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ZD6474 inhibits proliferation and invasion of human hepatocellular carcinoma cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is characterized by hypervascularization, neoangiogenesis formation and blood vessel invasion. Recently, it has been demonstrated that an inhibitor of the vascular endothelial growth factor (VEGF) receptor, ZD6474, may directly inhibit the growth of tumor cells. ZD6474 effectiveness was investigated on cell growth, apoptosis, adhesion, migration and invasion and related to the drug-dependent modulation of main molecular targets on HCC cells. ZD6474 inhibited HCC cell proliferation, however, such effect was reverted by Laminin-5 (Ln-5) but not by other extracellular matrix proteins (ECM). ZD6474 also inhibited HCC cell adhesion, migration and invasion, whereas the simultaneous treatment with the drug and Ln-5 strongly recovered those effects. Under the same experimental conditions, ZD6474 inhibited the expression of phosphorylated EGFR in all cell lines while the effect on p-Erk1/2 was dependent on cellular invasive characteristics. Nonetheless, co-incubation with Ln-5 completely recovered this effect. Our results support the hypothesis that ZD6474 could represent an interesting therapeutic opportunity for patients with HCC scarcely expressing the ECM protein, Ln-5.

1. Introduction

HCC represents one of the most common malignancies in the world and it has been estimated to further increase in the upcoming years [1]. In North American and European countries, it commonly develops in cirrhotic liver patients and is strongly associated to the hepatitis C virus (HCV) [2].

Prognosis and survival are still poor, mainly because of cancer reoccurrence and/or of cancer spread even in those patients who underwent to liver transplantation. In addition, the use of chemotherapeutic agents is strongly limited because of the reduced liver functionality as a consequence of the underlying cirrhosis and no other therapies are so far available to block or reduce the growth and/or the metastatic invasion of HCC [3]. Therefore, the development of novel therapeutic strategies for the treatment of HCC is strongly demanded by clinicians.

Tumor angiogenesis is currently a topic of intense research, since the formation of new blood vessels allows both local tumor growth and development of distant metastases [4]. Angiogenesis is the result of a multistep cascade modulated by positive soluble factors such as the vascular endothelial

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growth factor (VEGF) and the tumor growth factor (TGF)-α and negative soluble factors such as angiostatin and endostatin [5]. VEGF seems to play a pivotal role in the regulation of tumor angiogenesis and it is known to bind three different receptors designated as VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (Flt-4) [6]. The inhibition of angiogenesis, through blocking receptors, represents a promising therapeutic approach to reduce tumor growth and metastases formation. This new strategy seems particularly promising for the treatment of HCC because it is a hypervascular cancer with high levels of VEGF receptor expression in the tissue [7,8].

Recently, a better understanding in angiogenesis has allowed the development of various biological targeting therapies by using anti-VEGF monoclonal antibodies (Mabs) and low molecular weight inhibitors of VEGFR-2 tyrosine kinase activity [9]. ZD6474, an anilinoquinazoline derivative, has shown to be a selective inhibitor of the VEGFR-2 tyrosine kinase activity [10]. Additionally, ZD6474 inhibits EGFR tyrosine kinase activity, thereby inhibiting the production of pro-angiogenic growth factors such as VEGF and TGF-α [11] and currently it is in Phase II evaluation for the treatment of other tumors [12]. Experimental studies have shown that inhibition of VEFGR-1 or VEGFR-2 activity in vivo using antibodies (i.e. inhibiting angiogenesis) can inhibit HCC tumor growth [13].

HCC cells grow in island-like structures embedded in a tissue enriched of extracellular matrix proteins such as Laminin-1 (Ln-1), Collagen IV (Coll IV), Coll I, Fibronectin (Fn), Fibrinogen (Fg) and Vitronectin (Vn) as a consequence of the underlying cirrhosis. In addition, Ln-5, a member of the Ln family, has been reported to be involved in the metastatic process of different tumors including breast, colon and oral (reviewed in [14]). Furthermore, we have previously reported that Ln-5 is expressed in metastatic but not in the non-metastatic HCC tissue [15].

In this study, we investigated the direct effects of ZD6474 on HCC cells proliferation and invasion in vitro, taking in consideration the potential role of ECM proteins such as Ln-5.

2. Materials and methods

2.1. Reagents and antibodies

ZD6474 was kindly provided by AstraZeneca Pharmaceuticals. Stock solutions were prepared at 20 mM in dimethyl sulfoxide (DMSO) and stored in aliquots at −20 °C. Purified Ln-5 was prepared as previously described [16]. Working dilutions were made in culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50,000 U l^{-1} penicillin and 80 μM streptomycin.

Laminin-1 (Ln-1), and Collagen IV (Coll IV), was purchased from Sigma Chemical Company (St. Louis, MI), Coll I from BD Biosciences (Bedford, MA), Fibronectin (Fn) from Calbiochem (La Jolla, CA), Fibrinogen (Fg) and Vitronectin (Vn) was a gift of Dr. Felding-Habermann (TSRI, La Jolla, CA) [17].

Anti-phosphotyrosine polyclonal antibody PY99 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-EGFR monoclonal antibody from Becton-Dickinson (San Diego, CA), and the following monoclonal antibodies from Cell Signaling (Bverly, MA): anti-Akt, anti-phosphoAkt, anti-Erk1/2, and anti-Erk1/2 and anti-phospho-Erk1/2. Mouse and rabbit horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden) were used as secondary antibodies.

2.2. Cell cycle analysis

HCC cells were grown to subconfluence, serum-starved for 24 h, and incubated with ZD6474 (IC_{50}) or control medium for 1 day. After, cells were harvested, washed twice with ice-cold PBS (pH 7.4), fixed in 4.5 ml of 70% ethanol at −20 °C and washed with ice-cold PBS. Propidium iodine (50 μg/ml) was added, and cells were analyzed using a FACScan flow cytometer (Beckman Coulter, Fullerton, CA) and the data interpreted using the CellQuest software.

2.3. Inhibition of cell proliferation

ZD6474 determination of IC_{50} was performed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay. The human HCC cell lines Alexander, HepG2, SK-Hep1 and HLF were maintained in culture as previously described [18]. Briefly, 7500 cells in a volume of 200 μl were plated in each well on a 96-well plate in the presence of medium containing FBS 10%. After 24 h, cells were challenged for 3 days with ZD6474 at the following concentrations: 0.01, 0.1, 0.5, 1.0, 5.0, 10.0 and 50.0 μM. Each experiment was reproduced in six different wells, and repeated at least three times. Results were expressed as a dose–response curve, plotting the fraction of unaffected (surviving) cells versus the drug concentration. IC_{50} was defined as the drug concentration yielding 50% of affected (non-surviving) cells compared with untreated controls. In some experiments, Ln-1, Ln-5, Coll IV, Coll I, Fg, Fn and Vn (1 μg/ml) were added simultaneously to cells in the presence of ZD6474 IC_{50} and the cell growth inhibition was measured by the MTT assay.

2.4. Adhesion, migration and invasion assays

Adhesion assay was performed as previously described [19]. In some experiments, cells were previously treated with ZD6474 IC_{50} in the presence or absence of Ln-5 (1 μg/ml), for 1 or 3 days.

Briefly, 96-well plates (Sarstedt, Newton, NC) were coated with different ECM proteins (1 μg/ml). Cells were resuspended in a serum-free medium and seeded (80×10^4) in each well. Plates were then incubated for 30 min at 37 °C in a humidified incubator containing 5% CO_2. After removing non-adherent cells by washing with PBS, cells were fixed with 100% methanol, stained with crystal violet, solubilized with 1% SDS and quantified with an ELISA plate reader, at 540 nm.

Transwell haptotactic migration assay was performed as already reported [19]. Briefly, transwell filters (8.0 μm pore size, Corning, The Netherlands) were coated on the bottom compartment with Ln-5 (1 μg/ml) and 6×10^5cells were plated in the upper part of the chamber, under serum-free conditions. Filters were incubated for 16 h at 37 °C in a humidified incubator containing 5% CO_2, fixed and stained with 0.5% crystal violet in methanol. The non-migratory cells were removed with a cotton tip and the migratory cells were counted at a light microscope, with 400× magnification. Each experiment was run in duplicate, and four microscopic fields

from each of the two filters were counted in every case. Results were expressed as the mean number of cells counted in each field, ±standard deviation.

Chemo-invasion through a reconstituted Basement Membrane was performed as reported by Albini et al. [20]. Briefly, 8 μm pore size (Nucleopore, Cabin John, MD) polycarbonate filters were coated with reconstituted BM GFR Matrigel (8 μg/cm^2) according to the manufacturer's instructions. Cells (3×10^6), resuspended in serum-free medium, were plated in the upper part of the chamber. In the lower compartment, Ln-5 (1 μg/ml) was used as chemoattractant. Filters were then fixed and stained, and non-invasive cells were removed with a cotton tip, as described above. The total number of invasive cells was quantified by image-analysis software as previously reported [18]. For each condition, three filters were run in each experiment. Results show the mean number of counted cells ± standard deviation.

2.5. Western blot analysis

All experiments were carried out in the presence of medium containing FBS 10%. Protein samples were extracted after 6 h, 1 day and 3 days from ZD6474 (IC_{50}) and/or Ln-5 (1 μg/ml) treated cells (3×10^6 cells) after homogenization in RIPA buffer (0.5 M NaCl, 1% Triton X-100, 0.5% NP40, 1% deoxycolic acid, 3.5 mM sodium dodecyl sulfate (SDS), 8.3 mM Tris/HCl pH 7.4, 1.6 mM Tris base) and treated with a 20% protease inhibitor cocktail (Sigma). Protein concentration was determined by the Bradford method, samples were normalized for protein concentration (25–50 μg), and electrophoresis performed on 10–12.5% SDS-PAGE. The signal was detected by the chemoluminescence assay (ECL-Plus, Amersham Life Science, UK) and the expression level was calculated by densitometric analysis using QuantyOne software (Biorad, Hercules, CA), using β-actin expression as internal standard.

2.6. Immunoprecipitation analysis

All experiments were carried out in the presence of medium containing FBS 10%. HCC cells (3×10^6) were treated with ZD6474 (IC_{50}) and/or Ln-5 (1 μg/ml) for 24 h. Cells were lysed in RIPA-IP buffer (140 mM NaCl, 20 mM Tris/HCl, 10 mM EDTA pH8, 10% glycerol, 1% NP40, 1 mM Na-deoxycolic acid, 1 mM phenylmethylsulfonyl fluoride [PMSF]), passed through a 22-gauge syringe and cleared by centrifugation at 10,000 × *g* at 4 °C for 10 min. Proteins were immunoprecipitated by incubating 0.1–0.3 mg of total cell lysate with 0.2 μg of anti-phosphotyrosine antibody or anti-EGFR antibody for 1 h at 4 °C. Two to five microliters of protein A/G agarose (Santa Cruz Biotechnology) were incubated overnight at 4 °C. The cell suspension was centrifuged at 2600 rpm and the pellet was washed three times with phosphate buffered saline (PBS) and then resuspended in 10 μl of Laemli buffer. Each sample was separated on 10% acrylamide gel and Western blot was performed as described above.

2.7. Apoptosis analysis

Apoptosis detection was performed by Annexin V-FITC staining assays (Biovision, Palo Alto, CA, USA) and by propidium iodide (PI). In accordance with the manufacturer's instructions, HCC cells (5×10^5) were incubated with Annexin V at room temperature for 10 min. Apoptotic cells were detected by FACS analysis (Becton-Dickinson, Franklin Lakes, NJ) and quantified using cellquest software (Becton-Dickinson).

2.8. Statistical analysis

Results are expressed as the mean ± standard deviation (S.D.) and the statistical significance of the Ln-5 dependent reduction of ZD6474 activity was determined by Student's *t*-test with a 95% confidence interval.

3. Results

All the experiments with ZD6474 were evaluated after 1 and 3 days of continuous exposure, in the presence of FCS to ensure cell vitality, consistently with other studies [21–23].

3.1. ZD6474 anti-tumoral activity in HCC cell

We tested the effectiveness of ZD6474 in four different HCC cell lines, previously characterized as invasive (HLF and Sk-Hep1) and non-invasive (Alexander and HepG2). The drug was used at different concentrations and incubation times, to investigate different biological activities including proliferation, adhesion, migration and invasion, as well as the molecular pathways involved.

All the cell lines were investigated under the same experimental conditions to limit biological variability. As reported in Table 1, HCC cell lines showed similar ZD6474 IC_{50} values. As expected, no evident cell growth inhibition was observed after 1 day of drug exposure, while after 3 days the IC_{50} was in the μM range. Experiments were repeated at least three times, and the mean and standard deviation refer to all the experiments. Nevertheless, in all cell lines, 1 day ZD6474 exposure induced a slight accumulation of cells in G0/G1 phase (about 12–18% as respect to the untreated cells).

Apoptotic cells were quantified using Annexin V and cytoplasmic histone-associated-DNA fragmentation assays. After 3 days of ZD6474 exposure, there was no early or late apoptosis in either the invasive or the non-invasive cells (data not shown).

Table 1 – ZD6474 IC50 in HCC cell lines after 1 and 3 days incubation

Cell lines	IC50 (μM)	
	1 day	3 days
Alexander	>100	0.58 ± 0.28
HepG2	>100	3.16 ± 0.42
HLF	>100	1.62 ± 0.22
Sk-Hep1	>100	3.35 ± 0.6

Each experiment was carried out in triplicate.

Table 2 – Percentage of survived HCC cells treated with ZD6474 (IC50) in the presence of ECM proteins

	Alexander	HLF	Sk-Hep-1	HepG2
ZD6474	51.3 ± 4.8	45.2 ± 7.3	49.2 ± 0.1	48.6 ± 3.5
ZD6474+Ln-5	99.8 ± 1.2	80.7 ± 2.5	84.2 ± 0.07	88.2 ± 3.1
ZD6474+Ln-1	62.5 ± 5.4	52.2 ± 3.1	51.5 ± 5.7	55.4 ± 4.8
ZD6474+Fn	54.4 ± 7.2	57 ± 5.2	59.1 ± 5.5	56.8 ± 6.1
ZD6474+Fg	60.1 ± 6.7	48 ± 4.3	54.9 ± 6.7	54.3 ± 5.1
ZD6474+Vn	52.2 ± 5.3	51 ± 6.4	54.1 ± 4.7	52.4 ± 4.9
ZD6474+Coll I	51.6 ± 3.5	50.5 ± 4.1	49.6 ± 2.9	50.6 ± 3.8
ZD6474+Coll IV	55.3 ± 6.4	47.5 ± 3.5	51.8 ± 3.8	51.5 ± 4.5

Each experiment was carried out in triplicate.

3.2. *The effect of ECM proteins on inhibition of HCC cell proliferation by ZD6474*

To test the hypothesis that ECM proteins may affect the anti-tumor activity of ZD6474, HCC cells were incubated with ZD6474 IC_{50} in the presence of different ECM molecules (1 μg/ml) such as Ln-1, Ln-5, Coll I, Coll IV, Fg, Fn and Vn, commonly expressed in cirrhotic liver. As shown in Table 2, ZD6474-dependent inhibition of HCC cell proliferation (as determined by IC_{50}) was reduced by the presence of Ln-5, with a consequent significant increase of cell survival compared with ZD6474 alone ($p < 0.001$), whereas no effect was observed with the other ECM proteins used As control, Ln-5 alone did not affect HCC cells growth.

3.3. *ZD6474 affects adhesion, migration and invasion of HCC cells*

As previously reported, HCC cells adhere differently on ECM substrates [18]. After 1 day of pretreatment, ZD6474 strongly reduced adhesion of both HLF and Sk-Hep1 on Ln-5, by 27.1 ± 3 and 36.9 ± 1.2%, respectively, while pretreatment with ZD6474 and Ln-5 reversed the inhibitory effect. No adhesion was observed with Alexander and HepG2 on Ln-5, as previously reported [18]. Furthermore, no effect of ZD6474 was observed on other ECM proteins such as Ln-1, Coll I, Coll IV, Fn, Fg and Vn and similar results were obtained after 3 days of drug exposure (data not shown).

Based on the above-described data, migration and invasion were investigated on HCC invasive cells only (HLF and SK-Hep1). Consistent with the adhesion data, 1 day pretreatment with ZD6474 significantly reduced HLF and Sk-Hep1 migration on Ln-5 ($p < 0.01$) (Fig. 1). However, pre-treatment with both ZD6474 and Ln-5 reverted the drug inhibitory effect on migratory activity in a statistically significant manner ($p < 0.01$). As control, Ln-5 treatment did not show any effect on HCC cell migration. Similar results were obtained after 3 days of ZD6474 with or without Ln-5 (data not shown).

Finally, 1 day of ZD6474 pretreatment strongly inhibited HLF and Sk-Hep1 invasion through a reconstituted BM using Ln-5 as chemoattractant, whereas simultaneous pretreatment with the drug together with Ln-5 abolished the therapeutic inhibitory effect (Fig. 2). As control, Ln-5 treatment alone did not show any effect. Similar results were obtained incubating HCC cells for 3 days, data not shown.

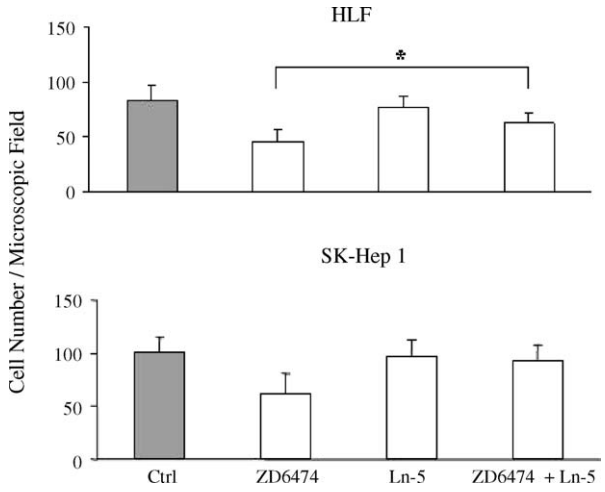

Fig. 1 – ZD6474 modulation of HCC migration onto Ln-5. One day's ZD6474 pretreatment inhibited migration of HLF and Sk-Hep1 onto Ln-5. Simultaneous pretreatment with both the drug and Ln-5 significantly ($p < 0.01$) restored the migratory activity.

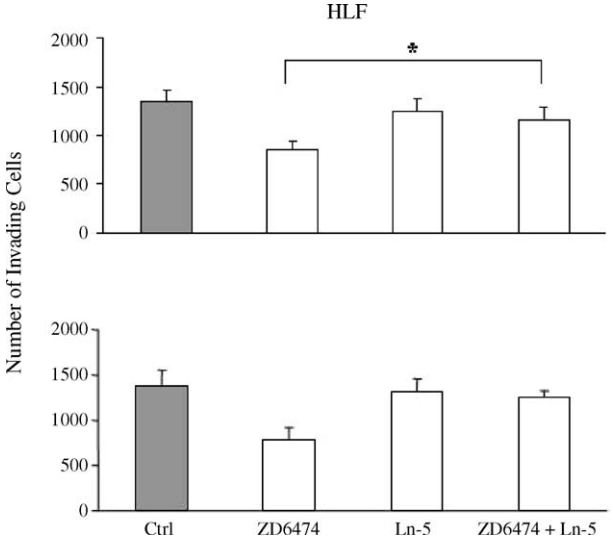

Fig. 2 – ZD6474 modulation of HCC invasion through a reconstituted basement membrane. One day's ZD6474 pretreatment inhibited invasion of HLF and Sk-Hep1 through a reconstituted basement membrane (Matrigel). Simultaneous pretreatment with both the drug and Ln-5 significantly ($p < 0.01$) restored the invasive activity.

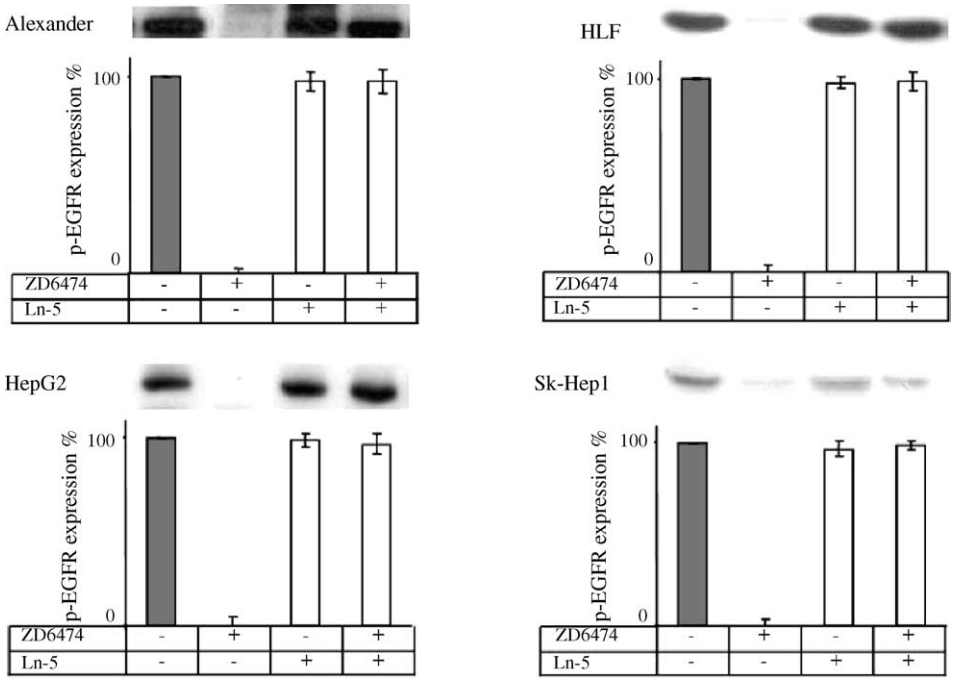

Fig. 3 – ZD6474 modulation of p-EGFR. Six hours' ZD6474 treatment completely inhibited p-EGFR expression on HCC cells. Simultaneous treatment with the drug and Ln-5 restored the phosphorylation form of the receptor.

3.4. *ZD6474 modulation of molecular targets*

To investigate the molecular mechanisms responsible for the biological effect of ZD6474 on HCC cells, we analyzed the modulation of drug epithelial cell targets such as EGFR, as well as the main steps of its signal transduction pathways, including Erk1/2 and Akt.

EGFR, Akt and Erk1/2 expression were not modulated by ZD6474 and Ln-5, as compared to controls using β-actin expression as internal standard (data not shown). On the contrary, drug treatment (at IC_{50} concentrations) reduced the levels of p-EGFR after 6 h to undetectable levels. Addition of Ln-5 to ZD6474 prevented the drug-related reduction in pEGFR levels. As control, Ln-5 alone did not affect p-EGFR levels (Fig. 3). After 1

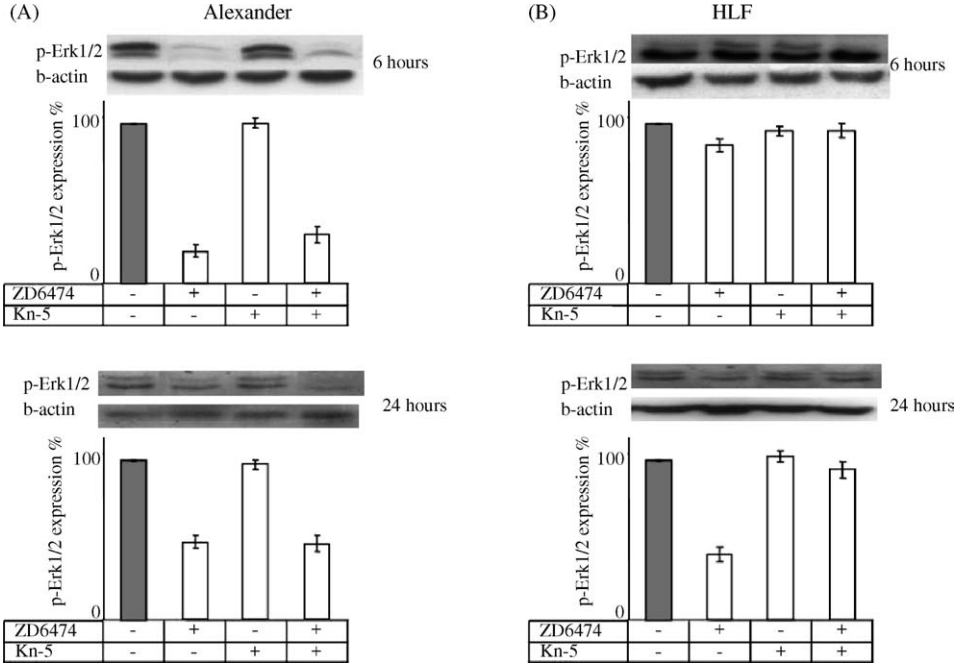

Fig. 4 – (A and B) ZD6474 modulation of p-Erk1/2. ZD6474 treatment inhibited p-Erk1/2 expression on HCC cells. Simultaneous treatment with the drug and Ln-5 restored the phosphorylation form of the effector.

day of ZD6474 treatment, p-EGFR were similar to controls, indicating that the effects of ZD6474 on p-EGFR were transient, data not shown. All the HCC cell lines had a similar behavior, as shown in Fig. 3.

ZD6474 treatment resulted in small reductions (ranging from 10 up to 20%) in p-Akt staining during the first day of drug exposure in all the HCC cell lines, and co-treatment with Ln-5 prevented these reductions (data not shown). In contrast, drug treatment inhibited p-Erk 1/2 with some differences among the invasive and non-invasive HCC cells. In both non-invasive Alexander and HepG2 (not shown) cells, after 6 h ZD6474 exposure, p-Erk1/2 was strongly inhibited, while after 24 h this effect was reduced. Ln-5 treatment did not affect recovery of p-Erk1/2 either after 6 and 24 h, and no effect of Ln-5 alone was observed (Fig. 4A). To explain the Ln-5 effect on cell growth recover after ZD6474 exposure, we analyzed p-Erk1/2 modulation after 3 days of drug exposure. Consistently with cell growth recover data, Ln-5 overcomes drug effectiveness (from 10 up to 95%) in function of time. In both invasive HLF and SK-Hep1 (not shown) cells, ZD6474 treatment produced a greater reduction of p-Erk1/2 levels at 24 h compared with 6 h, and co-treatment with Ln-5 prevented this effect (Fig. 4B).

4. Discussion

The underlying cirrhosis that discourages the use of chemotherapeutic drugs together with both the peculiar histological liver architecture lacking a BM tissue boundaries and the biological characteristics of the tumor make HCC a highly aggressive and lethal cancer. Therefore, significant effort is being applied to understanding the molecular mechanisms regulating HCC growth and invasion in order to identify potential new anti-cancer therapies. The increased vascularization and expression of VEGF, suggest the use of specific VEGF receptor inhibitors as potential therapy for HCC [24–27].

In this preclinical study, we report that ZD6474 blocks proliferation, adhesion, migration and invasion via EGFR pathway-inhibition of HCC cells. Our results suggest that ZD6474 preferentially inhibits the proliferation pathway through p-Erk1/2, and that this modulation could also be the basis for its effect on reducing cell adhesion, migration and invasion. In fact, the Erk/MAPK pathway is involved in cell adhesion and cell migration through direct cytosolic activation of protease calpain-2 and MLCK. In the specific context of cell motility, Erk1/2 can regulate the activities of Rac1 and RhoA, via transcriptional upregulation of the urokinase receptor (uPAR) and of the Fra-1 transcription factor, respectively. We can conclude that the evidence is strong enough to suggest that inhibition of EGFR activity through Erk/MAPK may provide a way to block cell motility [28,29]. These hypotheses are being investigated in the course of ongoing studies in our laboratories.

Moreover, ZD6474 was effective even in the presence of Ln-1, Coll I, Coll IV, Fg, Fn and Vn ECM proteins. This seems important because the surrounding microenvironment is involved in regulating neoangiogenesis, proliferation, invasion, etc., and because in HCC, tissue remodeling occurs as a consequence of the cirrhosis. Nevertheless, Ln-5 restores cell activities faster in invasive than in non-invasive cells and this result is consistent with findings in our previous study [30]. The molecular mechanisms of this protective role exerted by Ln-5 are still unknown, but a physical interaction between ZD6474 and Ln-5 can be excluded since p-Erk1/2 is still inhibited in the presence of Ln-5. On the other hand, it has been shown that Ln-5 directly phosphorylates EGFR because of its EGF-like domain [31], and this could justify the recovery of p-EGFR, previously inhibited by ZD6474 treatment. Therefore, Ln-5 could represent a negative predictive factor of ZD6474 effectiveness, particularly bearing in mind that it has been reported to be expressed in metastatic but not in non-metastatic HCC [15]. This could also contribute to elucidate the molecular mechanisms responsible for non-responsiveness to ZD6474 treatment in some patients.

The anti-proliferative effect of ZD6474 has been reported in different tumors including breast, lung, thyroid, colon and prostate cancer [32–35]; and recently, it has been reported the efficacy of ZD6474 as adjuvant to radiotherapy in an ''in vivo'' experimental model of lung cancer [36], The novelty of our work is the description of its activity against human HCC cells in a preclinical study. This is consistent with the findings in a murine experimental model, in which monoclonal antibodies against VEGFR inhibited HCC angiogenesis and tumor growth [13]. In addition, we report for the first time that ZD6474 also inhibits adhesion, migration and invasion, likely via the p-Erk1/2 pathway. This is consistent with other studies reporting that VEGF, whose release is inhibited by ZD6474 [37], disassembles intra-cellular tight junctions in HCC cells, thus allowing cancer diffusion [38].

In this study, for the first time we report the inhibition of HCC cell proliferation by ZD6474 in vitro In addition, we report a novel anti-invasive activity of ZD6474 in vitro. Since VEGF signaling inhibitors/anti-angiogenics (e.g. avastin and sorafenib (BAY43-9006)) and EGF-signaling inhibitors (e.g. gefitinib, erlotinib) are being investigated in clinical trials in HCC, and hold some promise, a molecule like ZD6474 currently in phase II of clinical trial, which combines both direct inhibition of tumor, cell growth (via EGFR inhibition) and inhibition of tumor angiogenesis (via VEGFR2 inhibition) would be particularly interesting to examine in HCC clinical trials. Finally, a potential association between increased Ln-5 expression and reduced clinical benefit in HCC patients treated with EGFR signaling inhibitors might be predicted by this (and previous gefitinib [30]) study, and therefore this warrants further investigation in the clinical setting.

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