
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31 studied. Cells were pretreated with atorvastatin for 1 hour and thereafter exposed with
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33 gemcitabine or 5-Fu for 48 hours. As expected, both drugs alone induced a significant
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35 inhibition of cell proliferation (Fig. 3A). As shown in figure 3A, 1 hour pretreatment with
36
37 atorvastatin strengthened the gemcitabine- and 5-Fu-induced inhibition of cell
38
39 proliferation over a broad concentration range. In these experiments low concentrations
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41 of gemcitabine and 5-Fu were used and the cells were incubated for 48 hours. The
42
43 pretreatment of atorvastatin combined with 0.01 μ M gemcitabine induced a similar
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45 inhibition of cell proliferation as 0.1 μ M. This means a 10 times increased efficiency in
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47 this experiment (Fig. 3A).
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55 We next studied the effect of atorvastatin on gemcitabine- and 5-Fu-induced apoptosis.
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57 Apoptosis was assayed by Hoechst staining and by cleavage of PARP. As shown in
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3 figure 3B, the combination of atorvastatin (4 μM) and gemcitabine (0.04 μM) or 5-Fu (5
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5 μM) significantly increased the level of apoptosis. 5 μM 5-Fu induced a minor effect on
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7 PARP (data not shown) and therefore higher concentrations were used to study the effect
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9 on PARP. As shown in figure 3C atorvastatin (4 μM), gemcitabine (0.04 μM) or 5-Fu (50
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11 μM) resulted in cleavage of PARP and this effect was strengthened by combining
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13 atorvastatin with cytostatic drugs. Figure 3C also shows the densitometric analysis of 85
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15 kDa PARP fragment.
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22 The effect of atorvastatin on the antiproliferative effect of LY294002 (PI3K inhibitor)
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24 was also studied in Panc-1 cells (Supplemental data 1). In these experiments low
25
26 concentrations of LY294002 (non-toxic concentration and without any detectable effect
27
28 on pAkt) were used. Treatment of cells with the combination of atorvastatin (1 μM) and
29
30 LY294002 (2.5 μM) significantly decreased the level of pAkt Ser473 and reduced growth
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32 of Panc-1 cells. Taken together, atorvastatin increased the effect 5-Fu or gemcitabine on
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34 cell proliferation and apoptosis in pancreatic cancer cells.
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41 **3.3 Statins decrease pAkt and increased the effect of gemcitabine in MIA PaCa-2,** 42 43 **but not in Capan-2 cells.** 44

45 To study the cell specificity of the effect of statins we also studied two additional
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47 pancreatic cancer cell lines, Capan-2 and MIA PaCa-2. As shown in figure 4A
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49 atorvastatin and pravastatin decreased the constitutive level of pAkt Ser473 in MIA
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51 PaCa-2 cells. Furthermore, in this cell line increased inhibition of cell proliferation was
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53 induced by atorvastatin and gemcitabine (Fig. 4B). Atorvastatin also induced PARP
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55 cleavage (Fig. 4C). In Capan-2 cells incubation with atorvastatin or pravastatin did not
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3 decrease the level of pAkt and no increased inhibition of cell proliferation was induced
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5 by statins in combination with low doses of gemcitabine (Fig. 4A and B). However
6
7 statins induced cleavage of PARP after 24 hours (Fig. 4C).
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10 11 **3.4 Effects of atorvastatin in Panc-1 and MIA PaCa-2 are mediated by P2X7 receptors.**

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13 We have previously shown that statins regulate nuclear pAkt via P2X7 purinergic
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15 receptor in lung cancer cells [20]. We also documented a statin-induced nuclear
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17 localization of the antigen in rat hepatocytes *in situ*, indicating that statins may affect the
18
19 P2X7 receptor *in vivo* [20]. We investigated the role of the P2X7 receptor in statin-
20
21 induced effects in Panc-1 cells. The P2X antagonist, oxidized-ATP (o-ATP), and a more
22
23 selective inhibitor of P2X7 receptor, KN-62, were tested [26]. As shown in figure 5A
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25 preincubation with both inhibitors abrogated the atorvastatin-induced effect on pAkt
26
27 Ser473. The inhibitors alone did not have any effect on pAkt (data not shown). Next, the
28
29 P2X7 agonists, ATP and a more selective agonist BzATP were tested. As shown in
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31 Supplemental data 2 the levels of pAkt was decreased in Panc-1 and MIA-PaCa-2, but
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33 not in Capan-2 cells. Further, BzATP increase the effect of gemcitabine in Panc-1, MIA
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35 PaCa-2, but not in Capan-2 cells (Supplemental data 2). Thus ATP and BzATP
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37 mimicked the effects of statins.
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48 In another series of experiments Panc-1, Capan-2 and MIA PaCa-2 cells were transfected
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50 with siRNA against P2X7 for 72 h and thereafter incubated with atorvastatin for
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52 additional 48 h. As shown in figure 5B siRNA transfection decreased the level of P2X7
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54 and prevented the effect induced by atorvastatin on cell proliferation in Panc-1 and MIA
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56 PaCa-2 but not in Capan-2 cells (Fig. 5B). Similar effects as induced by siRNA were
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3 induced by KN-62 (Fig. 5C). KN-62 did not affect the anti-proliferative effect of
4
5 gemcitabine (data not shown). To determine whether differences in response might be
6
7 explained by differences in receptor levels, P2X7 was analyzed by Western blotting and
8
9 immunocytochemistry. 2-fold, 4-fold and 10-fold dilutions of Panc-1 samples were ran
10
11 alongside to compare band intensities between different cell lines. Capan-2 cells, in
12
13 contrast to Panc-1 and MIA PaCa-2 cells, exhibited a low level of the P2X7 receptor
14
15 (Fig. 5D). The specificity of the antibody was confirmed by using HEK293 cells
16
17 heterologously expressing human P2X7 or P2X4 receptors (Fig. 5D). No binding was
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19 detected in HEK293 cells heterologously expressing human P2X4 receptors. This was
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21 also supported by immunocytochemical staining. As shown in figure 5E staining for P2X7
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23 was detected in Panc-1 and MIA PaCa-2, while a faint staining concentrated in droplets
24
25 in the cytoplasm was seen in Capan-2 cells. Several studies have shown nuclear
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27 localization of the P2X7 [27-28]. A recent study showed an intense cytoplasmic and
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29 plasma membrane staining, using the same antibody as employed here [29]. These results
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31 support the notion that the effects of statins on pAkt and cell proliferation were mediated
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33 by P2X7 receptor.
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43 To further confirm an involvement of P2X7 receptors in statin-induced inhibition of cell
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45 proliferation in P2X7 expressing Panc-1 and MIA PaCa-2 cells we used HEK293
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47 expressing human P2X7 or P2X4 receptors [30]. In our recent publication it was found
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49 that pAkt was induced by insulin in HEK295 P2X7 cells, and that atorvastatin decreased
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51 nuclear pAkt levels within 5 min. Furthermore, atorvastatin did not decrease pAkt in cells
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53 expressing P2X4 (20). Here we found that atorvastatin decreased cell proliferation in
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55 HEK293 P2X7 expressing cells, but had no effect on cells expressing P2X4 (Fig 5F).
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3 Activation of P2X7 receptors has been shown to induce blebs [31]. As shown in figure 5
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5 G, incubation of A549 cells with statins induced formation of blebs, indicating an
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7 activation of P2X7 receptors. Taken together, these data indicate an involvement of P2X7
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9 receptor in statin-induced inhibition of PI3K/Akt signaling in P2X7 expressing cells.
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13 14 **3.5 Atorvastatin affects Raf/MEK and NFκB pathways in Capan-2 cells**

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16 Raf/MEK and NFκB are two additional pathways commonly involved in regulating
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18 apoptosis in pancreatic cancer cells [12-13]. Both have been shown to be down-regulated
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20 by statins via an inhibited prenylation [32]. Thus, we evaluated the effect of atorvastatin
21
22 on Raf/MEK and NFκB in the three cell lines studied (Figure 6 A, B and C). As shown in
23
24 figure 6A an inactivating phosphorylation of Raf at Ser259 was induced and this
25
26 correlated to decreased levels of pMEK Ser217/221 in Capan-2 cells. Only small effects
27
28 in this pathway were detected in MIA PaCa-2 and Panc-1 cells. Further, in Capan-2
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30 cells, decreased levels of NFκB and its downstream target, survivin, were induced by
31
32 atorvastatin. These data suggest that under conditions used here atorvastatin readily
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34 induced effects that can be explained by an inhibited prenylation in Capan-2 cells [7], but
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36 not in the Panc-1 and the MIA PaCa-2 cell lines.
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46 **4. Discussion**

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48 Our data show that statins in pharmacologically relevant concentrations inhibit
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50 phosphorylation of Akt in Panc-1 and MIA PaCa-2 pancreatic cancer cells. This effect
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52 was associated with inhibition of cell proliferation and induction of apoptosis. In
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54 addition, statin increased the effect of cytostatic drugs commonly used to treat pancreatic
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3 cancer. We also present data indicating that these effects are mediated by the P2X7
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5 receptor.
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10 The Akt pathway is commonly altered in pancreatic cancer [12]. We found that
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12 constitutive levels of Akt as well as insulin-induced activation of Akt were inhibited by
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14 statins and that this correlated with decreased phosphorylation of the downstream targets
15
16 GSK3 β Ser9 and 70 S6K Thr386. We also found that combined treatment of cells with
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18 atorvastatin and gemcitabine or 5-Fu induced increased inhibition of pAkt and that this
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20 correlated to inhibition of cell proliferation and increased the level of apoptosis in Panc-1
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22 and MIA PaCa-2 cells. Inhibition of Akt has been shown to sensitize cancer cell lines to
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24 chemotherapeutic agents [33-35], and statins have been shown to sensitize pancreatic
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26 cancer cell lines to cytostatic treatment [36]. Our data is also in line with a recently
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28 published study showing that fluvastatin inhibits proliferation, induces apoptosis and
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30 potentiates the cytotoxic effect of gemcitabine in MIA PaCa-2 cells [5].
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38 Gemcitabine is the current standard chemotherapeutic drug for treatment of pancreatic
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40 cancer but with minor benefit [37]. However, we found that in combination treatment,
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42 atorvastatin increased the effect of gemcitabine. Similar results were also induced by
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44 combining atorvastatin and 5-Fu. These effects correlated to decreased Akt
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46 phosphorylation. The same response was detected with combination of 5-Fu and
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48 atorvastatin. We also found that PI3K inhibitor, LY294002, increased the effect of
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50 atorvastatin in Panc-1 cells. This effect suggests that statins act by inhibiting PI3K/Akt
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52 pathway.
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3 Several lines of evidence indicate that the statin-induced growth inhibition in Panc-1 and
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5 MIA PaCa-2 cells was mediated by P2X7. Thus, P2X7 inhibitors, KN-62 and o-ATP,
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7 abrogated the atorvastatin-induced effect on pAkt and cell proliferation. Our data also
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9 show that silencing of P2X7 with siRNA prevented the effect of atorvastatin on cell
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11 proliferation. These data are in line with our recently published data showing that the
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13 statin-induced effect on nuclear pAkt is mediated via P2X7 [20]. In addition, we showed
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15 that the natural ligand for P2X7, extracellular ATP, inhibited insulin-induced Akt
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17 signaling in epithelial cells via P2X7. These effects were induced more rapidly than can
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19 be expected if an inhibited prenylation was involved [33]. Taken together data presented
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21 here indicate that atorvastatin inhibit the growth of pancreatic cancer cells via P2X7
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23 signaling. However, a remaining question is how statins activate P2X7.
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30 P2X7 is an ATP-gated cation channel leading to Ca^{2+} release and to pleiotropic effects
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32 [38]. Activation of the receptor is able to stimulate the release of proinflammatory
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34 cytokines and P2X7 activation can thus be involved in inflammation [22]. Chronic
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36 pancreatitis is a risk factor for development of pancreatic cancer and recently, increased
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38 levels of P2X7 were detected in chronic pancreatitis and pancreas cancer [23]. P2X7
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40 might thus be involved in pancreatic cancer development and might also be an attractive
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42 target for chemoprevention.
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48 In Capan-2 cells we found a low level of P2X7 concentrated in cytoplasmic droplets. The
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50 picture resembled that found in cells where P2X7 has been internalized in a trafficking
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52 process activated by ATP [39]. It thus seems that P2X7 is aberrantly regulated in a cell
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54 line in which atorvastatin did not affect pAkt levels nor sensitized them to cytostatic
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56 drugs. However, in these cells statins affected apoptosis. Statins have been shown to
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3 reduce NF κ B activation by inhibiting prenylation [36-37] and our data show that in
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5 Capan-2 cells NF κ B was reduced. In the same time phosphorylation of Raf and MEK
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7 were affected, which can be explained by an inhibited prenylation of Ras [32-41]. The
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9 reason why Panc-1 and MIA PaCa-2 cells were less sensitive to these effects remains to
10
11 be studied. One possibility is that abundantly expressed P2X7 in Panc-1 and MIA PaCa-2
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13 cells in some way prevents these effects.
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19 Pancreatic ductal adenocarcinoma is a highly malignant neoplasm that still carries an
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21 extremely poor prognosis. The use of adjuvant chemotherapy has not improved much on
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23 survival. Our data suggests that in cells with functional P2X7, P2X7-Akt signaling is a
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25 useful target that can sensitize many pancreatic cancers to chemotherapy.
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Figure Legends

Figure 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, C**) or for times indicated (**D**). Samples were then analyzed by Western blotting, employing antibodies for Akt, pAkt Ser473, Cyclin D1, pGSK-3 β 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 \pm 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-3 β Ser9/Cdk2, p70 S6K Thr386/Cdk2 and Cyclin D1/Cdk2 from three independent experiments; mean \pm SD from three independent experiments; * significantly different from corresponding control, $P \leq 0.05$.

Figure 2. Atorvastatin, gemcitabine and 5-Fluorouracil induce increased effect on pAkt in Panc-1 cells. **A** and **B**, cells were treated with atorvastatin (1 μ M) for 1 h and thereafter with gemcitabine (0.04 μ M) or 5-Fu (50 μ M) 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 \pm SD from three independent experiments; * significantly different from corresponding control, $P \leq 0.05$.

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3 **Figure 3. Antiproliferative and apoptotic effects of combined treatment of**
4 **gemcitabine or 5-Fluorouracil and atorvastatin in Panc-1 cells.** Cells were treated
5 with atorvastatin (4 μM) for 1 h and thereafter with gemcitabine or 5-Fu for
6 concentrations indicated for 48 h (**A** and **B**) or with gemcitabine (0.04 μM) or 5-Fu (50
7 μM) for 24 h (**C**). Cell proliferation was estimated using MTT assay (**A**). Columns, mean
8 \pm SD from three independent experiments; # significantly different from corresponding
9 control, * significantly different from atorvastatin, $P \leq 0.05$. The percentage of apoptotic
10 cells was quantified using Hoechst staining after 24 h (**B**). Samples were also analyzed by
11 Western blotting, employing antibody for PARP (**C**). Cdk2 was used as a loading control.
12 In panel **C** columns show densitometric analysis; mean \pm SD from three independent
13 experiments; * significantly different from corresponding control, $P \leq 0.05$.
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31 **Figure 4. Statins decrease pAkt and increase the effect of gemcitabine in MIA PaCa-**
32 **2, but not in Capan-2 cells.** In **A** and **C**, cells were treated with atorvastatin or
33 pravastatin for 1 or 24 h in concentrations indicated. Samples were analyzed by Western
34 blotting, employing antibodies for pAkt Ser473, pGSK-3 β Ser9 and PARP. Cdk2 was
35 used as a loading control. In **B**, cells were treated with atorvastatin for 1 h and thereafter
36 with gemcitabine or 5-Fu for 48 h. Cell proliferation was estimated using MTT assay.
37 Columns, mean \pm SD from three independent experiments; # significantly different from
38 corresponding control, * significantly different from atorvastatin, $P \leq 0.05$.
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53 **Figure 5. Effects of atorvastatin cells are mediated by P2X7 receptors.** **A**, cells were
54 treated with o-ATP (250 μM) or KN-62 (100 nM) for 10 min and thereafter with
55 atorvastatin (1 μM) for 10 min. Samples were then analyzed by Western blotting,
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3 employing antibodies for Akt and pAkt Ser473. Cdk2 was used as a loading control.
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5 Panc-1 cells were transfected with siRNA against P2X7 (50 nM) for 72 h (**B**) or treated
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7 with KN-62 (100 nM) for 1 h (**C**) and thereafter with atorvastatin for 48 h. Cell
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9 proliferation was estimated using MTT assay. **B**, shows mean \pm SD from three
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11 independent experiments; significantly different from * siRNA control/siRNA P2X7 or #
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13 from siRNA control + atorvastatin, $P \leq 0.05$. **C**, column, mean \pm SD from three
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15 independent experiments; significantly different from * control/KN-62 or # from
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17 atorvastatin, $P \leq 0.05$. Samples were also analyzed by Western blotting, employing
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19 antibody for PARP. In **B** Western blotting showing the level of P2X7 in siRNA P2X7
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21 transfected Panc-1 cells. In **D** Western blotting (20, 10, 5 and 0.2 μ g protein) of P2X7
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23 expression in Panc-1, MIA PaCa-2, Capan-2 cells and HEK293 cells heterologously
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25 expressing human P2X7 or P2X4 receptors. Columns; mean ratio from densitometric
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27 analysis of P2X7/Cdk2 from three independent experiments. In **E** and **G**,
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29 immunocytological staining of P2X7 expression in Panc-1, MIA PaCa-2, Capan-2 and
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31 A549 cells. In **F**, HEK293 P2X7 and HEK293 P2X4 cells were treated with atorvastatin
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33 for 48 h in concentrations indicated. Cell proliferation was estimated by MTT assay.
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43 **Figure 6. Atorvastatin affects Raf/MEK- and NF κ B pathway in Capan-2 cells.**

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45 In **A** cells were treated with atorvastatin for 24 h in concentrations indicated. Samples
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47 were then analyzed by Western blotting, employing antibodies for pRaf Ser259, pMek
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49 1/2 Ser217/221, Survivin and NF κ B p65. Cdk2 was used as a loading control. **B** shows
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51 mean ratio from densitometric analysis of pRaf Ser259/Cdk2, pMEK1/2 Ser217/221 or
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53 NF κ B/Cdk2 from three independent experiments, mean \pm SD; * significantly different
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55 from control, $P \leq 0.05$. In **C** Panc-1 and Capan-2 cells were treated with atorvastatin (4
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3 μM) for 8 h and thereafter immunocytochemically stained for NF κ B. Columns show
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5 relative staining intensity (mean \pm SD from 100 cells). * significantly different from
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7 corresponding control, $P \leq 0.05$.
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Figure 1

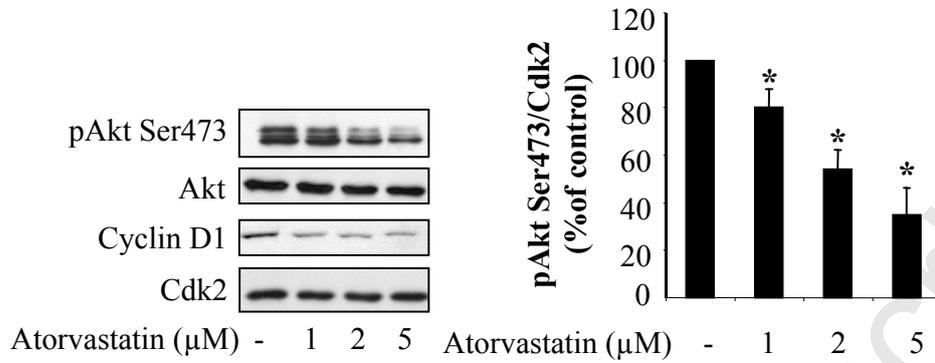
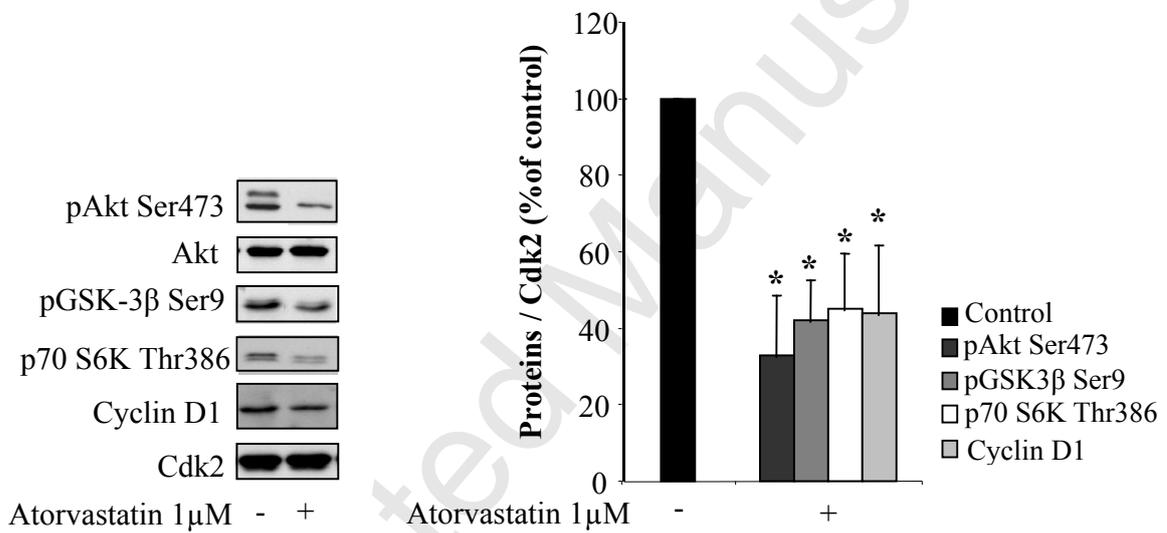
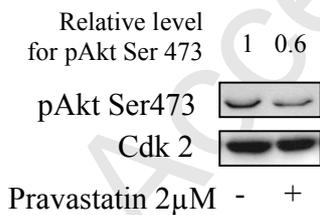
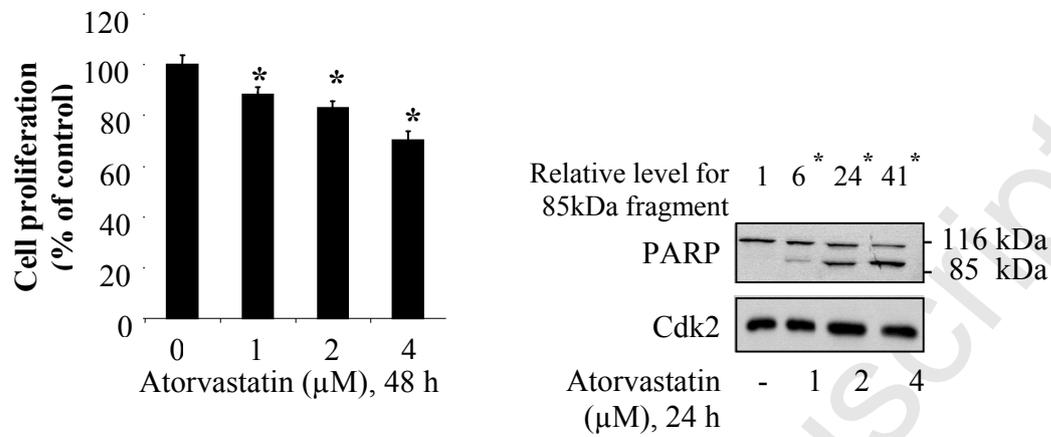
A. Panc-1**B. Panc-1****C. Panc-1**

Figure 1

D. Panc-1



E. Panc-1

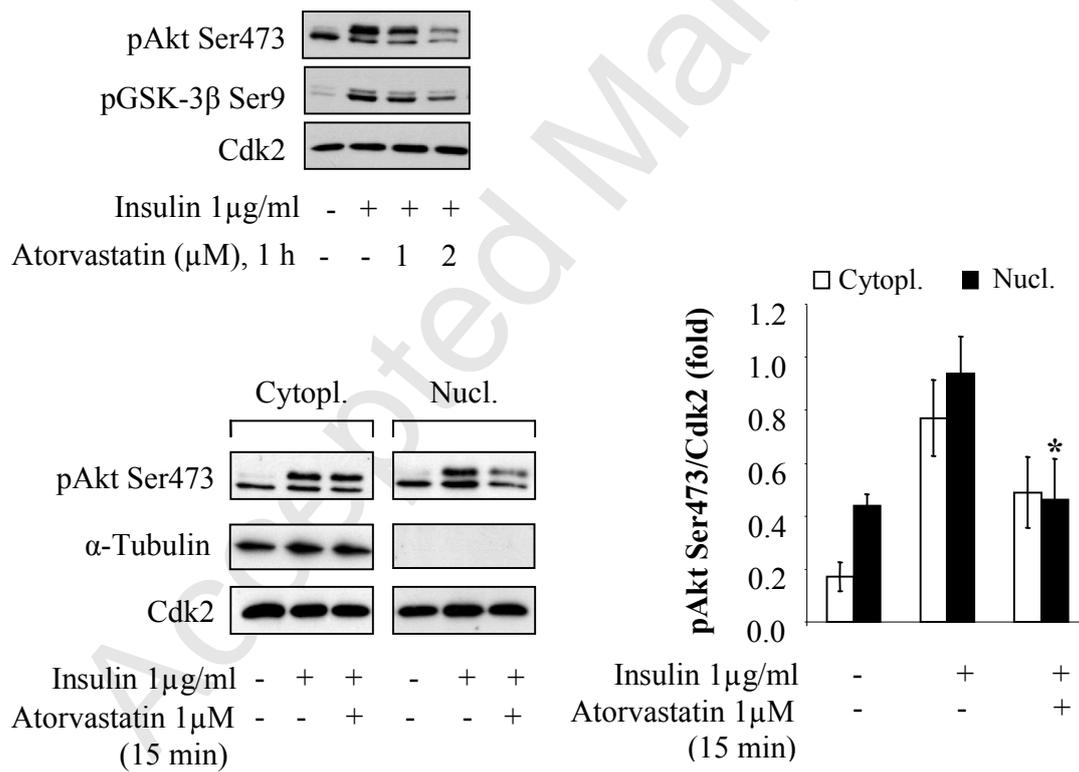
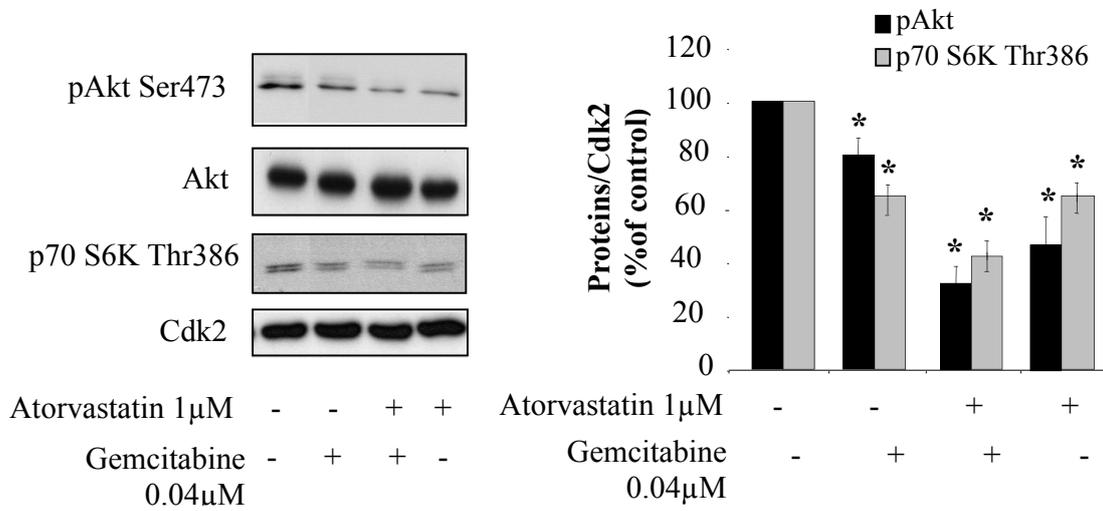


Figure 2

A. Panc-1



B. Panc-1

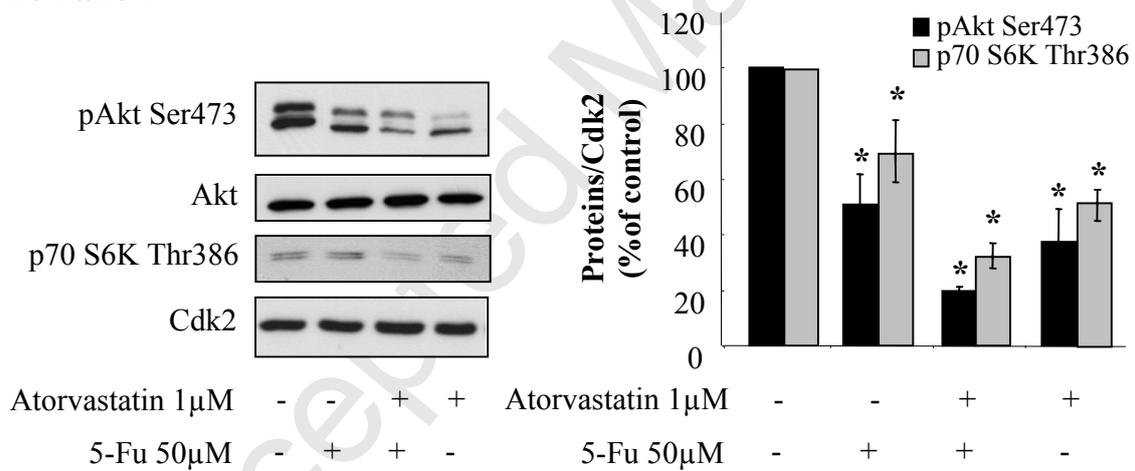
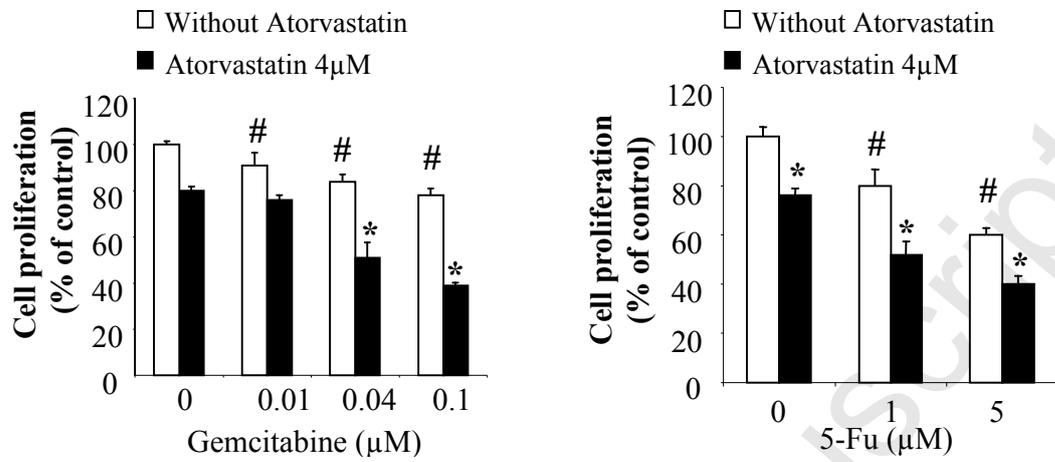
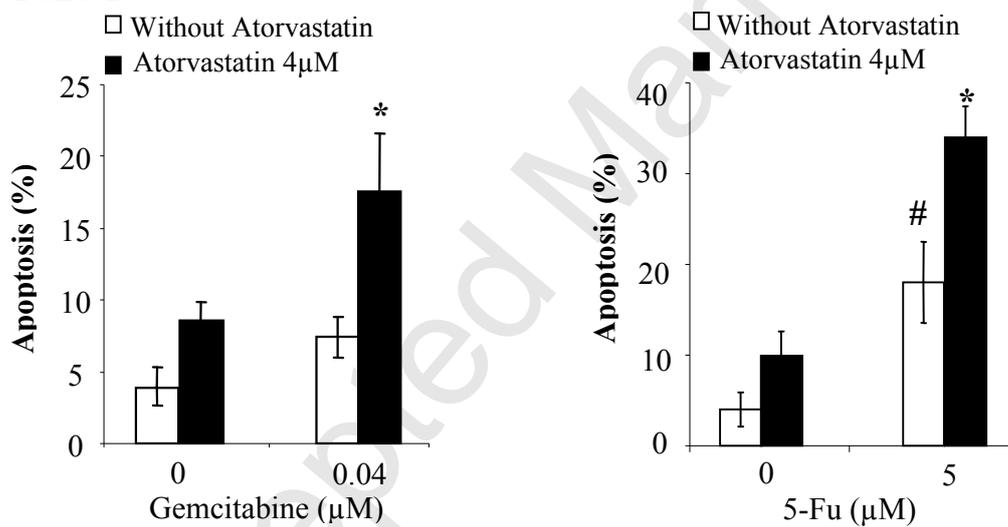


Figure 3

A. Panc-1



B. Panc-1



C. Panc-1

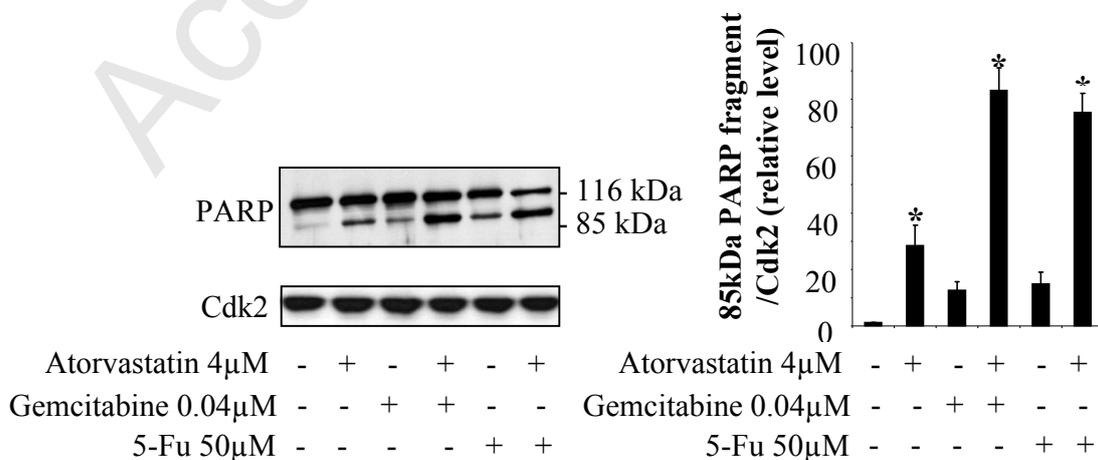
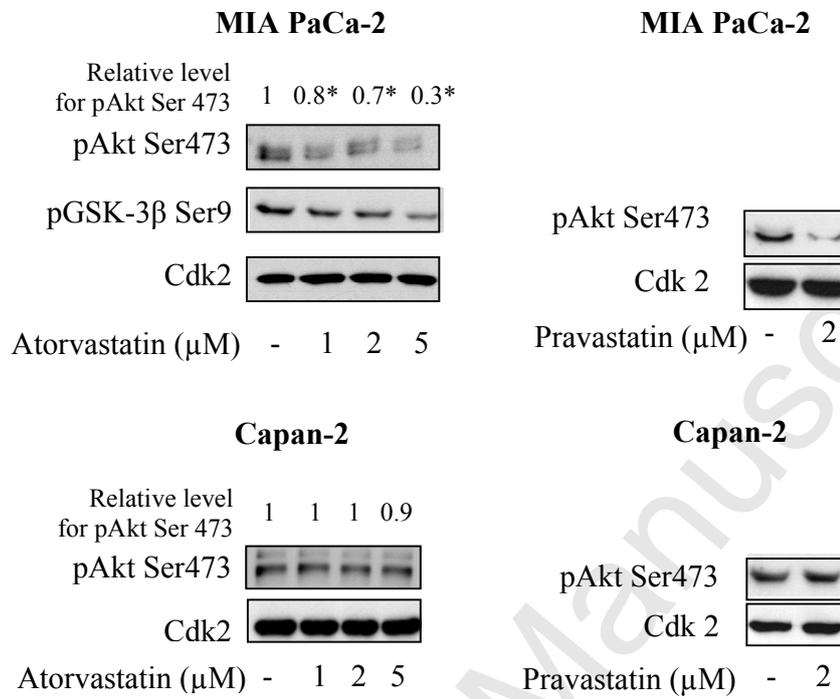


Figure 4

A.



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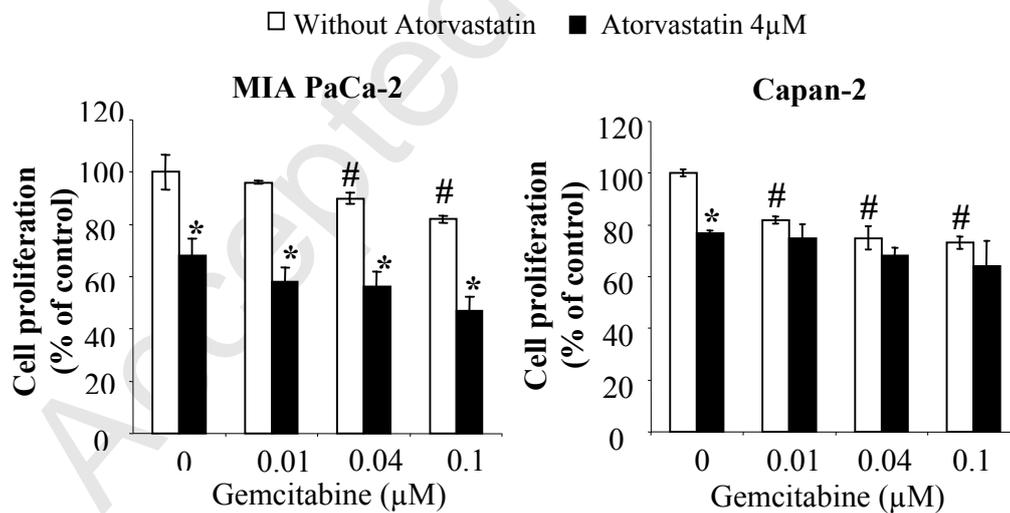


Figure 4

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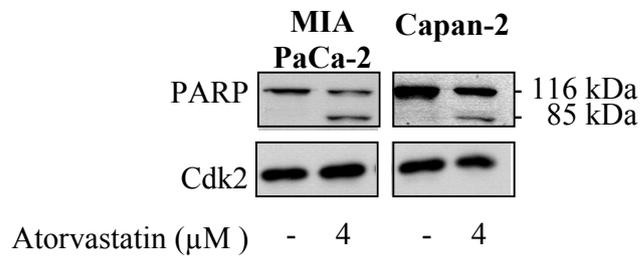
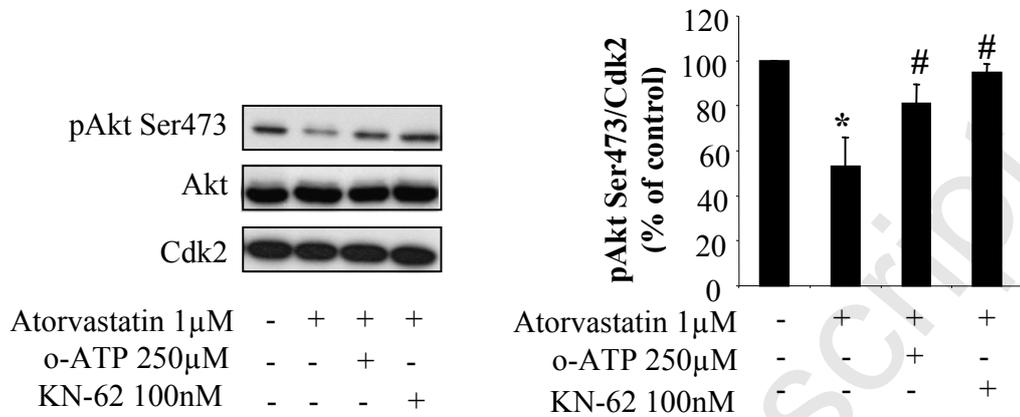
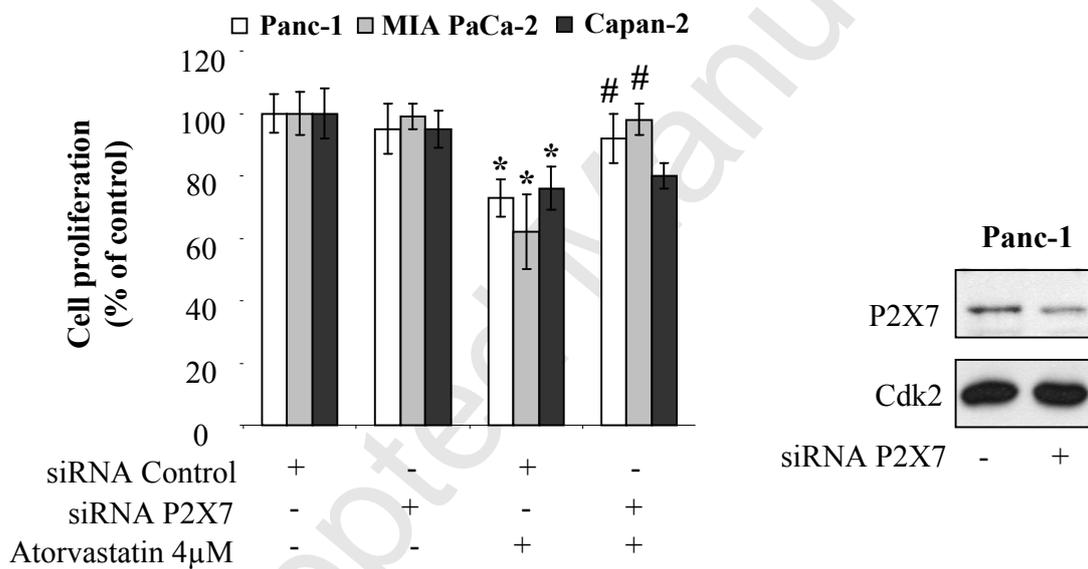


Figure 5

A. Panc-1



B.



C.

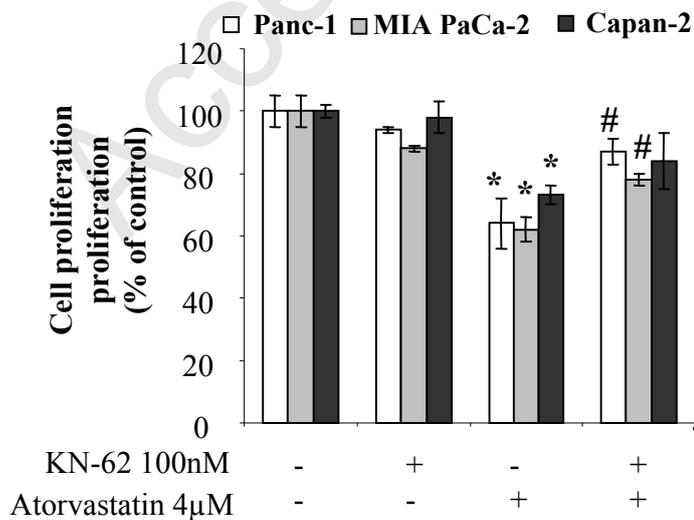
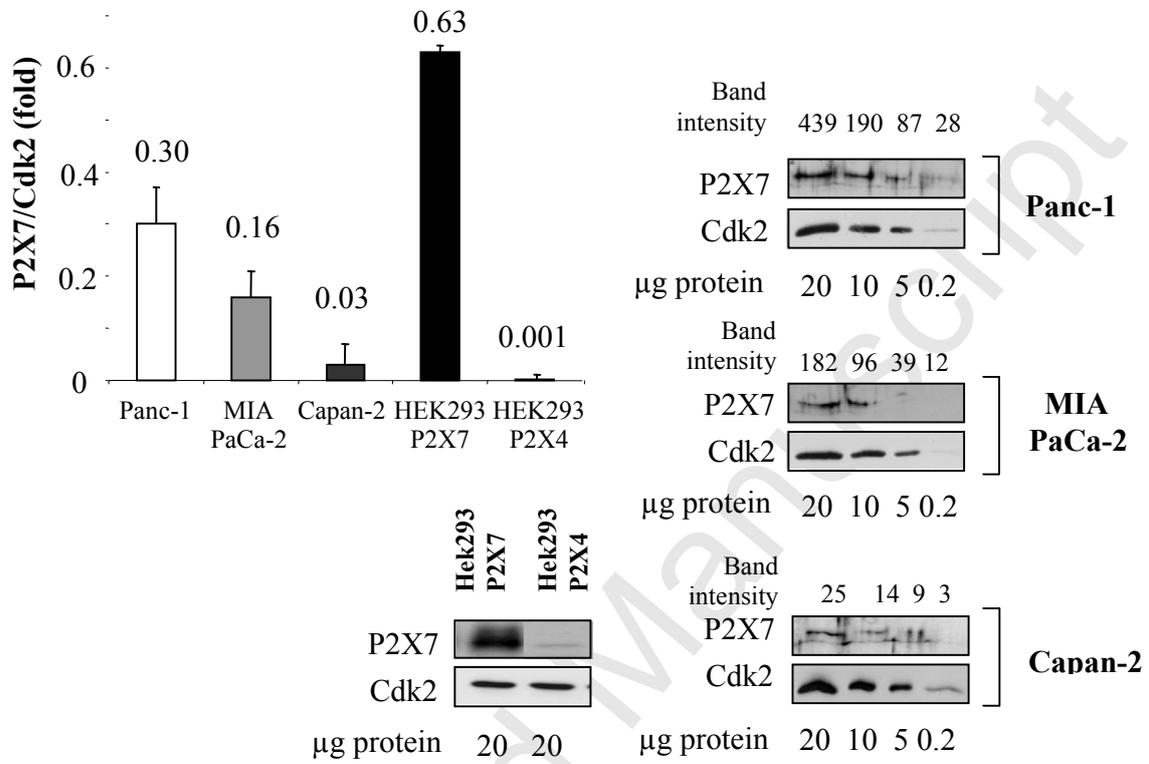


Figure 5

D.



E.

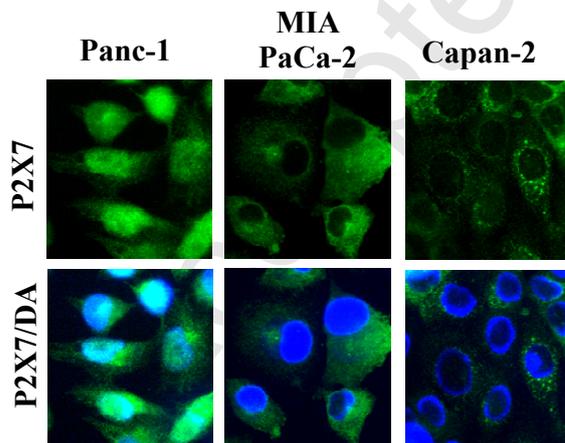
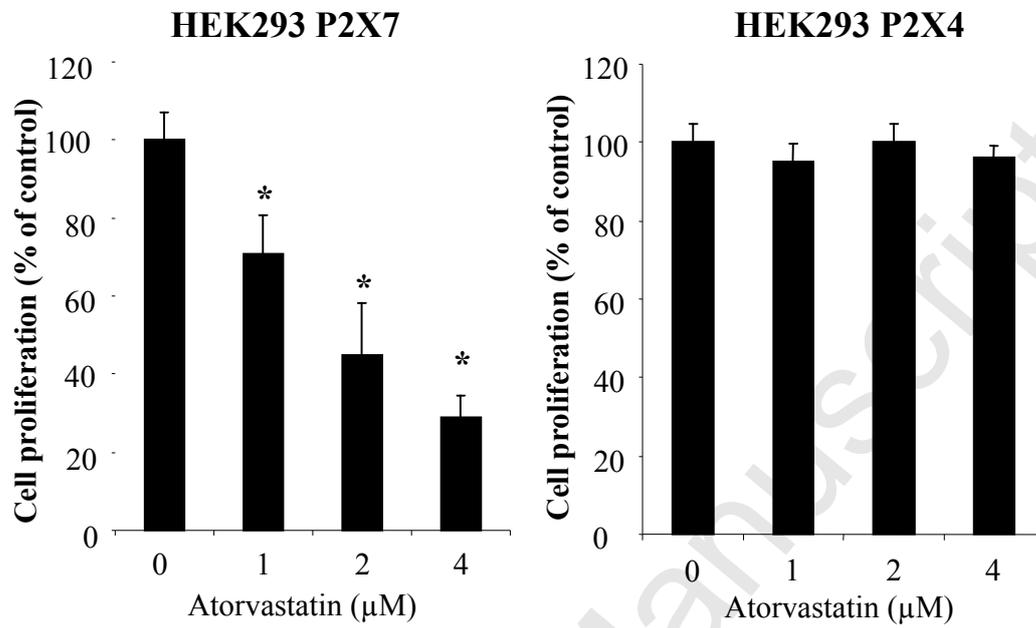


Figure 5

F.



G. A549-P2X7

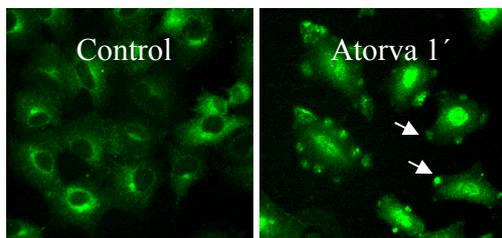
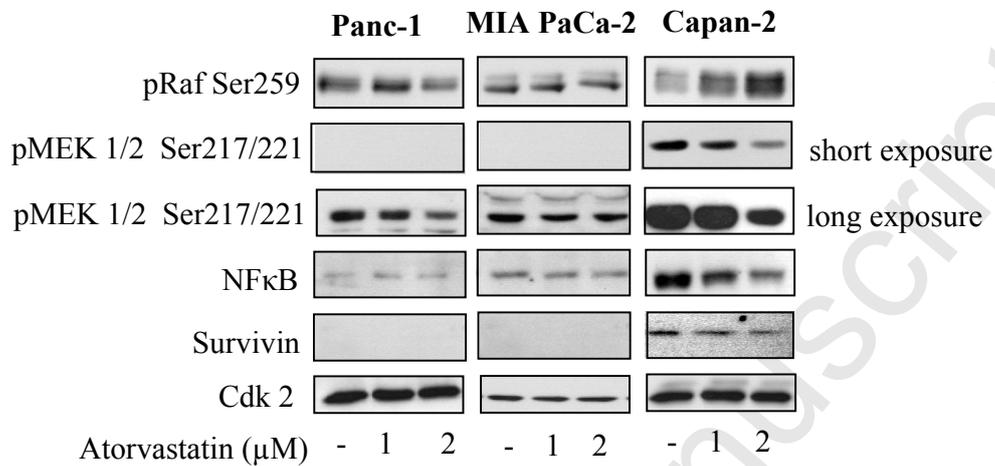


Figure 6

A.



B.

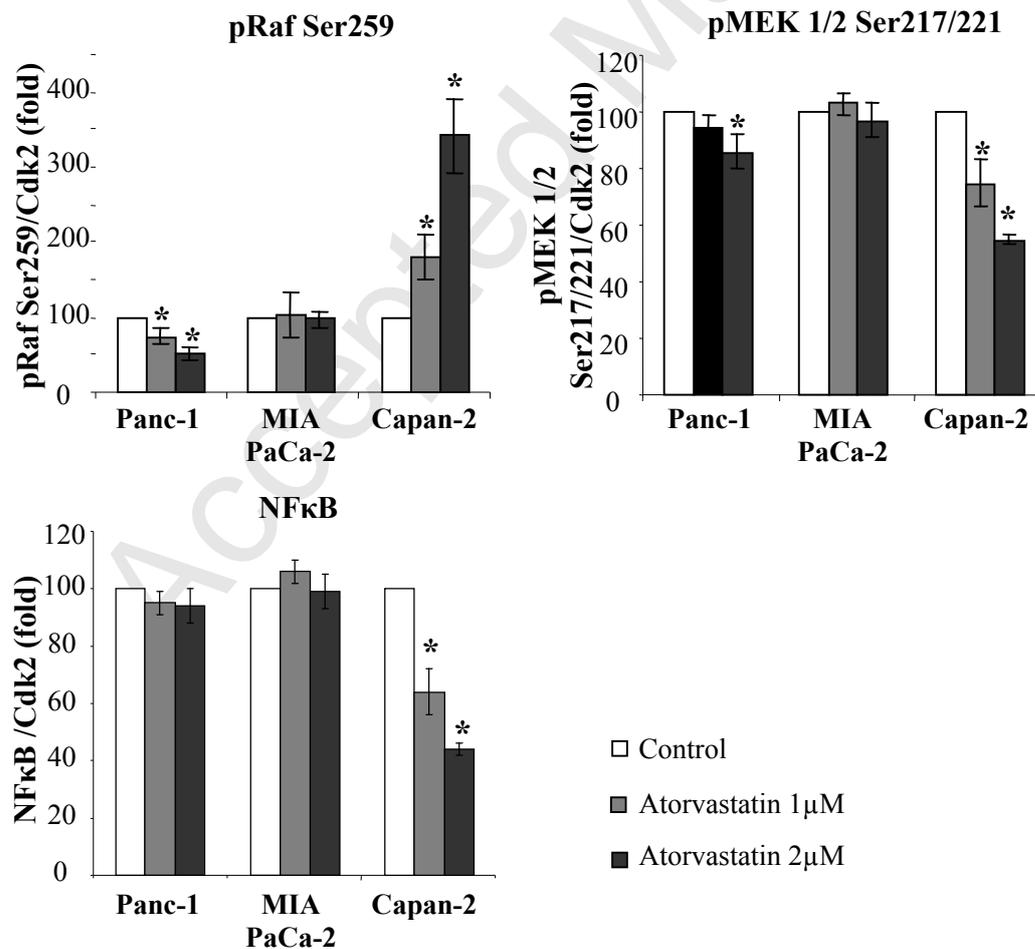


Figure 6

C.

