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The tyrphostin AG1478 inhibits proliferation and induces death of liver tumor cells through EGF receptor-dependent and independent mechanisms

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related death. Different signaling pathways are de-regulated in this pathogenesis, among them the epidermal growth factor receptor one (EGFR/Erb1). Here we show that blockage of this pathway by the tyrphostin 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) in different liver tumor cell lines promotes both inhibition of cell proliferation and induction of cell death, which are coincident with arrest in the G1 phase of the cell cycle, caspase-3 activation and DNA fragmentation. AG1478 up-regulates the expression of the pro-apoptotic member of the BCL-2 family BIM and down-regulates the expression of the anti-apoptotic BCL-XL and MCL1. Furthermore, it also decreases the levels of the caspase inhibitors HIAP2 and XIAP. The treatment of HCC cells with AG1478 enhanced the apoptosis induced by other pro-apoptotic stimuli, such as the physiological cytokine, TGF-β, highly expressed in liver tumors, or the chemotherapeutic drug doxorubicin. The effects observed by AG1478 were broader than the ones seen by silencing of the EGFR with siRNA, which indicates that this drug might act on other targets different from the EGFR. In this same line of evidence, AG1478 retained some cytotoxic effects in cells where EGFR has been targeted knock-down with shRNA. Interestingly, AG1478 preferentially acts on liver tumor cells, being untransformed cells much less responsive to its cytotoxic effects. In conclusion, AG1478 could be a potential therapeutic drug to be used in HCC.

1. Introduction

Human hepatocellular carcinoma (HCC) is one of the most common cancers and its prevalence is arising in the West. Surgical resection and liver transplantation are currently the best options to treat liver cancer. However, recurrence or metastasis is common in patients who have had a resection, and survival rate is 30–40% at 5 years postoperatively [1]. A relevant number of molecular mechanisms altered in HCC initiation and progression are compromising the balance between survival and apoptosis in the pre-neoplastic hepatocytes. Indeed, it has been shown that in HCC there is a disruption of the fragile balance between apoptotic and survival pathways; first, the expression of different proteins involved in the apoptotic machinery are down-regulated or their activity is impaired; second, various pro-survival signals are over-activated [2,3]. One of these de-regulated survival pathways is the epidermal growth factor receptor (EGFR) pathway [4,5]. On the one hand, different EGF-like ligands (*TGFA*, *AR*, and *HB-EGF*) are over-expressed in liver cirrhosis and HCC, contributing to EGFR activation during HCC progression [6–8]. On the other hand, it has been shown that human HCC tissues over-express EGFR and ERBB3 [9]. Furthermore, the expression and activity of TACE/ADAM17 (TNF-α converting enzyme), a metalloprotease responsible for EGF-like ligands shedding, is increased in cirrhotic and HCC samples, as compared to controls [10].

It is well known the relevance of EGFR signaling in liver regeneration [11]. Indeed, EGF is mitogenic in hepatocytes in

Abbreviations: TGF-β, transforming growth factor-beta; EGFR, epidermal growth factor receptor; HCC, hepatocellular carcinoma; TGF-α, transforming growth factor-alpha; HB-EGF, heparin binding-epidermal growth factor-like growth factor; AR, amphiregulin; HIAP, human inhibitors of apoptosis protein; TACE, TNF-alpha converting enzyme; HFH, human fetal hepatocytes; HBV, hepatitis B virus; HCV, hepatitis C virus.

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primary culture and induces survival signals that allow them to resists to apoptotic stimuli, such as those induced by the transforming growth factor-beta (TGF-β) [12,13]. Tyrphostins are protein tyrosine kinase inhibitors. The tyrphostin 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) is well known for its activity as an EGFR inhibitor. Previous studies from our group revealed that AG1478 has no cytotoxic effects per se in rat fetal and adult hepatocytes, while it prevents proliferation and induces apoptosis in the FaO rat hepatoma cell line [14,15]. Taking these results in consideration, the aim of this study was to analyze the effects of AG1478 in human liver tumor cells growth and death and compare them with its effects in human untransformed hepatocytes.

2. Material and methods

2.1. Materials

Human recombinant TGF-β1, AG1478 and doxorubicin were from Calbiochem (La Jolla, CA, USA). Fetal bovine serum (FBS) was from Sera Laboratories International (Cinder Hill, UK). Human heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor alpha (TGF-α) were from Sigma (Saint Louis, USA). The caspase-3 substrate Ac-DEVD-AMC was from Pharmingen (San Diego, CA, USA). The antibodies used were as follows: rabbit anti-phospho-EGFR (Tyr1068) (CS 3777), rabbit anti-EGFR (CS-2232), rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr 204) (CS-9101), rabbit anti-p44/42 MAP Kinase (137F5) (CS-9122), rabbit anti-phospho-AKT (Ser473) (CS-9271), and rabbit anti-AKT (CS-9272) were from Cell Signalling Technology (Beverly, MA, USA); rabbit anti-BIM and mouse anti-XIAP were from BD Pharmingen (San Diego, CA, USA); rabbit anti-BCL-XS/L (S-18) and rabbit anti-MCL1 (S-19) were from Santa Cruz Biotechnology (Santa Cruz, CA; USA); monoclonal anti-β-actin was from Sigma (Madrid, Spain); rabbit anti-HIAP1 and anti-HIAP2 were from R&D Systems (Minneapolis, USA). RT-MLPA, the SALSA MLPA KIT R011 Apoptosis mRNA, was from MRC-Holland (Amsterdam, The Netherlands).

2.2. Cell culture

Hep3B, HepG2, PLC/PRF/5 and SK-Hep1 cell lines were obtained from the European Collection of Cell Cultures (ECACC, UK). HepG2 and Hep3B were maintained in MEM medium, SK-Hep1 in MEM medium supplemented with 1 mM sodium pyruvate, and PLC/PRF/5 in DMEM medium. All media were supplemented with 10% FBS and cells were maintained in a humidified atmosphere of 37 °C, 5% CO_2. For experiments, cells at 70% confluence were serum-starved during 12 h before treatments. Human fetal hepatocytes were isolated and plated on collagen-coated plates using 10% FBS William's E medium, supplemented with different additives, as described [16]. Medium was changed every second day. After 10 days in culture, cells were starved overnight before adding AG1478. The HH4 cell line, an untransformed human hepatocyte cell line, was created by the introduction of the HPV E6 and E7 genes into hepatocytes isolated from a normal adult liver, followed by derivation of a clonal, immortalized cell line [17]. HH4 human hepatocyte cell line was cultivated in supplemented William's E medium [16] in collagen-coated plates. During the experiments, William's E medium was used without any protective additives in both HH4 and HFH cells.

2.3. Crystal violet staining

After treatments, cells were washed twice with phosphate-buffered saline and the remaining viable adherent cells were stained with crystal violet (0.2% in 2% ethanol) and analyzed spectrophotometrically, as described previously [18].

2.4. DNA synthesis assay

After incubation of the cells for 48 h, DNA synthesis was evaluated by [methyl-^{3}H]-thymidine (GE Healthcare, Barcelona, Spain) incorporation into TCA-precipitable material during the last 40 h, as described previously [19].

2.5. Analysis of caspase-3 activity

Fluorimetric analysis of caspase-3 activity was determined as described previously [20], with 20 μg protein extract. Fluorescence was measured in Microplate Fluorescence Reader Fluostar optima. A unit of caspase-3 activity is the amount of active enzyme necessary to produce an increase in 1 fluorescence unit in the luminescence spectrophotometer. Results are presented as units of caspase-3 activity/h/μg protein.

2.6. Analysis of DNA content by flow cytometry

Ploidy determination of cells was estimated by flow cytometry DNA analysis [21]. Cell cycle analysis was carried out using the software ModFit LTTM (Verity Software House, USA).

2.7. Analysis of gene expression

RNeasy Mini Kit (Qiagen, Valencia, CA) was used for total RNA isolation. Reverse transcription (RT) was carried out with Random primer using 1 μg of total RNA from each sample for complementary DNA synthesis. Quantitative RT-PCR was done with predesigned Taqman® primers for human and Taqman® Universal Master Mix was used for PCR reactions, which were performed in duplicate in a ABIPrism 7700 System; specific primers used were GAPDH, hs99999905_m1 and EGFR, hs01076092_m1. Results are expressed using the $2^{-\Delta\Delta C_T}$ algorithm to analyze the relative changes in gene expression. It requires the assignment of one housekeeping gene (when comparing among different cell lines 18S was used, when comparing samples of the same cell line GAPDH was used), which is assumed to be uniformly and constantly expressed in all samples, as well as one reference samples (when comparing among different cell lines HH4 cells were used as a reference sample, when comparing samples of the same cell line, the untreated sample was used as the reference sample). The expression of other samples is then compared to that in the reference sample [22].

Finally, when RNA was analyzed by reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) the SALSA MLPA KIT R011 Apoptosis mRNA from MRC-Holland (Amsterdam, The Netherlands), for the simultaneous detection of 38 messenger RNA molecules, was used and relative expression of each gene normalized by β-2-microglobulin expression [23].

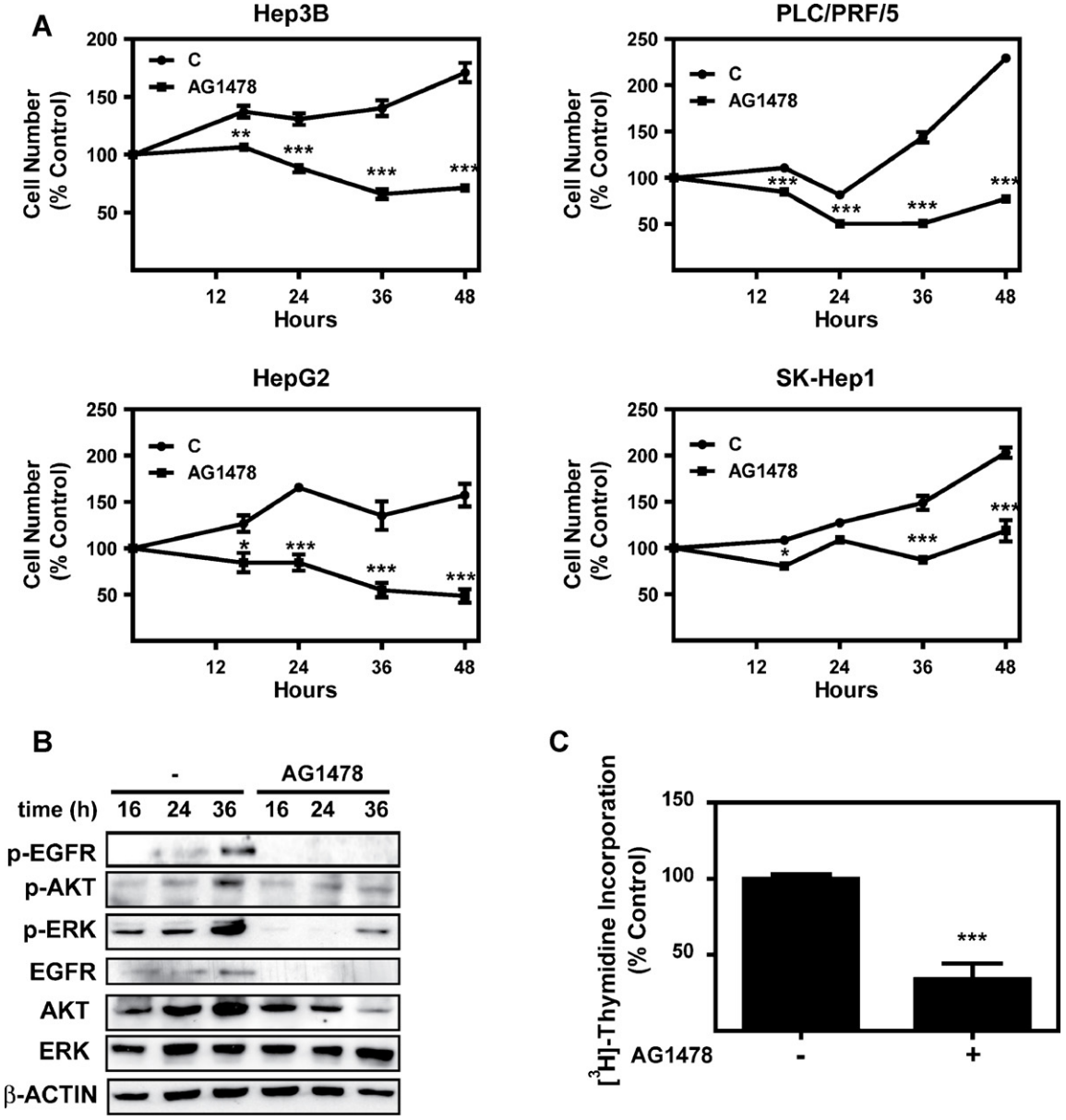
2.8. Western blot

Total protein extracts were obtained using a lysis buffer containing 30 mM Tris–HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycolate, 0.1% SDS and 10% glycerol. Pellets were incubated during 1 h in lysis buffer at 4 °C, and centrifuged at 13,000 rpm during 10 min at 4 °C. Western blot procedure was carried out as described previously [14]. β-Actin is shown as a loading control.

2.9. Targeting knock-down assays

For transient siRNA transfection, cells at 70% confluence were transfected using TransIT-siQuest (Mirus, Madison, USA) at 1:300 dilution in complete medium, according to the manufacturer's

recommendation, with a final siRNA concentration of 25 nM. Protocols used were previously described [24]. For all the cell lines excepting Hep3B, 24 h after transfection cells were trypsinized and plated for the different experiments; 24 h later, cells were serum deprived. In the case of Hep3B cells, they were transfected during 24 h and then serum deprived. Oligos were obtained from Sigma-Genosys (Suffolk, UK): Unsilencing: 5′-GUAAGACACGACUUAUCGC-3′, EGFR: 5′-GCGAUAAGUCGUGUCUUAC-3′. Unsilencing siRNA was selected from previous works [24]. Specific oligos with maximal knock-down efficiency were selected among three different sequences for each gene evaluated by the Dharmacon design algorithm (http://www.dharmacon.com/DesignCenter). For stable transfection of shRNA, against EGFR or control shRNA, cells at 50–60% confluence were transfected with MAtra-A reagent (IBA GmbH, Germany) at a dilution of 1:600 in complete media, according to the manufacturer's recommendation (15 min on the magnet plate),



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	G1	S	G2/M
Control	62.18 ± 1.51	24.76 ± 1.74	13.07 ± 0.94
AG1478	75.27 ± 3.00 **	13.00 ± 2.30 ***	11.80 ± 1.14

Fig. 1. AG1478 inhibits proliferation of HCC cell lines. (A) Viable cell number in serum-deprived cells cultured in the absence or presence of AG1478 (20 μM) after 16, 24, 36 or 48 h treatment was analyzed by crystal violet; results are expressed as percentage of the initial number of cells: Hep3B, PLC/PRF/5, HepG2 and SK-Hep1 cell lines. (B–D) Effects of AG1478 (20 μM) on serum-deprived Hep3B cells: (B) Western blot analysis after 16, 24 or 36 h treatment; a representative experiment of 3; (C) [^{3}H]-thymidine incorporation (48 h) expressed as percentage of control (untreated) cells; (D) analysis of cell cycle after treatment of serum-deprived cells with or without AG1478 during 24 h. Results are expressed as mean ± S.E.M. ($n = 3$, in triplicate for (A), (C) and (D)). Data are compared treated cells versus untreated cells. One-way ANOVA was used in (A), Student's t-test was used in (C) and (D): $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

using 2 μg/μl of shRNA plasmid, four different plasmids of EGFR shRNA were transfected separately or together (pool), as well as a control shRNA. After 24 h, media was changed to complete media, and selection of transfected cells was done with puromycin (for 50 days prior to experiments). shRNA plasmids were selected from Mission SH, Sigma (Madrid, Spain).

2.10. Statistical analyses

Statistical analysis was performed using GraphPad Prizm software (Graph-Pad for Science Inc., San Diego, CA, USA). Data are presented as mean ± S.E.M., with $p \leq 0.05$ considered to be statistically significant.

3. Results

3.1. AG1478 attenuates proliferation and promotes apoptosis of HCC cells

We have previously described that different HCC cell lines (Hep3B, PLC/PRF/5, HepG2 and SK-Hep1) show autocrine proliferation in the absence of serum [25]. As shown in the Suppl. Fig. 1,

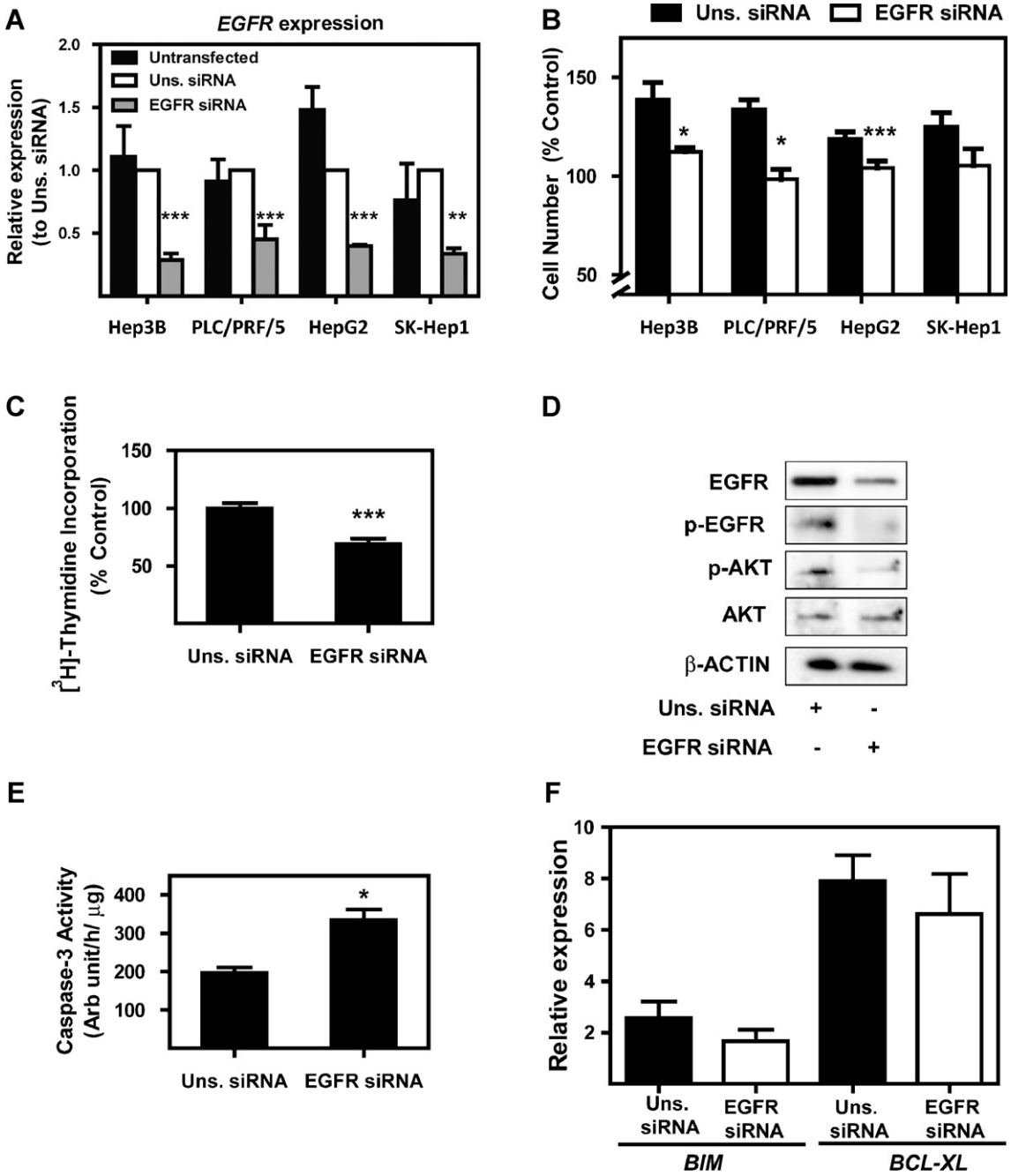

Fig. 2. EGFR knock-down decreases cell proliferation and induces cell death. Different HCC cell lines were transfected with either an unsilencing (Uns) siRNA or specific siRNAs for EGFR. 24 h after transfection, FBS was removed from culture medium. (A) *EGFR* transcript levels, analyzed by real-time PCR (24 h after serum deprival). Untransfected cells are included to show that transfection does not significantly affect EGFR expression. Results are expressed relative to the Uns. siRNA basal levels. (B) Effect in loss in cell viability (24 h after serum deprival), analyzed by crystal violet staining, results are expressed as percentage of the initial number of cells. (C–F) Effects of EGFR silencing on Hep3B cells: (C) [^{3}H]-thymidine incorporation (48 h after serum deprival) expressed as percentage of control; (D) Western blot analysis (24 h after serum deprival); (E) caspase-3 activity (24 h after serum deprival); (F) transcript levels of *BIM* and *BCL-XL* by RT-MLPA (16 h after serum deprival). Mean ± S.E.M. ($n = 4$). Data from cells transfected with EGFR siRNA are compared versus cells transfected with Uns. siRNA. One-way ANOVA was used in (A) and (B), Student's *t*-test was used in (C), (E) and (F): $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

addition of the EGFR inhibitor AG1478 to the culture provoked a decrease in the number of cells. HepG2 and PLC cells showed a maximal response at 10 μM, whereas Hep3B cells required higher doses. We fixed the concentration to 20 μM for the next experiments (Fig. 1A), which completely blocked EGFR phosphorylation in all the cell lines (results not shown), even in the most resistant one (Suppl. Fig. 1B). The ability of these human liver tumor cells to proliferate in the absence of serum correlates with high expression of at least one EGFR ligands (of the three we have analyzed: *AR*, *HBEGF* and *TGFA*) (Suppl. Fig. 2). Furthermore, HCC cells expressed increased levels (Suppl. Fig. 2) and activity (results not shown) of the metalloprotease responsible for their shedding, *TACE*/*ADAM17*, when compared to untransformed cells (primary cultures of HFH and HH4 cell line). In Hep3B cells, AG1478 impaired the phosphorylation of different EGFR downstream targets, AKT and ERKs, observed after serum deprivation (Fig. 1B), coincident with a decrease in DNA synthesis analyzed by [^{3}H]-thymidine incorporation and an increase in the percentage of cells in the cell cycle phase G1 (Fig. 1C and D). According to results previously published [26] and results presented here (Fig. 2A–D), EGFR knock-down by specific siRNA oligos attenuated not only the response to EGFR ligands (Suppl. Fig. 3), but also the autocrine proliferation observed in these cell lines in the absence of serum, consistent with the results obtained with AG1478. However, it is worthy to mention that AG1478 effects were higher than the ones observed by transient EGFR knock-down.

We also wanted to analyze whether EGFR inhibition could induce apoptosis. As shown in Fig. 3, AG1478 promoted an increase in the percentage of hypodiploid cells both in the presence and absence of serum, coincident with caspase-3 activation. In order to understand how the EGFR inhibition by AG1478 induced apoptosis we analyzed the expression of BCL-2 and IAP families, whose members are involved in regulating cell death [27,28]. On the one hand, AG1478 induced the expression of the pro-apoptotic member of the BCL-2 family BIM, both at the mRNA and protein level after 16 h of treatment (Fig. 4A and B). On the other hand, AG1478 decreased the expression at the mRNA level of the anti-apoptotic member of the BCL-2 family *BCL-XL* and impaired the increase of MCL1 protein observed after serum deprivation (Fig. 4A and B). Moreover, at the protein level we observed that AG1478 decreased the expression of two members of the caspase inhibitors IAP family, HIAP2 and XIAP (Fig. 4B), which revealed a possible post-transcriptional regulation of these genes. The apoptotic effects induced by AG1478 are much more pronounced than the ones induced by EGFR silencing. This might be due to the ability of AG1478 to regulate BCL-2 family expression, since EGFR knock-down does not modulate either *BIM* or *BCL-XL* mRNA levels (Fig. 2E and F). These results would indicate that AG1478 was inducing the intrinsic apoptotic

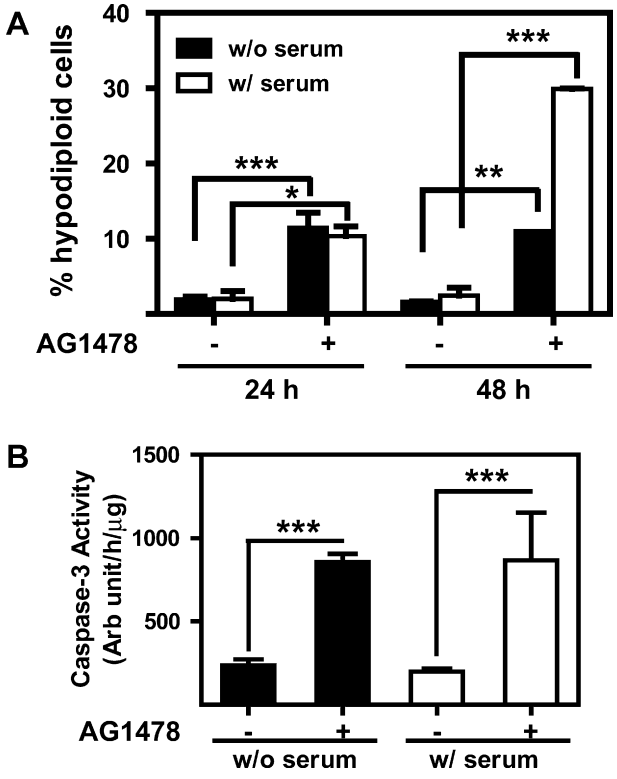

Fig. 3. AG1478 induces apoptosis in Hep3B cells. Cells were incubated with or without AG1478 (20 μM) in the presence or absence of serum. (A) Percentage of hypodiploid cells at the indicated times. (B) Caspase-3 activation (24 h). Results are expressed as mean ± S.E.M. ($n = 3$). Data are compared treated cells versus untreated cells. One-way ANOVA was used in (A), Student's *t*-test was used in (B): $^{**}p < 0.01$, $^{***}p < 0.001$.

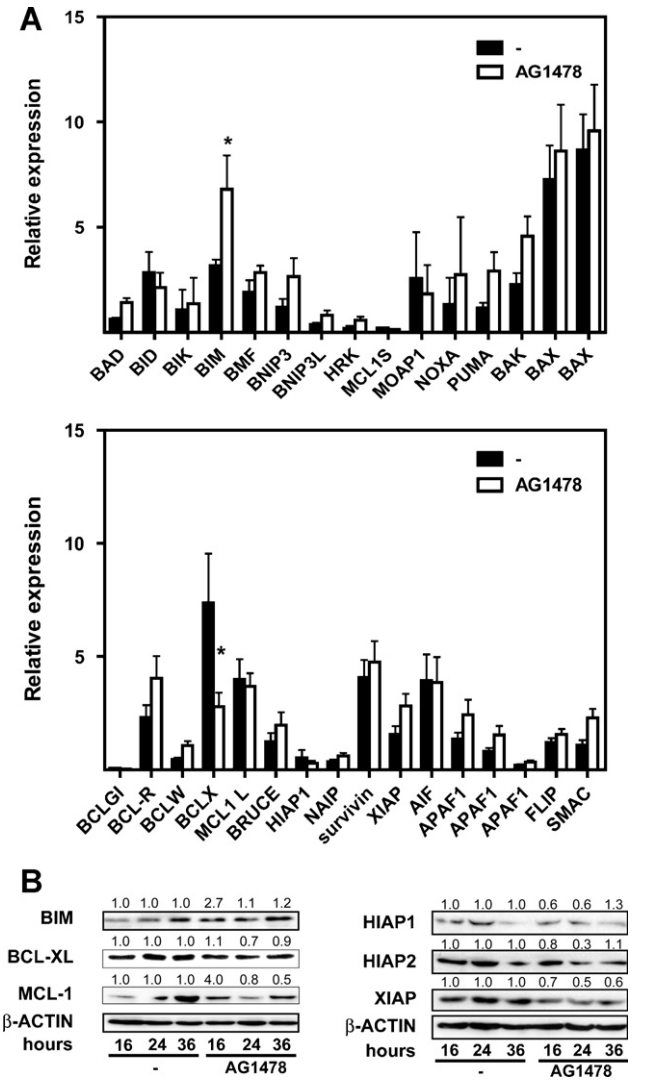

Fig. 4. AG1478 regulates the expression of apoptotic genes in Hep3B cells. Cells were incubated with or without AG1478 (20 μM) in serum-deprived cells. (A) Transcript levels of apoptotic genes by RT-MLPA (16 h). Mean ± S.E.M. ($n = 4$). Student's *t*-test was used comparing untreated cells versus AG1478-treated cells: $^{*}p < 0.05$. (B) Western blot analysis after 16, 24 or 36 h of treatment. Numbers above panels indicate the densitometric analysis of bands quantified as the ratio between the protein of interest and β-actin, and expressed as fold increase versus the respective control (untreated cells) at each of the analyzed times. A representative experiment of 2 is shown.

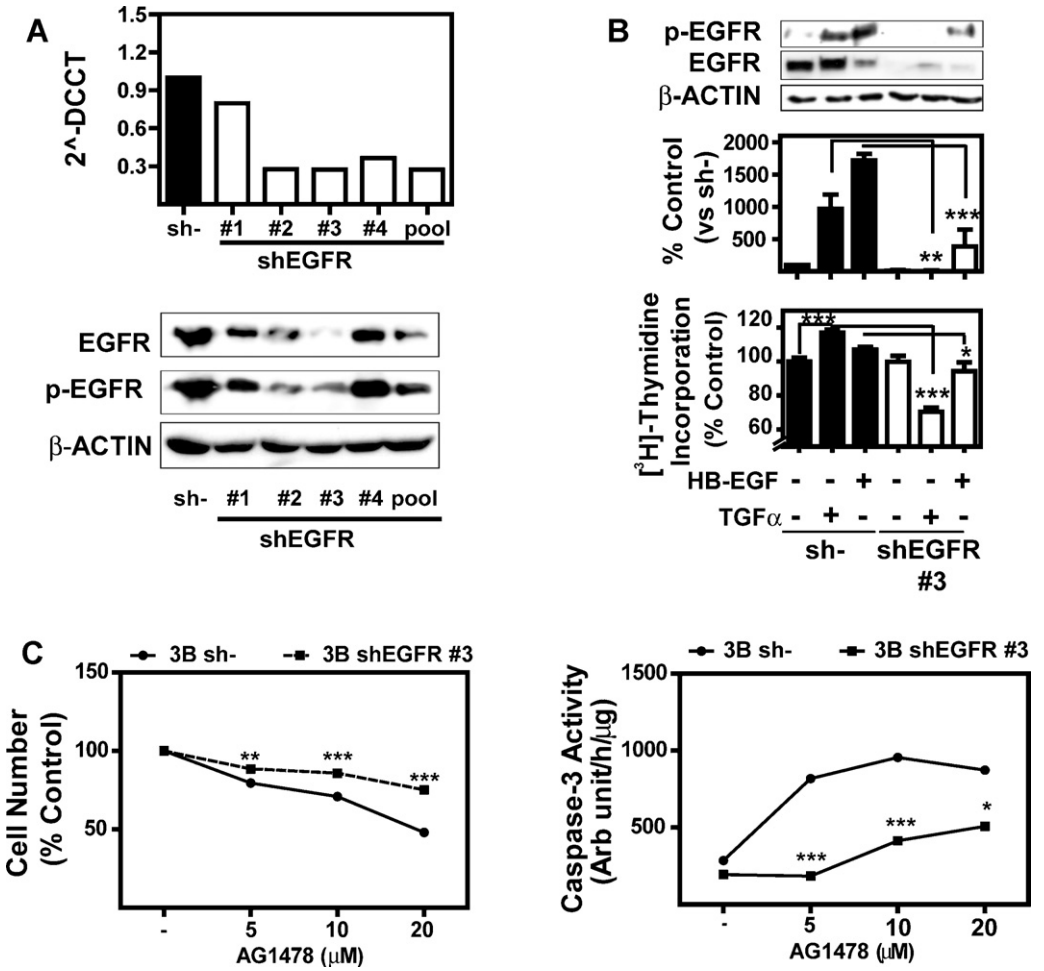

Fig. 5. AG1478 still has cytotoxic effects on Hep3B cells in which EGFR expression has been stably silenced with shRNA. (A) Analysis of EGFR expression. Top: assessment of EGFR mRNA levels by real-time PCR. Bottom: analysis of protein levels by Western blot. Comparison of Hep3B sh- versus Hep3B-shEGFR clone #3 in serum deprived cells: (B) cells were treated with/without TGF-α or HB-EGF (20 ng/ml). Top: phospho-EGFR was analyzed by Western blot, cells were treated for 15 min. Middle: densitometric analysis of *p*-EGFR levels versus β-actin. Bottom: [^{3}H]-thymidine incorporation (48 h) expressed as percentage of their respective control (untreated cells). (C) Cells were incubated with or without AG1478 at the indicated concentrations. Left: viable cell number (48 h), results are expressed as percentage of control. Right: caspase-3 activation (24 h). Results are mean ± S.E.M. Data from treated cells are compared versus untreated cells using One-way ANOVA in (B). In (C) a two-way ANOVA was used to compare the effect of AG1478 in 3B sh- versus 3B-shEGFR #3: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

pathway as it up-regulated the expression of pro-apoptotic genes and down-regulated the expression of anti-apoptotic members of the BCL2 family, increasing its effects by decreasing the expression of some IAP proteins responsible for the inhibition of caspase activity.

3.2. Role of EGFR as a target of AG1478 effects

EGFR transient silencing only renders a mild inhibition of proliferation and does not induce apoptosis when compared to the effects induced by AG1478. These results led us to think that EGFR might not be the only target of AG1478. In order to know whether AG1478 could have cytotoxic effects on cells which do not express EGFR, or only express very low levels of EGFR, we generated Hep3B cells stably expressing EGFR shRNA, using four different EGFR-shRNA plasmids. Three of the Hep3B-shEGFR clones showed lower EGFR expression, both at the mRNA and protein level, a lower basal phosphorylation of EGFR (Fig. 5A). For the following experiments the clone #3 was chosen. Ablation of EGFR in clone #3 almost completely impaired the ability of these cells to respond to EGF-like ligands, both in terms of EGFR phosphorylation and cell proliferation (Fig. 5B). Interestingly, in a dose response assay to AG1478, both the inhibition of proliferation and the apoptosis induced by AG1478 were significantly diminished in Hep3B-shEGFR when compared with control Hep3B cells, but a significant effect continued being observed (Fig. 5C). This suggests that AG1478 might be affecting other targets different from the EGFR.

3.3. Effect of AG1478 on human fetal hepatocytes (HFH) and the immortalized human adult hepatocyte cell line (HH4)

In the adult hepatocyte cell line, HH4, the addition of AG1478 reduced the number of viable cells either in the absence or in the presence of FBS (Fig. 6A), but effects were lower than those observed in HCC cells. Reduction in cell viability was accompanied by inhibition of DNA synthesis in FBS-stimulated cells (Fig. 6B), but no induction of apoptosis, analyzed by caspase-3, was observed (Fig. 6C). In proliferating human fetal hepatocytes in primary culture, AG1478 inhibited autocrine DNA synthesis, but the activation of caspase-3 was not significant (Suppl. Fig. 4). Therefore, untransformed and HCC cells showed a differential capacity to respond to AG1478: transformed HCC cell lines undergo inhibition of cell proliferation and apoptosis after AG1478 treatment, in contrast human adult hepatocytes and human fetal hepatocytes are more resistant to AG1478 cytotoxic effects.

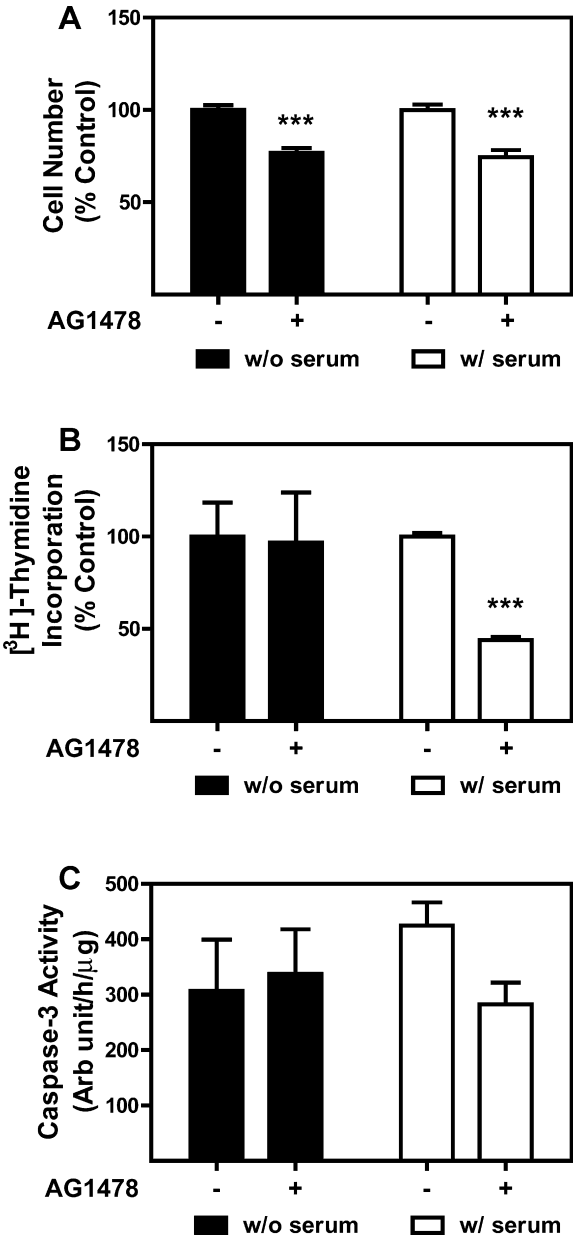

Fig. 6. Effects of AG1478 in non-transformed hepatocytes. Adult hepatocyte cell line, HH4, was incubated with or without AG1478 (20 μM) in the presence or absence of serum. (A) Viable cell number analyzed by crystal violet (48 h), results are expressed as percentage of control (untreated cells). (B) [^{3}H]-Thymidine incorporation (48 h) expressed as percentage of control (untreated cells). (C) Caspase-3 activation (24 h). Results are the mean ± S.E.M. ($n = 3$). Student's t-test, treated cells versus untreated cells: $^{***}p < 0.001$.

3.4. AG1478 potentiates cell death induced by physiological apoptotic stimuli and chemotherapeutic drugs

From previous results from our group we knew that TGF-β not only promotes apoptosis in liver tumor cells, but it is also able to induce survival signals through the activation of the EGFR pathway. Indeed, inhibition of EGFR enhanced TGF-β-induced cell death [14,20,26,29]. We wondered whether similar results were obtained in HCC cells using the tyrphostin AG1478. As observed in Fig. 7A, AG1478 was able to potentiate TGF-β-induced loss of viable cell number and to enhance caspase-3 activation. Moreover, AG1478 impaired TGF-β-induced expression of MCL1, and decreased the expression of BCL-XL (Fig. 7A). We also have observed that low concentrations of AG1478 were able to increase doxorubicin-induced apoptosis in human HCC cells (Fig. 7B).

In conclusion, all these results together support the potential effectiveness of AG1478 in suppress HCC growth, because it would impair cell proliferation, promote apoptosis, enhance the apoptotic response to other physiological factors, such as TGF-β, and increase the cytotoxic effects of the widely used chemotherapeutic agent doxorubicin.

4. Discussion

HCC is the main type of liver cancer, with nearly 600,000 deaths each year worldwide. Most cases of HCC are diagnosed in an advanced or unresectable stage of the disease. Even with the use of transarterial chemoembolization, intra-arterial or systemic chemotherapy, radiotherapy, immunotherapy or hormonal therapy, the 5-year relative survival rate for patients with HCC is only 7% [30]. In patients with advanced-stage of HCC, sorafenib is used; however, the benefit is small, and new therapies to combine with or replace sorafenib are urgently needed [31]. HCC pathogenesis is highly complex, and numerous molecular alterations have been described [32]. However, it is widely accepted that in all cases, there is an imbalance between proliferation and cell death, mainly caused by over-activation of survival pathways [2].

In HCC, EGFR ligands and the receptor itself are over-expressed [6–9] and activation of EGFR has been suggested to be involved in tumor progression [33]. Over the years our and other groups have accumulated evidence that supports the importance of EGFR signaling in hepatoma and HCC cells [4,14,15,26,34]. From previous studies and results presented here, we know that EGFR is important for their autocrine proliferation. Different evidences support this hypothesis. First, human HCC cells show over-expression of at least one of the EGFR ligands and all of them show higher expression of the metalloprotease responsible for their shedding (TACE/ADAM17), when compared to untransformed cells (Suppl. Fig. 2). Second, EGFR transient silencing in human HCC cells attenuates proliferation and induces apoptosis [26] (Fig. 2). Furthermore, drugs blocking EGFR pathway attenuate in vitro growth of liver tumor cells [15,26,35–38]. EGFR blockage, either by the use of inhibitors that bind to the adenosine triphosphate binding site of the receptor's tyrosine kinase domain (gefitinib, erlotinib) or using antibodies that block the receptor (cetuximab and panitumumab), has proved to be useful in the treatment of advanced colorectal, head and neck, lung and pancreatic cancers [39,40]. Clinical trials have been also designed with some of these molecules to check their efficiency in HCC [41–43]. However, success has been modest and more preclinical investigation is necessary to better understand the relevance of the EGFR in liver tumor progression, as well as to explore the effectiveness of new potential EGFR inhibitors.

The tyrphostin AG1478 is widely considered as an EGFR inhibitor and, although it has not been proposed as a potential anti-neoplastic drug in HCC yet, previous studies have shown that it can be administrated either by subcutaneous injection or by oral administration reaching the liver [44]. In this work we have tested the effects of AG1478 in human HCC cells, comparing its effects to those observed in untransformed human hepatocytes. As shown in Figs. 1 and 3, AG1478 blocks cell proliferation and promotes apoptosis in HCC cells, coincident with attenuation of EGFR signaling. Proliferation inhibition correlates with an increase in the percentage of cells in G1 phase of the cell cycle. Apoptosis is evidenced by the increase in the percentage of hypodiploid cells coincident with caspase-3 activation. These results are in concordance with reports from other groups which have shown that the use of EGFR antisense in hepatoma cells has a growth inhibitory effect [34] and the EGFR tyrosine kinase inhibitors

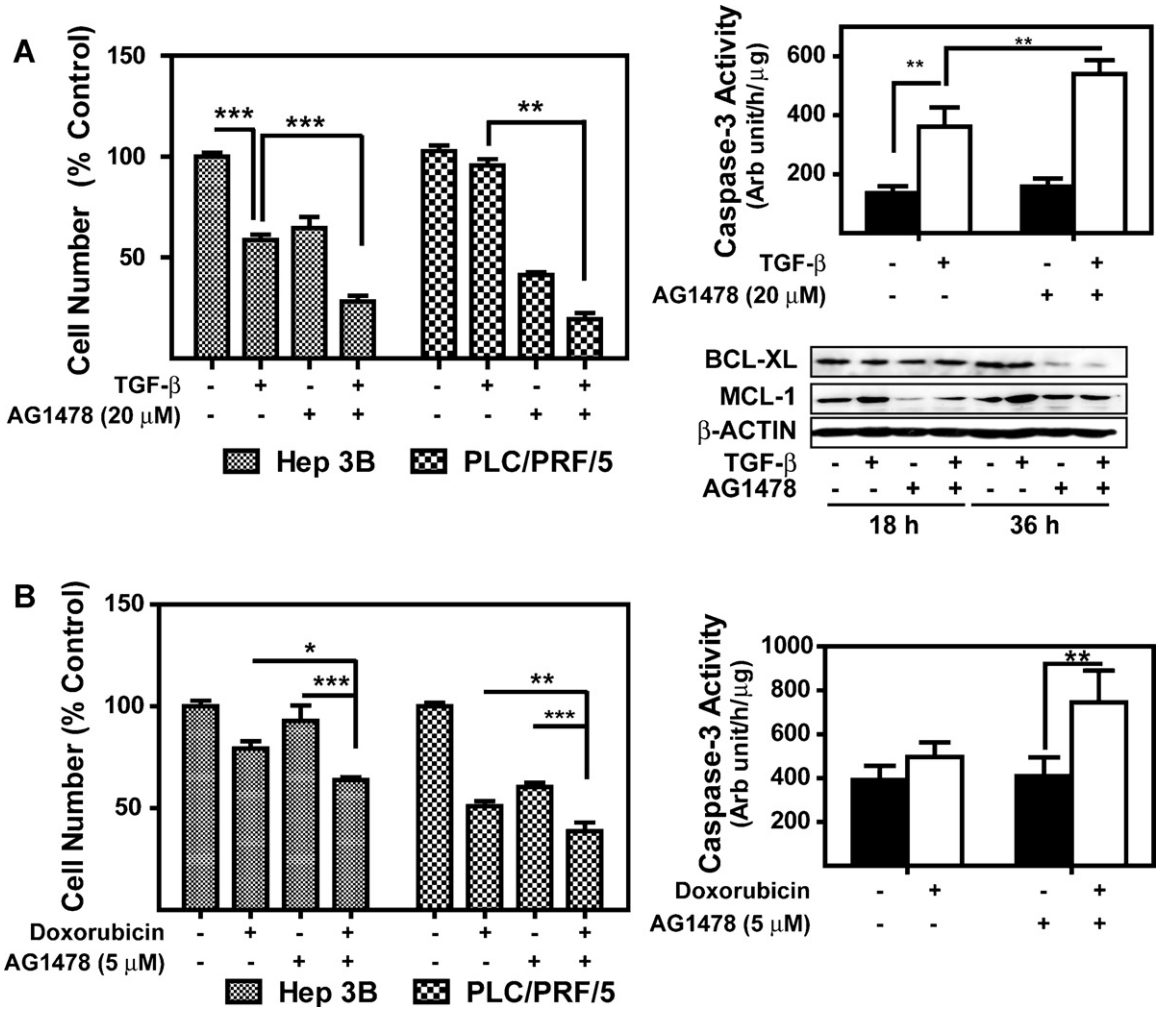

Fig. 7. AG1478 enhances the effect of different apoptotic stimuli in HCC cells. (A) Cells were incubated with or without AG1478 (20 μM) in the presence or absence of TGF-β (2 ng/ml). (B) Cells were incubated with or without AG1478 (5 μM) in the presence or absence of doxorubicin (0.25 μM). In the left column, number of viable cells analyzed by crystal violet is shown (48 h). In the right column, caspase-3 activity is shown in Hep3B cells ((A) 16 h; (B) 24 h). In (A), Western blot analysis of BCL-2 family is shown, representative of 3. Data are the mean ± S.E.M. ($n = 3$, in duplicate/triplicate). Data are compared as indicated in each figure, one-way ANOVA was used: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

gefitinib or erlotinib are able to induce cell cycle arrest and apoptosis in HCC cells, enhancing chemosensitivity towards cytostatics [35,37].

The high efficiency of AG1478 in inducing apoptosis might be explained by its capacity to regulate the expression of different members of the BCL-2 and IAP families (Fig. 4). Preliminary results from our group in FaO rat hepatoma cells had indicated that AG1478 decreased the expression of both MCL1 and BCL-XL, which is in agreement with the more extended study presented here [15]. It is important to highlight that EGF up-regulates the expression of BCL-XL in hepatocytes [13], which would explain the efficiency of AG1478 in decreasing its levels in HCC cells. Additionally, AG1478 induces the expression of the pro-apoptotic member of the BCL-2 family BIM, both at the mRNA and protein level (Fig. 4). In agreement with this result, previous studies indicated that EGFR inhibitors might promote the intrinsic pathway of apoptosis by modulating the expression of pro-apoptotic members of the BCL-2 family in non-small-cell lung cancer or oral squamous cell carcinoma [45,46]. In fact, up-regulation of BIM has been associated with the efficiency of EGFR inhibitors in inducing apoptosis in lung tumor cells [47,48]. Interestingly, results presented here also indicate that AG1478 decreases the expression, at the protein level, of two members of the IAP family, HIAP2 and XIAP (Fig. 4B), inhibitors of caspase activity. The expression of anti-apoptotic members of the BCL2 family is increased in HCC [2,49], as well as the expression of different members of the IAP family, such as HIAP1, HIAP2, survivin and XIAP [50–52], particularly linked to HBV (hepatitis B virus) or HCV (hepatitis C virus) infection [50,53]. Thus, AG1478 is counteracting the anti-apoptotic army of HCC cells at different levels. It is worthy to note that untransformed hepatocytes are much less sensitive to AG1478 cytotoxic effects than HCC cells. Human fetal hepatocytes (HFH) show inhibition of autocrine proliferation in response to AG1478 and the immortalized human hepatocytes HH4 show attenuated FBS-induced growth; however none of them shows significant apoptosis in response to this drug (Fig. 6). These results show that the use of AG1478 would have major cytotoxic effects in tumoral than in untransformed cells, which favors the use of this tyrphostin as a therapeutic drug in HCC treatment.

Interestingly, AG1478 antiproliferative and pro-apoptotic effects are much higher than those observed by transient silencing of the EGFR expression (Fig. 2). In this same line of evidence, gefitinib, another EGFR inhibitor, and cetuximab (a neutralizing antibody) show modest effects inducing apoptosis when compared with that observed with AG1478 ([26] and results not shown). Furthermore, here we show that AG1478 is still able to inhibit proliferation and to promote apoptosis in cells in which EGFR expression has been stably targeted knock-down by shRNA (Fig. 5),

although at a lower extent. These results indicate that this tyrphostin might exert some actions in an EGFR (Erb1)-independent manner. It has been suggested that small-molecule EGFR inhibitors, included AG1478, might be able to prevent phosphorylation of other members of the erbB family [54]. Thus, we cannot exclude the possibility that AG1478 can target other EGF receptors, although unpublished results from our group indicate that tyrphostins that target other members of the HER family, such as HER-2, have no effects on hepatocytes or liver tumor cells. Another possibility should be that AG1478 might have other targets different from the EGF receptors. It has been recently proposed that AG1478 might have EGFR-independent activity in disassembling the Golgi in human cells, through inhibiting the activity of a small GTPase ADP-ribosylation factor [55]. To confirm whether this could occur in HCC cells, we have evaluated the effects of AG1478 on Golgi in AG1478-treated Hep3B cells. Preliminary results have indicated that AG1478, but not EGFR silencing, induces Golgi disassembling in these cells (Suppl. Fig. 5). The fact that AG1478 might be able to target other proliferation and survival signals different from the EGFR makes it potentially efficient in counteracting growth of both tumor cells highly dependent on EGFR and tumor cells with lower activation of this receptor.

AG1478 might also enhance the apoptotic effects of the physiological pro-apoptotic cytokine TGF-β and the cytostatic drug doxorubicin in human HCC cell lines (Fig. 7). In both cases AG1478 enhances the loss of viable cells and the increase in caspase-3 activation induced by TGF-β or doxorubicin alone. TGF-β is highly expressed in HCC [56,57] and this cytokine plays a dual role inducing both pro- and anti-apoptotic signals, the balance among them decides cell fate [14,20,58]. Cells that survive undergo epithelial-mesenchymal transitions [21,59], which might contribute to the acquisition of a pro-fibrotic and migratory phenotype [60,61]. It is worthy to note that TGF-β-induced anti-apoptotic signals are mediated by the EGFR pathway [20,62,63], which might explain the effectiveness of AG1478 in balancing the HCC cells response to TGF-β towards its pro-apoptotic effects. Results observed with TGF-β might suggest that if HCC cells express higher autocrine TGF-β expression than untransformed HH4 cells, this could mediate the observed HCC cytotoxic response to AG1478. Preliminary results have indicated us that this is not the case. Although TGF-β expression is clearly higher in Hep3B and SK-Hep1, the presence of the TGF-β receptor I inhibitor SB-431542 did not impair AG1478 effects on cell number in Hep3B cells (Suppl. Fig. 6).

Interestingly, AG1478 could also contribute to improve the response to chemotherapeutic drugs, such as doxorubicin, which is widely used in the treatment of HCC alone or in combination with other treatments [30]. This effect might be mediated through both EGFR-dependent and -independent mechanisms, since AG1478 has been shown to inhibit the ATP-binding cassette (ABC) transporters, ABCB1 and ABCG2 [64,65], which might reverse ABC-mediated drug resistance. Therefore, AG1478 apart from inducing apoptosis per se would be increasing its effectiveness by enhancing the apoptosis induced by physiological cytokines, such as TGF-β, or chemotherapeutic drugs, such as doxorubicin.

In conclusion, AG1478 could be a potential therapeutic drug to be used in HCC, since it inhibits cell cycle and induces apoptosis. Its effects are greater than those observed by simple EGFR silencing, which indicates that it is also acting on other targets, which makes it suitable to be used in tumors that lack alterations in the expression of EGFR. It enhances the apoptotic effects of other agents, such as TGF-β, whose expression is up-regulated in HCC, or doxorubicin, a widely used chemotherapeutic drug in HCC. Interestingly, it preferentially acts on liver tumor cells, being untransformed cells much less responsive to its cytotoxic effects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.08.011.

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