

## Modulation of Doxorubicin Actions in Hepatocellular Carcinoma Cells by Insulin-Like Growth Factor-I

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### Abstract

Hepatocellular carcinoma (HCC) is typically advanced at presentation and doxorubicin is the chemotherapeutic agent most frequently used in chemoembolization. Tumor microenvironment contains many cell types, including platelets, as well as cytokines and growth factors, potentially capable of modulating doxorubicin actions. Insulin-like growth factor I (IGF1) and its receptor (IGF1-R) signaling is important in cellular proliferation and prevention of apoptosis and the acquisition of chemotherapy resistance. IGF1 is present in platelets, which have been shown to alter doxorubicin actions in HCC cells. The effects on growth and motility after IGF1 pre-treatment in doxorubicin-treated HCC cells were thus examined, as well as the IGF1-R downstream pathways, PI3K/Akt and MAPK kinases. We found that IGF1 antagonized the doxorubicin-mediated decrease in cell growth and motility, as well as the doxorubicin-mediated decrease in levels of the proliferation-associated proteins phospho-IGF1-R, phospho-ERK, phospho-p38 and phospho-STAT3. The simultaneous induction of PI3K/Akt pathway, mediated by IGF1 receptor, regulated an increase in levels of Bcl-2, Bcl-xL and survivin through the Akt activation. Furthermore, PI3K/Akt signaling modulated several downstream targets, including phosphorylation levels of 4EB-P1, p70S6K and GSK-3 $\beta$ .

Up-regulated IGF1 signaling antagonized the doxorubicin-mediated changes cell proliferation, motility and apoptosis, thus contributing to drug resistance. Therefore IGF1-R may be a promising target for HCC management.

**Keywords:** Drug resistance; IGF1; PI3K/Akt pathway; Intracellular signaling; Doxorubicin

### Introduction

Doxorubicin, an anthracycline antibiotic with anti-proliferative and cytostatic effects, is widely used in the treatment of inoperable hepatocellular carcinoma (HCC) patients, especially as part of chemoembolization procedures. Despite its high efficacy, the doxorubicin-based chemotherapy is associated with multiple and severe side effect to non-tumors tissues, especially in heart, liver and kidney, restricting its clinical application. Furthermore, several studies have demonstrated that drug resistance decreases susceptibility to chemotherapeutic agent effectiveness [1-3]. We previously reported that platelets and their products, as major components of the HCC microenvironment, could affect cell growth and motility of HCC cells, thus modulating doxorubicin actions [4]. The role of increased blood platelet counts in various clinical aspects and prognosis of HCC has been recently shown [5,6]. In particular, it has also been reported that several growth factors have anti-apoptotic action and are able to antagonize doxorubicin-mediated growth inhibition and apoptosis and autophagy induction [7-11].

Among these products, insulin-like growth factor I (IGF1) and its receptor (IGF1-R) signaling has been increasingly seen to be important in hepatocarcinogenesis. The IGF1-R signaling triggered by IGF1 is involved in cellular proliferation, cell cycle progression and in the prevention of apoptosis, leading to the maintenance of the tumorigenic phenotype [11,12]. Moreover over-expression of IGF1-R has been demonstrated to reduce the dependence of the tumor cell growth from IGF1 and the cellular susceptibility to apoptosis [12]. It was also recently shown that IGF signaling is involved in the acquisition of chemotherapy resistance associated with several events including altered apoptotic signaling, induction of anti-apoptotic protein and over-expression of multidrug resistance (MDR) gene protein Pgp

[1,12]. Therefore, chemotherapy combined with modulation of the IGF1-R pathway may be beneficial in human liver cancer therapy [12,13].

In the present study we have examined the mechanisms underlying the interference of doxorubicin with the two major intracellular pathways induced by IGF1-R, PI3K/Akt and MAPK, in human HCC cell lines.

### Materials and Methods

#### Cells and drugs

Doxorubicin was purchased from Pfizer Labs (NY, USA), dissolved in 0.9% of NaCl and diluted in the appropriate concentrations. Recombinant human IGF1 was purchased from Prospec (Ness Ziona, Israel), GSK1838705A from Selleckchem (Houston, TX, USA). PLC/PRE/5 and HepG2 human HCC cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The culture medium was Dulbecco's Modified Eagle's Medium (DMEM). All cell culture components were purchased from Sigma- Aldrich (Milan, Italy).

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## Cell culture

HepG2 and PLC/PRF/5 cell lines were cultured in DMEM in monolayer culture, and supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. Since PLC/PRF/5 cells express high affinity IGF1-R which may mediate the stimulatory effects of exogenous IGF1 [14], we chose to represent the proliferation results in both cell lines and all the subsequent experiments in PLC/PRF/5.

## IGF1 assay

Human Platelet Lysates (hPLs), obtained as previously described [4], was used for the *in vitro* quantitative determination of human IGF1 by the human IGF1 ELISA kit (Wuhan Boster Biological Technology LTD, Wuhan, China). Serial dilutions of hPL or FBS, used as control, were prepared and processed according to the user's guide.

## Proliferation and apoptosis

The cells were cultured in 1% FBS medium containing IGF1 40ng/ml, the concentration was derived from the IGF1 ELISA dosage in hPLs, or hPLs corresponding to  $3.75 \times 10^7$  platelets/ml, or equivalent concentration of FBS, in presence of 0.1 and 1µM of doxorubicin. In the same condition HCC cell lines were cultured in presence of IGF1-R inhibitor 1µM GSK1838705A. After a defined time of incubation, the proliferative response was estimated by colorimetric 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. The trypan blue exclusion test was used to evaluate cell viability. Each experiment was performed in triplicate and repeated three times.

The Muse Annexin V/Dead Cell Assay Kit (Millipore, Darmstadt, Germany) for quantitative analysis of live, early/late apoptotic and dead cells was used with a Muse Cell Analyzer (Millipore). Briefly, the assay utilizes Annexin V to detect PS on the external membrane of apoptotic cells. A dead cell marker (7-AAD) is also used. HepG2 cell line, including positive and negative controls, were cultured in 1% FBS medium in presence of doxorubicin 0.1 or 1µM alone (control cells) or in combination with 40ng/ml IGF1 with/without 1µM GSK1838705A. The cells were then processed as described in the user's guide.

## Migration and invasion assays

A scratch assay was performed as previously described [15,16]. Briefly, a wound was generated with a pipette tip the on cells grown until it reaches confluence in multiwell plates, after rinsing, medium containing 40ng/ml IGF1 or 1% FBS (control) alone or in combination with 0.1 or 1µM of doxorubicin with/without 1µM GSK1838705A. Photographs were taken of each well immediately (T0) and after 24h (T1), 48h (T2) and 72h (T3). The values were expressed as percentage of migration, with 100% being when the wound was completely closed. The results were representative of three independent experiments.

Cell invasion assays were performed using Matrigel (BD Transduction, San Jose, CA, USA)-coated Transwells (8 µm pore PET membrane, Millipore, Billerica, MA, USA) as previously described [16]. Briefly, 0.1 or 1 µM of doxorubicin-treated cells were suspended in low serum medium. Medium containing 40ng/ml IGF1 or FBS with/without 1µM GSK1838705A was added to the bottom wells. After incubation of 24h, the invading cells were fixed and stained. The images were acquired and analyzed counting the cells with Image J Software (National Institute of Health, USA). Values obtained were expressed as percentage of invading cells, setting the cell counts of control cells as 100%. Results were representative of three independent experiments.

## Western blots

PI3K/Akt signaling and apoptosis markers in cells treated with doxorubicin 1µM alone or in combination with 40ng/ml IGF1, with/without 1µM GSK1838705A, were analyzed by Western Blots as previously described [16]. Briefly, cells were washed twice with cold PBS and then lysed in RIPA buffer (Sigma-Aldrich, Milan; Italy). After quantization of protein concentration, equal amount of protein (50 µg) were resolved on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) filters. The blots were blocked with 5% (w/v) nonfat dry milk for 2h at room temperature and then probed with primary antibody overnight at 4°C. The primary antibodies were directed against the following proteins: IGF1-R, PI3K and phospho-PI3K (P-PI3K tyr458/tyr199), Akt and phospho-Akt (P-Akt thr308), GSK-3β and phospho-GSK-3β (P-GSK-3β ser9), phospho-4EBP (P-4EBP), p70S6K and phospho-p70S6K (P-p70S6K tyr389), ERK and phospho-ERK (P-ERK thr202/tyr204), p38 and phospho-p38 (P-p38 thr180/tyr182), STAT3 and phospho-STAT3 (P-STAT3 ser727), Bax, Bad, Bid, Bcl-2, Bcl-xL, survivin and β-actin (Cell Signaling, Beverly, MA, USA). After three washes, incubation was followed by the reaction with horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. The immunoreactive bands were visualized and analyzed using enhanced chemiluminescence detection reagents, according to the manufacturer's instructions, and chemiluminescence detection system (ChemiDoc XRS apparatus and software, Bio-Rad).

The densitometric analysis was expressed as target protein/actin ratio for IGF1-R, P-4EBP, Bax, Bad, Bid, Bcl-2, Bcl-xL and survivin, while for the remaining proteins as the phospho-protein/total protein ratio.

## Statistical analysis

GraphPad Prism 5.0 software (La Jolla, CA, USA) was used for all statistical analysis. Mann-Whitney nonparametric test was employed to assess the statistical significance of differences between two groups.  $P < 0.05$  was considered statistically significant. All experiments were done in triplicate and data are presented as mean ± standard deviation (SD).

## Results

### IGF1 role in doxorubicin-mediated inhibition of HCC cell growth

We previously reported that doxorubicin could mediate growth inhibition, migration and invasion in HCC cells [4]. We now examined whether hPL, a key component of the microenvironment and itself stimulant of HCC growth [17], might modify doxorubicin actions.

HCC cells that had been pre-treated for 48h with hPLs corresponding to  $3.75 \times 10^7$  platelets/ml, or an equivalent concentration of FBS (controls), were cultured for the next 24h in presence of doxorubicin (0.1 and 1µM). We found that doxorubicin-mediated growth inhibition was antagonized by an average of 30% (0.1µM doxorubicin) or 55% (1µM doxorubicin) when the cells received hPLs pre-treatment (Figures 1A-1B). In the same experimental conditions, the protective role of 40ng/ml IGF1, a potent HCC mitogen that is found in platelets was next tested. The doxorubicin-mediated growth inhibition was decreased by an average of 36% (0.1µM doxorubicin) or 48% (1µM doxorubicin) when the cells received IGF1 pre-treatment (Figures 1C-1D). The effects of both hPLs and IGF1 (Figures 1A-1D) were strongly antagonized by GSK1838705A, an inhibitor of IGF1-R. GSK1838705A was used at a non-toxic concentration (1µM) that did

not affect the proliferation by itself. The effects of GSK1838705A on hPLs thus provide evidence for the protective effects of hPLs to be mediated by IGF1.

### IGF1 role on doxorubicin-mediated induction of apoptosis

We next investigated the protective role of IGF1 on doxorubicin-mediated apoptosis, using the same experimental conditions as for the growth experiments. Doxorubicin caused an increase (about 22% and 51% respectively, at either 0.1 or 1  $\mu$ M) in cellular Annexin V compared with untreated cells (11%). When HCC cells were pre-treated with IGF1, a significant decrease in doxorubicin-mediated apoptosis induction was found (19% and 23% respectively, for doxorubicin 0.1 or 1  $\mu$ M). Moreover the IGF1 blocking action was abrogated by concomitant addition to the cultures of IGF1 receptor inhibitor 1  $\mu$ M GSK1838705A (Figure 2A). These findings were confirmed by Western Blot analysis in which the expression levels of apoptosis inducers (Bax, Bad, Bid) and suppressors (Bcl-2, Bcl-xL, survivin) were evaluated. As shown in Figures 2B-2C, the levels of anti-apoptotic markers were decreased using doxorubicin, compared to the untreated control cells. This effect was antagonized by IGF1 pre-treatment, which caused an increase in all the apoptosis suppressors that were analyzed. The balance between pro and anti-apoptotic factors, expressed as Bax/Bcl-2 protein ratio, indicated that the induction of apoptosis observed in doxorubicin-treated cells was counteracted by IGF1 pre-treatment. The specificity of this IGF1 action was shown by using 1  $\mu$ M GSK1838705A which significantly blocked the IGF1 actions.

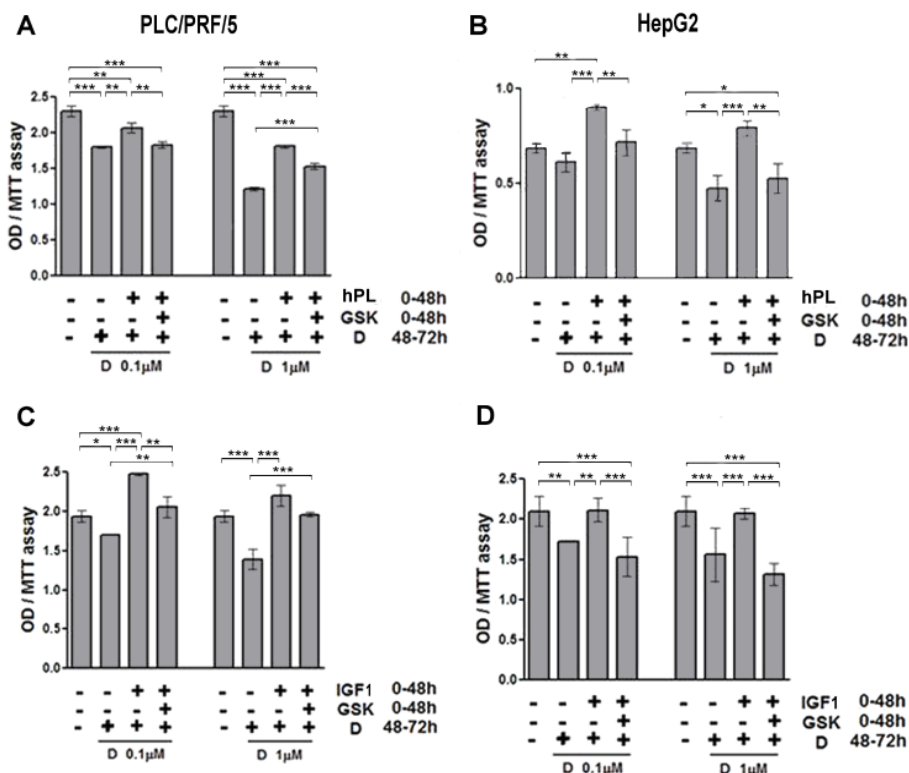
### IGF1 antagonism of doxorubicin-mediated inhibition of cell migration and invasion

IGF1 has been shown to stimulate cell motility [18,19]. The protective role of IGF1 on cell migration and invasion was then examined, under the same experimental conditions used in the growth experiments. We found that doxorubicin inhibited cell motility in a reversible manner at low concentrations (0.1  $\mu$ M), at which IGF1 exerted a strong protective effect if added before doxorubicin, but only partially reversed the doxorubicin-mediated inhibition if added after drug treatment (supplementary information 1A-1B). Conversely high doxorubicin concentration (1  $\mu$ M) irreversibly inhibited cell migration and invasion and IGF1 showed only a protective effect (Figure 3A). Comparable results were also obtained with cell invasion (Figure 3B) assays. We also found that IGF1 effects on cell motility were abrogated by the IGF1 receptor antagonist GSK1838705A.

### Changes in PI3K/Akt and MAPK signaling

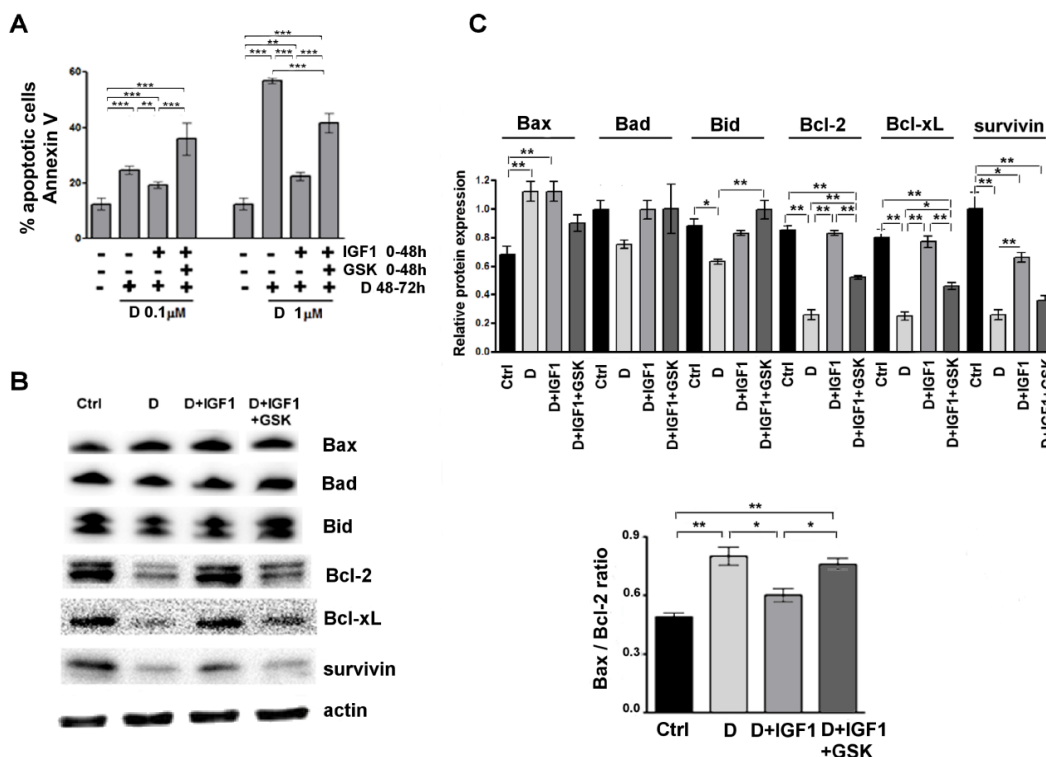
We examined the levels of total and phosphorylated proteins involved in PI3K/Akt and MAPK signaling by Western Blot analysis, since these are known targets of doxorubicin action, using cells without or with IGF1 pre-treatment.

We found that doxorubicin decreased cellular IGF1-R levels and this decrease was blocked by IGF1 pre-treatment of the cells (Figure 4A). The results were similar for the phosphorylation levels of PI3K and Akt proteins and for those of 4EB-P1, p70S6K and GSK-3 $\beta$ , some of the principal downstream targets of Akt (Figure 4B). In parallel,



**A-B.** PLC/PRF/5 and HepG2 cells pre-treated for 48h with hPLs corresponding to  $3.75 \times 10^7$  platelets/ml or FBS (controls) +/- 1  $\mu$ M GSK1838705A, were subsequently cultured for the next 24h in presence of two different concentrations of doxorubicin (D 0.1  $\mu$ M and 1  $\mu$ M). **C-D.** In the same experimental condition 40ng/ml IGF1, instead of hPLs, was tested. MTT assay was assessed after 72h. The results of three independent experiments are expressed as means  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.005$ ; \*\*\* $P < 0.0005$ .

**Figure 1:** Protective role exerted by IGF1 in doxorubicin-mediated inhibition of HCC cell growth.



**A.** PLC/PRF/5 cells pre-treated for 48h with 40ng/ml IGF1 with/without 1μM GSK1838705A, were next cultured for 24h in presence of two different concentrations of doxorubicin (D 0.1μM and 1μM). The Muse Annexin V kit was used to evaluate the percentage of apoptotic cells in three independent experiments. **B.** Representative Western Blot of pro and anti-apoptotic markers Bax, Bad, Bid, Bcl-2, Bcl-xL and survivin in presence of doxorubicin (D 1μM). **C.** The densitometric analysis of each apoptotic marker, expressed as target protein/actin ratio (arbitrary units) (up) and Bax/Bcl-2 protein ratio (down) in three independent experiments. All results are expressed as mean ± SD. \**P* < 0.05; \*\**P* < 0.005; \*\*\**P* < 0.0005.

**Figure 2:** Protective role exerted by IGF1 in doxorubicin-mediated induction of HCC apoptosis.

we evaluated the phosphorylation levels of ERK and its downstream kinases p-38 and STAT3 after doxorubicin treatment in cells pre-treated with IGF1. As shown in Figure 4C, doxorubicin decreased the phosphorylation levels of these proteins and this effect was blocked by IGF1 pre-treatment. All these IGF1 effects were antagonized by 1μM of the IGF1-R inhibitor, GSK1838705A.

## Discussion

Although doxorubicin is a potent cancer chemotherapy drug, its clinical use can be limited by its toxicity and the development of multidrug resistance [3]. The growth of HCC cells and the effects of chemotherapy agents can be modulated by the microenvironmental milieu, including platelets, which can contribute to the stimulation of HCC [4,6,17,20,21]. Platelets synthesize several HCC mitogens, some of which have been shown to be involved in chemotherapy drug resistance [22-24]. Among the secreted mitogens, IGF1 plays a role in the hepatocarcinogenic process, and can negatively regulate apoptosis [11,25]. Our previous results showed that human platelet lysates (hPLs) could modulate the growth-inhibitory effect of doxorubicin [4]. This effect was due at least in part to the anti-apoptotic action revealed by the induction of anti-apoptotic Bcl-xL and survivin levels and the decrease of pro-apoptotic tBid and Bim levels.

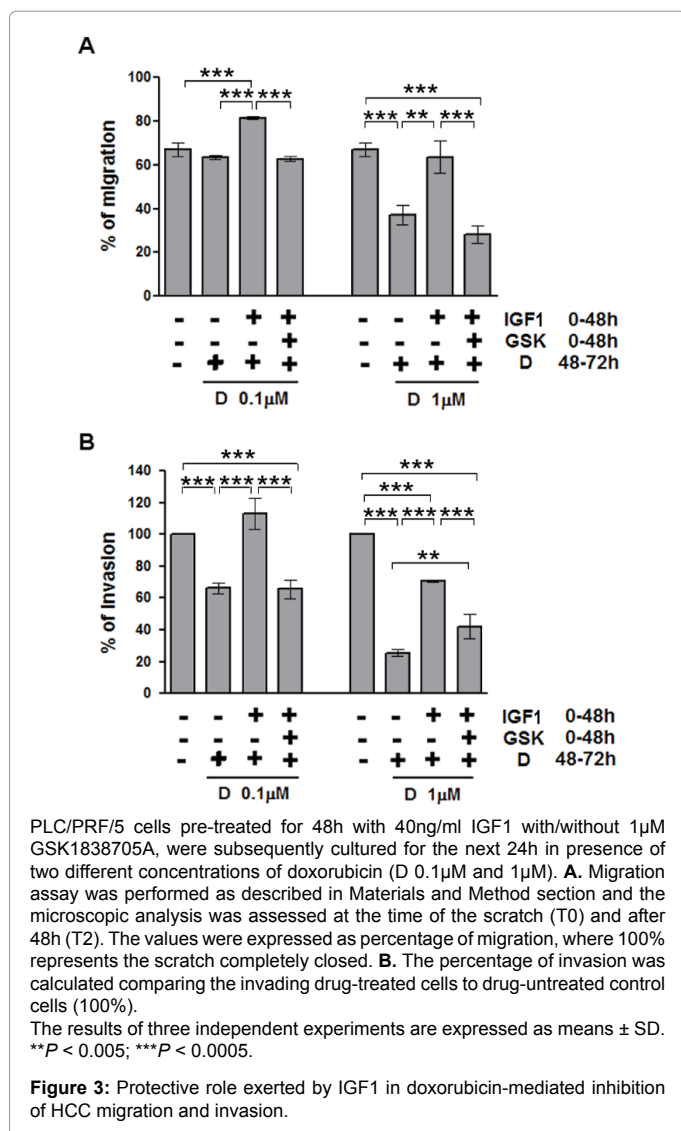
Moreover the protective effect of hPL on HCC cell growth was also significant at high doxorubicin concentrations (1μM) [4]. In the present study, we identified IGF1 as a major mediator of the stimulatory effect exerted by hPLs. The involvement of this growth factor was

demonstrated by using GSK1838705A, a specific IGF1-R inhibitor, that antagonized the effects of both hPL and of IGF1. Moreover we found a decrease of IGF1-R in doxorubicin-treated cells, but an increase in cells that had IGF1 pre-treatment. Since it has been shown that doxorubicin decreases tumor cell growth, mainly by induction of apoptosis, we focused our investigation on apoptosis and the role of IGF1 [13].

We previously demonstrated that although doxorubicin used at concentration of ≤ 0.1μM weakly affected cell growth, it was effective in long duration of cell treatment [26]. Moreover, we showed that the effect on apoptosis process of these low doxorubicin concentrations was blocked by subsequent hPL treatment [4]. Since doxorubicin concentrations ≥ 1μM can be toxic, in the current experiments, we used two different doxorubicin concentrations (0.1 and 1μM) and showed that the protective effects of hPLs and of IGF1, were significant even at the higher doxorubicin concentration.

At the lower doxorubicin concentration (0.1 μM) the growth-inhibitory effect was minimal in both HCC cell lines analyzed and the proliferative rate was completely restored at the control levels by hPLs or IGF1 administration. On the contrary, at the higher doxorubicin concentration (1 μM), the strong inhibitory effect was only partially restored by hPLs or IGF1 administration, although in these condition the percentage of antagonism was higher.

Our data showed that IGF1 pre-treatment exerted a significant protective effect in decreasing the percentage of cells in apoptosis in doxorubicin-treated cells, through the activation of survival



pathways. Our results indicate an increase of levels of Bcl-2, Bcl-xL and survivin though the activation of Akt phosphorylated in thr308. PI3K/Akt signaling modulates several downstream pathways. Among these we found a stimulation of mTORC1 by the increased level of phosphorylation of 4EB-P1 and p70S6K. These are involved in protein synthesis and cell growth, as well as a stimulation of glucose metabolism by blocking of GSK-3β that directly regulates Glycogen Synthase activity. Overall these IGF1 effects, antagonizing the doxorubicin action, lead to an increase HCC cell growth.

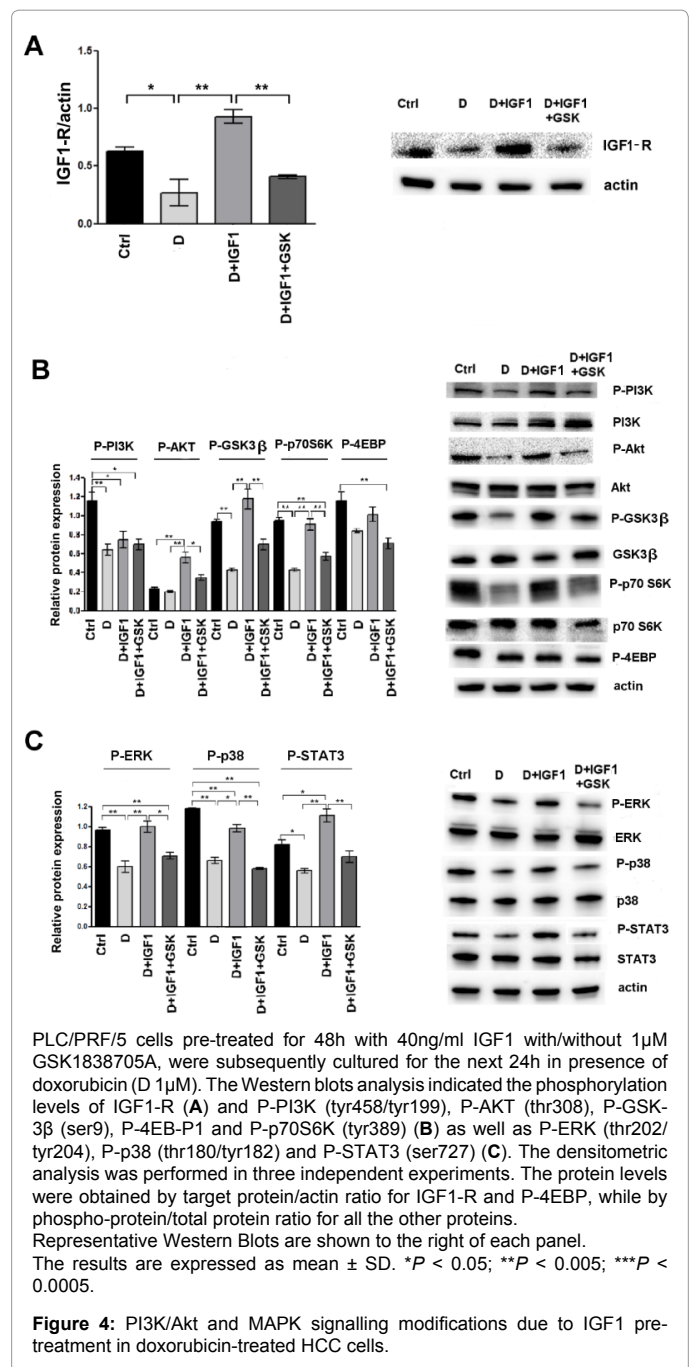
Recent data correlate platelet counts and the ensuing higher levels of IGF1, with portal vein tumor thrombosis (PVTT) that is a major cause of intra-hepatic metastasis and a major poor prognosis factor for HCC [27,28]. Moreover, HCC patients with thrombocytosis are considered at risk for extra-hepatic metastasis [27]. The activation of PI3K/Akt pathway, mediated by IGF1-R, regulates the assembly and re-organization of the actin cytoskeleton and motility by activating Rac/Cdc42 [29-32]. We report here findings concerning potential motility in doxorubicin-treated HCC cells, in which the stimulatory role of IGF1 pre-treatment was evident.

Strong antagonism was exerted by IGF1 on the inhibitory effects

of doxorubicin on P-ERK and P-p38 levels, as well as on the p38 downstream target, P-STAT3 [4]. These are important molecules in mediating cell proliferation and play a role in the induction of anti-apoptosis mediators.

We also found that the IGF1 signaling pathway plays a crucial role in several aspects of cellular biology. The up-regulated IGF1 signaling, causing enhanced cell proliferation and motility and reduced apoptosis, could be considered a major event responsible for drug resistance.

Therefore our results support the idea that IGF1-R might be an excellent molecular target for HCC management [33-35]. For this purpose several preclinical studies based on the use of anti-IGF1-R antibody [36-38] IGF1-R tyrosine kinase inhibitor [34,39] and RNA



interference [40] in combination of systemic chemotherapy appear to be promising HCC therapeutic strategies.

There are at least ten IGF1-R inhibitors in clinical trials, but only one, AVE1642, has been assessed for liver metastases [41]. Given our present data about GSK1838705A and the results of others showing the importance of IGF1 in mediating HCC growth and invasion, we think that clinical trials of this class of agent in HCC are warranted.

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