Atorvastatin Decreases HBx-induced phospho-Akt in Hepatocytes via P2X Receptors

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Running title: Statin Therapy Inhibits pAkt via P2X Receptors

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Hepatocellular carcinoma (HCC) is rated as the fifth most common malignancy and third in cancer related deaths worldwide. Statins, HMG-CoA reductase inhibitors, are potent cholesterol lowering drugs and recent epidemiological evidence suggests that statins prevent aggressive HCC development. Previous experiments revealed that statins downregulate phosphorylated Akt (pAkt). Here, it is demonstrated that atorvastatin decreases nuclear pAkt levels in pancreatic and lung cancer cell lines within minutes and this rapid effect is mediated by the purinergic P2X receptors. Akt is up-regulated by hepatitis viruses and has oncogenic activity in HCC; therefore, we tested the possibility that the P2X-Akt pathway is important for the anticipated anticancer effects of statins in hepatocytes. Atorvastatin decreased hepatitis B virus X protein- (HBx) and insulin-induced pAkt and pGsk3β (Ser9) levels. Furthermore, Akt-induced lipogenesis was counteracted by atorvastatin, and these statin-induced effects were dependent on P2X receptors. Statin also decreased proliferation and invasiveness of hepatocytes. These data provide mechanistic evidence for a P2X receptor-dependent signaling pathway by which statins decrease pAkt, its downstream phosphorylation target pGsk3β, and lipogenesis in hepatocytes.

Implications: The Akt pathway is deregulated and may act as a driver in HCC development, the P2X-Akt signaling pathway may have a role in anticancer effects of statins.
Introduction

Hepatocellular carcinoma (HCC) is rated as the fifth most common malignancy and third in cancer related death worldwide. There are several risk factors for HCC such as liver cirrhosis, alcohol, aflatoxins, obesity, lipogenesis and viral infections. Hepatitis B viral (HBV) infection accounts for 50-80% of all HCC cases [1], and recent data show that the relative risk for viral hepatitis increased between 1990-2013 and was the seventh leading cause of death worldwide [2].

The viral nonstructural gene hepatitis B virus X protein (HBx) has been shown to activate the PI3K/Akt pathway, which affects cellular and pathological processes such as invasion and metastases [3]. The molecular mechanism behind the HBx-induced activation of Akt pathway is not fully understood but evidence indicate that HBx induces activating Akt phosphorylation’s and downstream effects of Akt [4, 5]. Of perhaps particular interest are reports indicating increased activity and expression of several lipogenic enzymes in cancer cells [6, 7] and evidence identifying the Akt pathway as a major regulator of the lipogenic phenotype in liver cancer [8]. Data indicate that the Akt pathway induces lipogenesis both via transcriptional and Gsk3β-dependent post-transcriptional downregulation of sterol regulatory element-binding proteins (SREBPs) [8]. Activation of the PI3K/Akt signaling pathway has also been seen in HCC and Akt activation is strongly associated with HCC aggressiveness [9]. The PI3K/Akt axis has been suggested as a promising target for HCC treatment [10, 11].

Several epidemiological studies indicate a chemo-preventive effect of statins (3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors) against HCC [12, 13], including hepatitis B virus-related HCC [14]. Statins have also been shown to inhibit early phases of obesity-related
experimental liver tumorigenesis [15]. Anticancer effects in HCC and other cancer types are often explained as an on-target effect of statins on HMG-CoA reductase, which leads to depletion of the mevalonate pathway and an inhibited prenylation of signaling proteins [16].

Previously we have found that statins deplete nuclear pAkt via purinergic receptor-dependent signaling in liver, lung, prostate and pancreatic cells [17-19]. We provided evidence for P2X7 receptor-dependent rapid depletion of nuclear levels of activated/phosphorylated Akt (pAkt) in cells stimulated by insulin [17, 20, 21]. Akt is expressed mainly in the cytoplasm, but as recently highlighted [22], it is also expressed in the nucleus and there are data indicating that the nuclear localization of Akt is important for its oncogenic activity [23]. This suggested that statin-induced purinergic receptor signaling could be of importance for its cancer chemopreventive effects. Our interest in statin-induced P2X7 signaling is also stimulated by recent data showing that P2X7 affects tumor growth [24] and invasion [21].

Against this background we studied the effect of statins on pAkt in hepatocytes further. We here present evidence that atorvastatin decreases HBx-induced pAkt levels in hepatocytes. We also document downstream events such as decreases protein levels of lipogenic enzymes, and we show an involvement of P2X receptors in these effects, indicating an off-target effect of atorvastatin.
Material and methods

Cell culture

The Hepatocellular carcinoma HepG2 and Huh7 cells were purchased from American Type Culture Collection, ATCC (Manassas, VA). TRL-1215 cells were provided as a generous gift from Dr. Michael P. Waalkes from the National Cancer Institute. This non-tumorigenic cell line was originally derived from the livers of 10-day-old Fischer F344 rats [25].

HepG2 cells were grown in MEM, with Earl's salts and L-glutamine supplemented with 1mmol/L of sodium pyruvate, nonessential amino acids, 10% inactivated fetal bovine serum (FBS), and penicillin-streptomycin. The Huh7 cells were grown in D-MEM, with 10% inactivated FBS, penicillin-streptomycin and 1mM sodium pyruvate. The TRL-1215 cells were grown in William’s E+GlutaMax™-I with penicillin/streptomycin and 10% inactivated FBS. There are reports that HepG2 and Huh7 cells do not express P2X7 mRNA [26]. However, regarding HepG2 cells this is contradicted by other data [27, 28]. The latter shows initial low levels but rapid induction of P2X7 mRNA in HepG2 cells.

Reagents

Atorvastatin, insulin, KN-62 and adenosine 5’-triphosphate periodate oxidized sodium salt (o-ATP) were purchased from Sigma–Aldrich (St. Louis, MO). The atorvastatin concentration used was 1μM. Within 24h this concentration decrease cholesterol ester levels by 50% in HepG2 cells [29].

Western blotting
Cells were washed twice with PBS and lysed in IPB-7 with protein degradation inhibitors (1mg/mL phenylmethylsulfonyl fluoride, 0.1 mg/mL trypsin inhibitor, 1mg/mL aprotinin, 1mg/mL leupeptin, 1mg/mL pepstatin, 1mmol/L Na3VO4, and 1mmol/L NaF). The samples were subjected to SDS–PAGE and blotted onto a PVDF membrane (Bio-Rad, Hercules, CA). Some samples were sub fractionated. The protein bands were probed using antibodies against, Cdk2, HBx, P2X7, α-tubulin, total Akt, Akt phosphorylated at residues Ser473 and Thr308 from Santa Cruz (Santa Cruz, CA); Gsk3β Ser9, ACAC, MMP2 and MMP9 from Cell Signaling (Beverly, MA); FASN from BD Bioscience (BD Bioscience, Sweden). Proteins were visualized with ECL procedure (Amersham Biosciences, Sweden). The Western blotting results were analyzed with NIH Image 1.62 software.

**Immunocytochemical staining**

Cells were fixed in 3.7% formaldehyde. After fixation the cells were stained with polyclonal antibodies against phosphorylated Akt at residue Thr308 (Santa Cruz, Santa Cruz, CA). 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 nuclear staining intensity was analyzed with NIH Image 1.62 software.

**Small Interference RNA transfection**

Cells were transfected with P2X7, LXRβ or control small interference RNA (siRNA) (Santa Cruz Biotechnology, Santa Cruz, CA) for 40h according to the TranIT-TKO protocol (Lipofectamine™ 2000, Invitrogen).

**Plasmid transfection**
The expression vector for wild-type HBx was provided as a generous gift from Dr. Vijay Kumar at International Centre for Genetic Engineering and Biotechnology, India. The HBx plasmid has been described previously [30]. P2X7 plasmid and its empty vector were provided as a generous gift from Dr. Stéphane Chevalier (INSERM U1069, University of Tours, France). pEGFP-N1-mAKT and its empty vector were provided as a generous gift from Dr. Arthur Weiss at University of California, San Francisco. Cells were transfected with plasmids according to the Lipofectamine™ 2000 (Invitrogen) protocol. Cells were transfected for 2.5 µg plasmid per 60mm dish (according to the TranIT-TKO protocol) and for 40h or for times indicated in the figures.

Cell invasion assay

Cell invasion assay was performed using 8µm pore size Transwell Biocoat Control inserts (Becton Dickinson, Bedford, MA) according to the manufacturer’s instructions. The cells were fixed with methanol and thereafter stained with Toluidine Blue from Merck (Darmstadt, Germany). The number of transmembrane cells was counted.

MTT assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-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 4h. The cells were then lysed in DMSO. The absorbance was measured at 570–620 nm.

Statistical analysis
Statistical analysis was conducted using student’s t-test. 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 ≤ 0.05.

**Results**

*Atorvastatin rapidly decreases HBx-and insulin-induced pAkt.*

In line with previously published studies [4, 5], we showed that overexpression of HBx in HepG2 cells enhanced the level of pAkt Ser473. Total Akt was not affected. We also found that the Akt-induced Ser9 inactivating phosphorylation of Gsk3β was increased in HepG2 cells (Fig. 1A). We then investigated the effect of HBx overexpression on the pAkt level in Huh7 cells. We found that HBx overexpression enhanced the levels of pAkt Ser473 and pGsk3β Ser9 also in this human hepatocyte cell line (Fig. S1A).

Our earlier studies show that statins deplete nuclear pAkt without affecting total Akt levels in many epithelial cell types [17, 31]. The effect of atorvastatin on HBx-induced pAkt was now studied in HepG2 cells and we found that atorvastatin decreases HBx-induced pAkt Ser473 (Fig. 1B). The effect of atorvastatin on the insulin-induced pAkt level was also examined. We found that incubation of cells with atorvastatin (1μM) decreased the insulin-induced pAkt Ser473 (significant at 1 and 3h) and pGsk3β Ser9 (significant at 3h) in HepG2 cells (Fig. 1C). We also used non-transformed TRL-1215 rat liver epithelial cells. We found that atorvastatin decreased the insulin-induced pAkt Ser473 in TRL-1215 cells as well. The effect on pGsk3β Ser9 was non-significant (Fig. 1D). Taken together, we show that statins decrease HBx- or insulin-induced pAkt in hepatocytes.
Nuclear translocation of Akt is important for its activity [22, 23], and we have shown previously that statin decreases the nuclear pAkt within minutes [17]. Overexpression of HBx vector increased nuclear pAkt and atorvastatin lowered HBx-induced nuclear pAkt in HepG2 cells (Fig. 2A). This effect was confirmed by immunofluorescence microscopy (Fig. 2B). Employing the same technique we also found that atorvastatin depleted insulin-induced nuclear pAkt in HepG2 and TRL-1215 cells (Fig. 2C and D). As shown in figure 2E and F, fractionation experiments confirmed decreased levels of nuclear pAkt by showing decreased levels of pAkt Ser437. No concomitant increase of pAkt in the cytoplasmic fraction was seen. The time span used in some of our experiments (15min, Fig. 2C - F) tend to exclude an involvement of mevalonate depletion, since the effects of statins on mevalonate pathway are not detected until 2 - 4h [17]. In sum, these data indicate that atorvastatin deplete HBx- and insulin-induced nuclear pAkt in hepatocytes via mechanisms that are not dependent on mevalonate depletion.

*Effects of atorvastatin on Akt and lipogenic enzymes are mediated by P2X receptors.*

We have previously shown an involvement of the purinergic P2X7 receptor in pAkt depletion induced by statins [21], and that P2X7 co-localize with P2X4 upon addition of xenobiotics [32]. The role of P2X in HBV-induced pathogenesis is largely unknown but significant increase of purinergic receptor levels has been reported in hepatic cells stably expressing HCV structural proteins [33]. We now investigated the role of the P2X receptor in statin-induced effects in HepG2 cells. The P2X antagonist, oxidized-ATP (o-ATP) and a selective inhibitor of P2X7 receptor, KN-62, were tested. We found that atorvastatin-induced depletion of pAkt and pGsk3β Ser9 was inhibited by P2X7 inhibitors (Fig. 3A). Next, we investigated an involvement of P2X7 receptor in insulin-induced pAkt. As shown in Fig. 3B and C, preincubation with o-ATP, or KN62 abrogated the atorvastatin-induced effect on pAkt Ser473 in insulin-treated HepG2 and
TRL-1215 cells. o-ATP, or KN62 alone did not have any effect on pAkt (Fig. 3B and C). In addition, the siRNA against P2X7 was tested in TRL-1215 cells. As shown in Fig. 3D the atorvastatin-induced depletion of insulin-induced pAkt Ser473 was completely blocked by siRNA P2X7. To further investigate the role of P2X7 receptor, we tested the effect of statin on P2X7 negative Huh7 cells [33] (Fig. 3E). We found that atorvastatin had no effect on insulin-induced pAkt Ser473 in Huh7 cells, but that atorvastatin depleted pAkt Ser473 after transfection of P2X7 (Fig. 3E), confirming a dysfunctional P2X7 in this cell line as well as a role for P2X7 in atorvastatin-induced effects.

Gsk3β may downregulate SREBPs and lipogenesis [34, 35], and Akt-induced Gsk3β Ser9 phosphorylation inhibits this downregulation. Thus Akt overexpression enhances the levels of several proteins involved in lipogenesis [8]. We studied the effect of atorvastatin on lipogenic enzymes induced by Akt overexpression. We employed HepG2 cells transfected with HBx. However, this did not give clear enough effects on lipogenic enzymes. Instead we transfected HepG2 cells with Akt which resulted in clearly increased levels of FASN and ACAC levels (Fig. 4A). Similar effects were seen in TRL-1215 cells (data not shown). We also treated the Akt-overexpressing HepG2 cells with atorvastatin and found that Akt-induced FASN was decreased significantly by atorvastatin. This effect was seen after 24h of treatment (Fig. 4A), and also after 3h of atorvastatin treatment (data not shown). No effect of statin on ACAC was seen. We then investigated levels of HMG-CoA reductase, the rate limiting enzyme in cholesterol synthesis which is targeted by statin therapy [36]. We found that Akt transfection induced HMG-CoA reductase protein levels in HepG2 cells and that atorvastatin reduced these levels. We also found that KN62 counteracted this effect of atorvastatin (Fig. 4B), indicating an involvement of P2X7. A role for P2X receptors was confirmed by using Huh7 cells and the P2X4 inhibitor TNP-ATP
As shown in Fig S1B, also this inhibitor counteracted the statin-induced effect on HMG-CoA reductase protein levels.

It was recently shown that FASN deletion or overexpression may influence Akt activation via Rictor in mouse liver [37]. In control experiments employing HBx transfected cells (data not shown) we observed no effects of atorvastatin on Ricor levels, indicating that atorvastatin did not affect pAkt levels via downregulating Rictor.

*Atorvastatin reduces HBx-induced cellular invasiveness and proliferation.*

We have earlier shown that statin-induced pAkt depletion is associated with decreased invasiveness and proliferation of different cancer cell lines [21]. We have also shown that P2X7 is involved in invasive growth and that atorvastatin inhibits invasiveness through a P2X7-Akt axis [21]. The expression of matrix metalloproteinase (MMP) family has been shown to have a role in cellular invasion and metastases. Two particular genes of the MMP family, MMP-9 and MMP-2, have been reported to be increased by HBV infection [38]. MMP-9 is activated through activation of ERKs and PI3K-Akt pathways [39]. We found that HBx overexpression enhanced MMP-9 and MMP-2 protein levels in the HepG2 cells. Treatment of cells by atorvastatin for 24h decreased HBx-induced MMP-9 and MMP-2 levels significantly (Fig. 5A). Further, we found that HBx transfection induced invasiveness and that atorvastatin counteracted this effect. The inhibiting effect shown in Fig. 5B correlated with decreases in MMP levels (Fig. 5A) and with inhibition of cell proliferation (Fig. 5C). As shown in Fig. S2A and B, atorvastatin also induced a significant inhibition of hepatic cell proliferation. Atorvastatin have even a stronger anti-proliferative effect on HBx overexpressing cells than on un-transfected cells (Fig. 5C).
together, we found that atorvastatin decrease HBx-induced cellular invasiveness and proliferation in HepG2 cells, and that was correlated to decrease in MMP2 and MMP9 levels.

**Discussion**

In this study we show that atorvastatin decreases nuclear pAkt levels in hepatocytes. We employed insulin, the oncogenic virus protein HBx, and Akt transfection to increase pAkt levels, and a generalized ability of atorvastatin to abrogate Akt activation in hepatocytes was indicated. A mitigated inhibition of Gsk3β, decreased levels of lipogenic enzymes, decreased invasive growth and decreased cell proliferation, all possible downstream effects of decreased pAkt levels, were also documented. Typically we used 1µM concentration of atorvastatin in our experiments, but previously we also reported pAkt depletion at 50nM [20, 21] so an effect on pAkt by statin therapy in humans can be expected [40].

The time frame for many of our experiments indicates that decreased pAkt levels are not caused by an inhibited mevalonate pathway as components in this pathway are not affected until 2-4 hours after statin addition [41]. Purinergic receptors, on the other hand, induce rapid effects on cell signaling [17] and our data showing an involvement of P2X receptors is consistent with a rapid effect on pAkt levels. Our data thus indicate that the pAkt effects shown here can be delineated as an off-target effect of atorvastatin, and, as we argue below, statin-induced pAkt depletion may lead to effects mediated by Gsk3β and SREBP2 as well as to other effects discussed in our previous studies [17-21].

There are ample evidence that PI3K/Akt regulates SREBPs via Akt phosphorylation of Gsk3β, and also that PI3K/Akt inhibitors downregulate the mevalonate pathway [42-44]. We found that Akt overexpression increased FASN and HMG-CoA reductase protein levels, and we found that
atorvastatin counteracted the Akt-induced effects via a P2X-dependent mechanism. The effect of Akt overexpression is in line with earlier studies showing that Akt increases the expression of HMG-CoA reductase and FASN genes [45-47]. Atorvastatin may thus not only act as a competitive inhibitor of HMG-CoA reductase, but also decreased its protein levels by down-regulating Akt via an off-target effect. The regulation of HMG-CoA reductase is very complex and there are reports that statins up-regulate un-induced HMG-CoA reductase expression (see e.g. [29]). However, we have found no literature data on how statins affect Akt-induced HMG-CoA reductase levels.

In experimental studies of statins and cancer chemopreventive a role for depleted metabolites in the mevalonate pathway is often demonstrated by adding mevalonate in high concentrations (see e.g. [16]). Our data suggest that mevalonate may counteract not only the competitive inhibition of HMG-CoA reductase [16], but also the off-target effect on lipogenic enzyme expression, including a decreased HMG-CoA reductase expression as shown here. Consequently, counteracting effects of mevalonate addition may not exclude an involvement of an off-target effect on pAkt by statins.

In our previous work on statins and their effect on pAkt levels we discussed anticancer effects other than a decreased HMG-CoA reductase expression and an inhibited mevalonate pathway [17-19, 21]. For example and as mentioned above, we recently suggested a statin-induced pAkt-dependent effect on MMP-9 activity [21]. Of further interest is that in a well characterized 3D model of mammary glands mutant p53 caused malignant phenotypes by up-regulating SREBP2 and the mevalonate pathway (i.e. a mechanism similar to the one induced by pAkt), and that simvastatin very effectively inhibited this effect [16]. In fact, the authors comment that simvastatin in some aspects had more dramatic effects than deleting mutant p53 [16]. This latter
observation leads us to speculate that simvastatin might have decreased pAkt levels and that additional targets [9], besides SREBP2 and the mevalonate pathway, were affected by pAkt depletion. This 3D model might thus be suitable for further characterization of off-target effects of statins.

Epidemiological studies indicate that statins prevent HBV-induced HCC [14] as well as perhaps any type of HCC [13, 48]. Our data suggest that statin-induced downregulation of pAkt might be part of an explanation of this chemopreventive effect. Of perhaps particular interest is the question if a downregulation of lipogenic enzymes via pAkt depletion is of relevance for a chemopreventive effect on NASH and NASH-related HCC. Further studies are warranted as e.g. FASN has been implicated in this type of hepatocellular carcinogenesis [8]. For example, recent work indicates that e.g. FASN ablation prevents HCC development in Akt-overexpressing mice [37].

Our data indicate a role for P2X receptors in atorvastatin-induced effect in hepatocytes. P2X receptors are mainly studied in inflammatory and mesenchymal cells, and less is known about their function in epithelial cells even though P2X receptors are expressed in these cells [49]. In HCC, high P2X7 expression in peritumoral hepatocytes, but not in tumor tissue, correlates with poor prognosis for HCC patients [50]. It correlated to aggressive clinic-pathological features, especially high rates of vascular invasion, a marker of HCC invasiveness [50]. The role of P2X resoprtors in HBV-induced pathogenesis is unknown but significant increase of purinergic receptor levels in hepatic cells stably expressing HCV structural proteins has been reported [33].

Invasion and metastases are crucial events in HCC progression and our findings complements growing evidence that HBx-overexpression is associated with increased levels of MMPs and
contributes to cell invasion. HBx-induced invasiveness correlates to the PI3K/Akt pathway activity [3], and our finding that HBx transfection induced invasiveness support a role for Akt in HBx-induced HCC.

In summary, our data show that atorvastatin counteracts HBx-induced pAkt levels in human hepatocytes. In line with this, inhibiting effects on cellular invasiveness and proliferation were seen in HBx overexpressing cells, and on lipogenic enzyme expression in Akt-overexpressing cells. We also provide evidence that the atorvastatin-induced downregulation of pAkt was mediated by P2X receptors, and our data suggest that this effect might work in parallel with a statin-induced competitive inhibition of HMG-CoA reductase to counteract HCC development as documented in epidemiological studies.

References

Figure 1. Atorvastatin decreases HBx- and insulin-induced pAkt levels in hepatocytes.
A, HepG2 cells were transfected with HBx expressing plasmid for 40h. B, HepG2 cells were transfected with HBx expressing plasmid for 40h, serum starved (24h) and thereafter treated for atorvastatin (1µM) for additional 24h. C, serum starved HepG2 cells were treated with insulin (1µg/ml) for 15min and thereafter with atorvastatin for 1 or 3h. D, serum starved rat primary liver TRL-1215 cells were treated with 1µg/ml insulin for 15min and thereafter with atorvastatin (1µM) for 1 or 3h. A-D, cell lysates were analyzed for Akt1, pAkt Ser473, pGsk3β Ser9 and HBx by Western blotting. Cdk2 was used as loading control. Bars represent the mean ±S.D. from three independent experiments. * Significantly different from control cells, ¤ Significantly different from HBx overexpressed cells, # Significantly different from insulin-treated cells, p < 0.05.

Figure 2. Atorvastatin decreases nuclear pAkt in hepatocytes.
A, HepG2 cells were transfected with HBx vector for 40h, serum starved for 24h and thereafter incubated with 1µM atorvastatin for 3h. In B, HepG2 cells were transfected with HBx vector for 40h followed by starvation for 24h and thereafter treated with 1µM atorvastatin for 3h, and the cells were stained for pAkt Thr308. C-F, serum starved cells were incubated with 1µg/ml insulin for 15min and thereafter with 1µM atorvastatin for 15min. C and D, cells were stained for pAkt Thr308. E and F, Western blots and densitometric analysis of nuclear and cytoplasmic fractions are shown. A-F, Bars represent the mean ±S.D. from three independent experiments. Cdk2 was used as a loading control. *Significantly different from control cells, # significantly different from insulin-treated cells, ◇ significantly different from HBx vector transfected cells, p < 0.05.

Figure 3. P2X receptors mediates the atorvastatin-induced effect on HBx-and insulin-induced pAkt in hepatocytes.
A, HepG2 cells were transfected with HBx vector for 40h, followed by 24h starvation and thereafter incubated with o-ATP (250µM) or KN-62 (100µM) for 10min, and thereafter with atorvastatin (1µM) for 3h. B and C, serum starved cells were treated with Insulin (1µg/ml) for 15min followed by treatment with P2X inhibitors (o-ATP, 250µM or KN-62, 100µM) for 10min, and thereafter with atorvastatin (1µM) for 3h. D, TRL-1215 cells were transfected with siRNA against P2X7 (50nM) for 40h and thereafter serum starved (24h) and incubated with insulin (1µg/ml) for 15min then with atorvastatin (1µM) for 3h. E, Huh7 cells were transfected with P2X7 expressing plasmid for 40h and thereafter serum starved (24h) and treated with insulin (1µg/ml) for 15min then atorvastatin 1µM for 3h. A-E, Western blots and densitometric analysis of total fractions are shown. Bars represent the mean ±S.D. from three independent experiments. Cdk2 was used as a loading control. *Significantly different from control cells, # Significantly different from insulin-treated cells, p < 0.05.
Figure 4. Atorvastatin decreases Akt-induced lipogenesis enzymes. A, HepG2 cells were transfected with Akt expressing plasmid for 40h, serum starved (24h) and thereafter treated with atorvastatin (1μM) for additional 24h. In B, HepG2 cells were transfected with Akt vector for 24h followed by serum starvation (24h) and treatment with KN-62 100µM for 10min then by treatment with atorvastatin (1µM) for 3h. A-B, Western blots and densitometric analysis of total fractions are shown. Bars represent the mean ±S.D. from three independent experiments. Cdk2 was used as a loading control. *Significantly different from control cells, # Significantly different from Akt overexpressed cells, p < 0.05.

Figure 5. Atorvastatin decreases HBx-induced invasiveness and proliferation in HepG2 cells. A and B, HepG2 cells were transfected with HBx expressing plasmid for 24h, serum starved for 40h and thereafter treated with atorvastatin (1μM) for additional 24h. B, cells analyzed by invasion assay. C, HepG2 cells were transfected with HBx expressing plasmid for 40h. The cells were treated during the transfection with atorvastatin (1μM) for 24h and the cell proliferation was measured by MTT assay. A-C Bars represent the mean ±S.D. from three independent experiments. Cdk2 was used as a loading control. *Significantly different from control cells, # Significantly different from HBx overexpressing cells. ●Significantly different from empty vector overexpressing cells, □ Significantly different from empty vector overexpressing cells treated with atorvastatin. p < 0.05.
Figure 2  Author Manuscript Published OnlineFirst on February 16, 2017; DOI: 10.1158/1541-7786.MCR-16-0373

A. HepG2

B. HepG2-pAkt Thr308

C. HepG2-pAkt Thr308

D. TRL-1215-pAkt Thr308

E. HepG2

F. TRL-1215

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
**Figure 3**

### A. HepG2

- **pAkt Ser473**
- **pGsk3β Ser9**
- **Cdk2**

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**Protein levels**

- 2.5
- 2.0
- 1.5
- 1.0
- 0.5
- 0.0

### B. HepG2

- **pAkt Ser473**
- **Cdk2**
- **Insulin**
- **Atorvastatin**

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### C. TRL-1215

- **pAkt Ser473**
- **Cdk2**

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### D. TRL-1215

- **pAkt Ser 473**
- **pGsk3β Ser9**
- **Cdk2**
- **P2X7**

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<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>sRNA Control</td>
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<tr>
<td>sRNA P2X7</td>
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</table>

### E. Huh7

- **pAkt Ser473**
- **pGsk3β Ser9**
- **Cdk2**
- **P2X7**
- **Akt1**

- 3.5
- 3.0
- 2.5
- 2.0
- 1.5
- 1.0
- 0.5
- 0.0

**Conditions**

- Empty Vector
- P2X7 Vector

- Control
- Ins
- ins + At
**A. HepG2**

- FASN
- ACAC
- pAkt Ser473
- Akt
- CDK2

<table>
<thead>
<tr>
<th></th>
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<th>Akt vector</th>
<th>Akt vector + Atorvastatin</th>
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<tr>
<td>FASN</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>1.8 ± 0.3</td>
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<tr>
<td>ACAC</td>
<td>0.8 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>1.4 ± 0.3</td>
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<tr>
<td>pAkt Ser473</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.6 ± 0.3</td>
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</table>

**B. HepG2**

- 97kDa
- HMG-CoA
- Akt
- Cdk2

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<th>Akt vector</th>
<th>Akt vector + Atorvastatin</th>
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<tr>
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<td>-</td>
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<tr>
<td>Akt vector</td>
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<td>Atorvastatin (h)</td>
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Molecular Cancer Research

Atorvastatin Decreases HBx-induced phospho-Akt in Hepatocytes via P2X Receptors
Aram Ghalali, Javier Martin-Renedo, Johan Hogberg, et al.

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