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Inhibition of the EGF receptor blocks autocrine growth and increases the cytotoxic effects of doxorubicin in rat hepatoma cells
Role of reactive oxygen species production and glutathione depletion

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ABSTRACT

FaO rat hepatoma cells show increased levels of the epidermal growth factor receptor (EGFR) ligands, when compared with adult normal hepatocytes, and higher activity of the TNF-alpha converting enzyme (TACE/ADAM17), which is required for EGFR ligand proteolysis and activation. In this work we have analysed the consequences of inhibiting the EGFR in FaO rat hepatoma cells, focusing the attention on autocrine growth and protection from apoptosis. Results have indicated that FaO cells show overactivation of the EGFR pathway, which induces basal growth (in the absence of serum) and protection from pro-apoptotic agents, such as doxorubicin, generating drug resistance. Treatment of cells with the combination of doxorubicin and the tyrphostin 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478, a potent and specific inhibitor of EGFR tyrosine kinase) potently inhibits autocrine growth and induces apoptosis. The apoptotic effect correlates with high expression and activation of the pro-apoptotic Bax and decreased transcript and protein levels of the anti-apoptotic Mcl-1 and Bcl-x_L. Furthermore, the combination of AG1478 and doxorubicin induces reactive oxygen species (ROS) production and glutathione depletion in FaO cells, coincident with up-regulation of the NADPH oxidase NOX4 and down-regulation of the gamma-glutamylcysteine synthetase (γ-GCS), a key regulatory enzyme of the glutathione synthesis. Incubation of cells with glutathione ethyl ester attenuates the apoptosis induced by the combination of doxorubicin and AG1478, which indicates that glutathione depletion is required for an efficient cell death. In conclusion, targeting EGFR combined with other conventional pro-apoptotic drugs should potentially be effective in antineoplastic therapy towards liver cancer.

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Abbreviations: DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; GEE, glutathione ethyl ester; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; γ-GCS, gamma-glutamylcysteine synthetase; HCC, hepatocellular carcinoma; HB-EGF, heparin-binding epidermal growth factor-like growth factor; ROS, reactive oxygen species; TACE/ADAM17, TNF-alpha converting enzyme; TGF-α, transforming growth factor-alpha; TGF-β, transforming growth factor-beta.

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1. Introduction

The epidermal growth factor receptor (EGFR) is a tyrosine kinase of the ErbB receptor family, which is frequently activated in many epithelial tumors [1], leading to enhanced growth and other tumor promoting activities [2]. Targeting this receptor has been proved as an antineoplastic therapeutic tool, although success has not covered the initial expectative [3]. There is a need for improved strategies to integrate anti-EGFR agents with conventional therapies and to explore combinations with other molecular targeted approaches [2,3].

Liver cancer, in particular, hepatocellular carcinoma (HCC), is one of the major cancer killers [4]. There is not firstline option for patients with advanced HCC. Systemic doxorubicin provides partial responses, but no clear survival advantages, and well-known treatment-related complications [4]. Several other treatments, such as immunotherapy, internal radiation, tamoxifen, or anti-androgen agents, have not shown any relevant anti-tumoral effect [4]. Dysregulation of the balance between proliferation and cell death represents a protumorigenic principle in human hepatocarcinogenesis [5]. Although some physiological pro-apoptotic molecules are down-regulated or inactivated in HCC, dysregulation of the balance between death and survival is mainly due to overactivation of anti-apoptotic signals. Thus, some growth factors that mediate cell survival are up-regulated in HCC, as well as the molecules involved in the machinery responsible for cleavage of their proforms to an active peptide [5]. This is the reason why new drugs, such as tyrosine kinase inhibitors, are currently being tested. It has been suggested that targeting the EGFR by inhibitors of the EGFR tyrosine kinase activity, or monoclonal antibodies directed against the EGFR, might arrest cell cycle and enhance chemosensitivity towards cytostatics in liver cancer cells [6–10]. Interestingly, gefitinib (ZD1839, Iressa), a selective EGFR inhibitor, prevents hepatocellular carcinoma development in the rat liver with cirrhosis [11] and shows antimetastatic activity in a hepatocellular carcinoma model [12]. However, further work is required to elucidate the molecular mechanisms by which EGF contributes to liver tumor cell growth and survival, which might open perspectives in the design of new molecular targets for antineoplastic therapy.

We had previously described that the epidermal growth factor (EGF) protects rat hepatocytes (primary culture) from apoptosis by a phosphoinositide 3-kinase-dependent pathway [13]. EGF is able to maintain elevated levels of Bcl-x_L, an anti-apoptotic member of the Bcl-2 family [13]. Recent results have indicated that impairment of the transforming growth factor-beta (TGF-β)-induced apoptosis by EGF in hepatocytes correlates with an inhibition of the mechanisms that address the induction of a member of the NADPH oxidase family (*nox4*), involved in generating reactive oxygen species (ROS) [14]. Indeed, EGF is counteracting the oxidative stress caused by some pro-apoptotic agents.

FaO rat hepatoma cells express increased levels of EGFR ligands, when compared with adult normal hepatocytes [15] and, even more interestingly, the activity of the TNF-alpha converting enzyme (TACE/ADAM17), which is required for EGFR ligands proteolysis and activation, is much higher in FaO rat hepatoma cells than in adult hepatocytes [15]. Thus, FaO cells are a good model to analyse the involvement of the EGFR pathway in liver tumor progression. According to this, the aim of this work was to study the relevance of the EGFR pathway in FaO rat hepatoma cells, focusing the attention on autocrine growth and protection from apoptosis. Furthermore, we have analysed whether EGFR inhibition might enhance the cell response to chemotherapeutic drugs, such as doxorubicin, and the potential molecular mechanisms involved.

2. Materials and methods

2.1. Cell culture

FaO rat hepatoma cells, obtained from the European Collection of Cell Cultures (ECACC), were cultured in F12 Coon's Modified medium (Invitrogen-Gibco, Carlsband, CA), supplemented with 10 % fetal bovine serum (Sera Laboratories International, Cinder Hill, UK) and maintained in a humidified atmosphere of 37 °C, 5% CO_2. Medium was replaced by serum-free F12 Coon's Modified medium 18–24 h before the different treatments (cells at 70 % confluence). When cells were pre-treated with AG1478 + doxorubicin (both from Calbiochem, La Jolla, CA), AG1478, at a concentration of 20 μM [15], was added 30 min before doxorubicin. Adult hepatocytes were isolated from male Wistar rats, as described [15]. All animals received humane care according to the criteria outlined in the ''Guide for the Care and Use of Laboratory Animals''. Cells were plated in M-199 medium supplemented with 20% FBS, 100 μM insulin and maintained in a humidified atmosphere of 37 °C, 5% CO_2. After 24–48 h, medium was replaced by serum-free, insulin-free, M-199 medium, 12–24 h before cell treatments.

2.2. Analysis of the number of viable cells

After treatments, cells were washed twice with PBS and the remaining viable adherent cells were stained with crystal violet (0.2% in 2% ethanol), as described previously [13].

2.3. Analysis of DNA content by flow cytometry

The ploidy determination of FaO rat hepatoma cells was estimated by flow cytometry DNA analysis, as described previously [13]. A DNA content lower than 2C (hypodiploid cells) is considered a feature of apoptosis. Cell cycle analysis was carried out using the software ModFit LT™ (Verity Software House).

2.4. Immunofluorescence microscopy analysis

For fluorescence microscopy studies, cells were plated on gelatin-coated glass coverslips. Cells were fixed with 4 % paraformaldehyde for 12 min at RT, permeabilized in PBS containing 0.1% Triton X-100, 0.1% BSA for 3 min, blocked with 1% BSA and 10% goat serum in PBS for 1 h and then incubated with anti-EGFR rabbit polyclonal antibody (from Cell Signaling, Beverly, MA, USA: CS-2232) (1:100) and anti-HB-EGF goat polyclonal antibody (M-18) (from Santa Cruz Biotechnology, Santa Cruz, CA: SC-1414) (1:100) diluted in 1% BSA, overnight at 4 °C. After several washes with PBS, the samples were

incubated with fluorescent-conjugated secondary antibodies (1:500 for Cy3-conjugated anti-rabbit, 1:200 for Alexa Fluor 488-conjugated anti-goat) for 1 h at RT and embedded in Vectahield with DAPI (Vector Laboratories, Burlingame, CA). Cells were visualized in an Olympus BX-60 microscope.

2.5. DNA synthesis assay

Cells were incubated for 24 h in the absence or in the presence of the different factors and DNA synthesis was evaluated by [methyl-^{3}H] Thymidine (GE Healthcare) incorporation into TCA-precipitable material during the last 20 h, as described previously [16].

2.6. Western blots

Protein extracts were obtained as described previously [17]. Proteins were separated by SDS electrophoresis on 10–12% polyacrylamide gels and transferred to membranes that were then blocked in TTBS containing 5% non-fat dried milk. Membranes were incubated overnight with the corresponding primary antibody at 4 °C in TTBS containing 0.5% non-fat dried milk (all antibodies diluted 1:1000, with the exception of anti-β-actin that was used at 1:3000). After washing, the membranes were incubated with peroxide-conjugated secondary antibody for 1 h at room temperature (1:5000 in TTBS 0.5% non-fat dried milk). Anti-β-Actin, anti-Akt (CS-9272), anti-phospho Akt (Ser473) (CS-9271), anti-Phospho-p44/42 MAPK (Thr202/Tyr 204) (CS-9101), anti-p44/42 MAP Kinase (137F5) (CS-9122), anti-Phospho-Src (CS-2101), anti-EGF Receptor (CS-2232) and anti-phosho-EGF receptor (Tyr1068) (CS-2236) antibodies were from Cell Signaling (Beverly, MA, USA). Anti-Mcl-1, and anti-Bcl-x_L were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bax antibodies were from BD-Pharmingen (San Diego, CA). Anti-gamma-glutamylcysteine synthetase (γ-GCS) (ab17926) antibody was from ABCAM (Cambridge, UK). Antibody binding was visualized using ECL (Amersham Biosciences, UK). Protein concentration was measured using the BioRad protein reagent following the recommendations of the supplier.

2.7. Reverse transcriptase-polymerase chain reaction analysis (RT-PCR)

Total RNA was obtained using the RNeasy Kit (Qiagen). Complementary DNA was generated by the SuperScript First-Strand Synthesis System for RT-PCR using oligo (dT) as primer.

Rat specific primers sequences for PCR reactions were used:

bax: 5′-GTTACAGGGTTTCATCCAGGATCG-3′ (forward)
5′-AGCCACAAAGATGGTCACTGTCTG-3′ (reverse)
bcl-x$_L$: 5′-CGTGGAAAGCGTAGACAAGG-3′ (forward)
5′-GAGCCCAGCAGAACTACACC-3′ (reverse)
mcl-1: 5′-TTCTTGTAAGGACGAAGCGGG-3′ (forward)
5′-GCCAGCAGCACATTTCTGATG-3′ (reverse)
nox 4: 5′-TTACTACTGCCTCCATCAAGC-3′ (forward)
5′-GGAATGATTGGATGTCTCTGC-3′ (reverse)
albumin: 5′-CTGCCGATCTGCCCTCAATAGC-3′ (forward)
5′-GTGCCCACTCTTCCCAGGTTTCT-3′ (reverse)

Products were obtained after 30–35 cycles of amplification and 60–65 °C of annealing temperature and were electrophoresed in 0.8–1.2% agarose gels.

2.8. Analysis of caspase-3 activity

Fluorimetric analysis of caspase-3 activity was determined as described previously [13], with 10–20 μg protein extract. The caspase-3 substrate Ac-DEVD-AMC was from Pharmingen (San Diego, CA). Fluorescence was measured in a Microplate Fluorescence Reader Fluostar Optima. Protein concentration of the cell lysates was measured using the BioRad protein reagent.

2.9. Determination of the percentage of cells containing active Bax

Cells were fixed during 15 min with 4% paraformaldehide, permeabilized 2 min with 0.1% Triton X-100 on ice and blocked with BSA 1% during 1 h. After incubation for 1 h at 4 °C with the anti-Bax antibody 6A7 clone (BD Pharmingen), and for 1 h at 4 °C with Alexa 488-conjugated anti-mouse IgG antibody (Invitrogen), the fluorescence was estimated by flow cytometry.

2.10. Measurement of intracellular redox status

The oxidation-sensitive fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and MitoSOX™ Red (both from Invitrogen, Carlsbad, CA, USA) were used to analyse the total intracellular content of ROS and mitochondrial superoxide production, respectively. To analyse intracellular glutathione, monochlorobimane (Sigma–Aldrich) was used. After treatments, cells were incubated with 2.5 μM DCFH-DA (30 min, 37 °C), 5 μM MitoSOX™ Red (10 min, 37 °C) or 2 mM monochlorobimane (60 min, 37 °C) in HBSS without red phenol. Then, cells were lysed with 25 mM HEPES, pH 7.5, 60 mM NaCl, 1 mM MgCl, 0.2 mM EDTA and 1 % Triton X-100 (10 min, 4 °C) and transferred in triplicate into a 96-well plate. Fluorescence was measured in a Microplate Fluorescence Reader Fluostar Optima and expressed as percentage to control (untreated cells) after correction with protein content. Wavelength filters used were: for DCFH-DA, exc. 485 nm, em. 510; for monochlorobimane, exc. 355 nm, em. 440 nm; for MitoSOX™ Red, exc. 510, em. 590.

3. Results

3.1. Overactivation of the EGFR pathway in FaO rat hepatoma cells: effects on autocrine cell growth

FaO rat hepatoma cells express high levels of the EGFR ligands transforming growth factor-alpha (TGF-α) and heparin-binding epidermal-growth-factor-like growth factor (HB-EGF) transcripts, much higher than those found in adult hepatocytes (Fig. 1A). Levels of EGFR mRNA are lower than in adult hepatocytes, but expression of the TACE/ADAM17, which is required for EGFR ligands proteolysis and activation, is much higher, in agreement with previous reported data [15]. Correlation of HB-EGF transcript and protein levels was

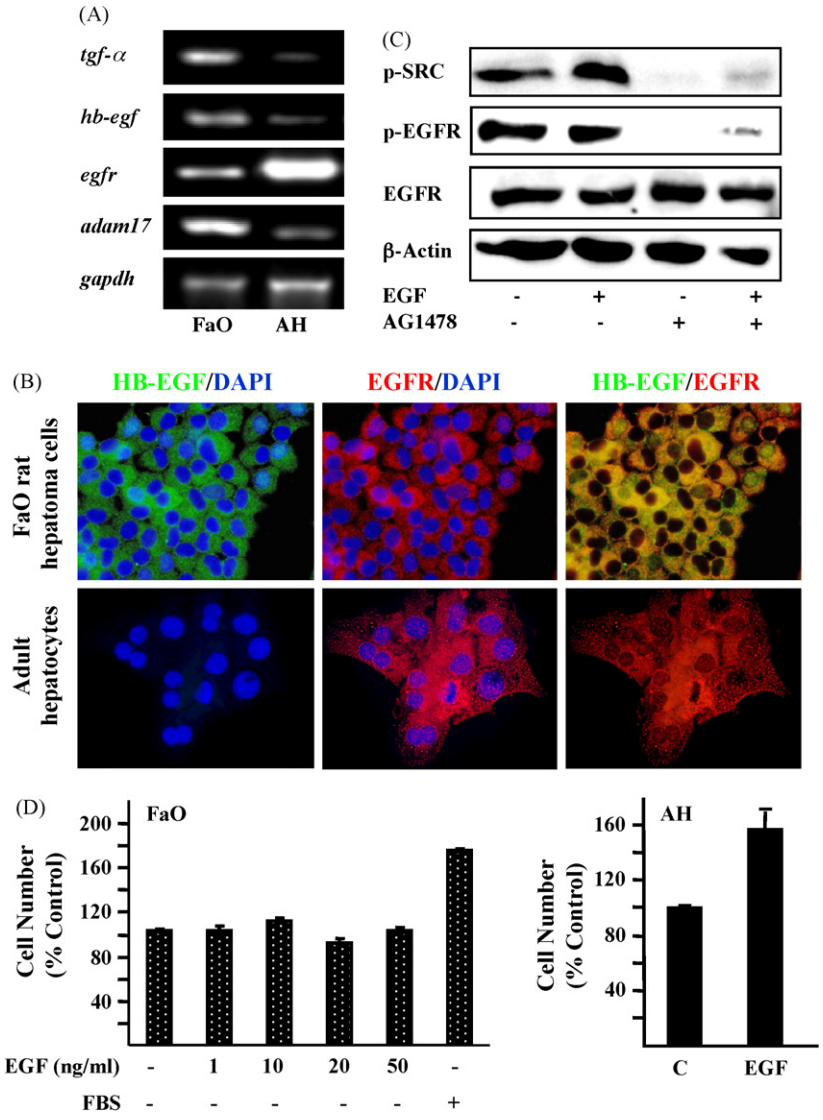

Fig. 1 – Autocrine activation of the EGFR pathway in FaO rat hepatoma cells. (A) Comparative analysis of basal levels of *tgf-α*, *hb-egf*, *egfr* and the *tace/adam17* metalloprotease transcripts in FaO rat hepatoma cells and adult rat hepatocytes. Analysis by semiquantitative RT-PCR. *Gapdh* was used as loading control. (B) Immunofluorescence study of the basal expression of EGFR (red) and HB-EGF (green) in FaO cells and adult hepatocytes. Blue signal represents the nuclear DNA staining with DAPI. (C) Analysis by Western blot of the phosphorylation status of the EGF receptor (EGFR) and c-Src (SRC) in FaO rat hepatoma cells after 18 h in the absence of serum. Cell response to EGF (20 ng/ml, 15 min). Treatment with the EGFR inhibitor AG1478 at 20 μM for 30 min greatly attenuates the phosphorylation of both proteins. (D) Number of viable cells, analysed by crystal violet staining. (Left) FaO rat hepatoma cells were incubated for 36 h without or with EGF or 10% FBS, as indicated. (Right) Adult hepatocytes (AH) were incubated for 48 h without (Control: C) or with EGF. In A, B and C, a representative experiment of at least three is shown. In D, results are expressed as percentage of the initial number of cells, and are the mean + S.E.M. (n = 3, in triplicate).

demonstrated in immunofluorescence studies, with specific antibodies. HB-EGF protein is barely detected in adult hepatocytes, but FaO cells express high levels of this protein (Fig. 1B). EGFR was present in both adult hepatocytes and FaO cells. Similar results were obtained for TGF-alpha (results not shown). The consequence of this specific expression pattern is a constitutive phosphorylation of the EGFR and its downstream effector c-Src [18] (Fig. 1C), which is inhibited by the presence of the tyrphostin 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478), a potent and specific inhibitor of EGFR tyrosine kinase whose favourable preclinical profile supports progression towards clinical trials [19]. Interestingly, extracellular EGF did not significantly increase phosphorylation of the EGFR (Tyr1068) and only slightly increased c-Src phosphorylation, which indicates almost maximal autocrine stimulation of this pathway. In agreement with this result, FaO cells barely responded to extracellular EGF in terms of growth, although they clearly increased proliferation in

response to fetal bovine serum (Fig. 1D). In contrast, adult hepatocytes showed a great response to EGF.

FaO rat hepatoma cells showed autocrine growth in the absence of serum (Fig. 2A), which was impaired when the culture medium was changed every 6 h (Fig. 2B). This indicates that some factors, secreted by the cells, are required for autocrine growth and survival. The presence of the EGFR inhibitor completely blocked the increase in cell number in the absence of serum (Fig. 2C). In agreement with this, in the presence of AG1478, a significant decrease in the percentage of cells in the S phase of the cell cycle and an increase in the percentage of cells in G1 phase were observed (Table 1). Furthermore, this inhibitor induced cell loss at long times (longer than 24 h), which indicate that long exposures to AG1478 might induce cell death. In contrast, adult hepatocytes did not show autocrine growth (Fig. 2C) and AG1478 barely induced cell loss after 48 h of treatment. FaO cells showed constitutive phosphorylation of Akt and ERKs, which was inhibited in the presence of AG1478 (Fig. 2D). It is worthy to note that in the case of ERKs, the EGFR inhibitor almost completely blocked their phosphorylation, which might indicate their strong dependence on the EGFR pathway.

All these results together indicate that FaO rat hepatoma cells show autocrine growth that is mostly dependent on the EGFR pathway.

3.2. *Inhibiting EGFR pathway enhances the cytostatic and cytotoxic response to doxorubicin in FaO rat hepatoma cells*

Next, we decided to analyse whether inhibiting the EGFR pathway might enhance the capacity of a cytostatic agent,

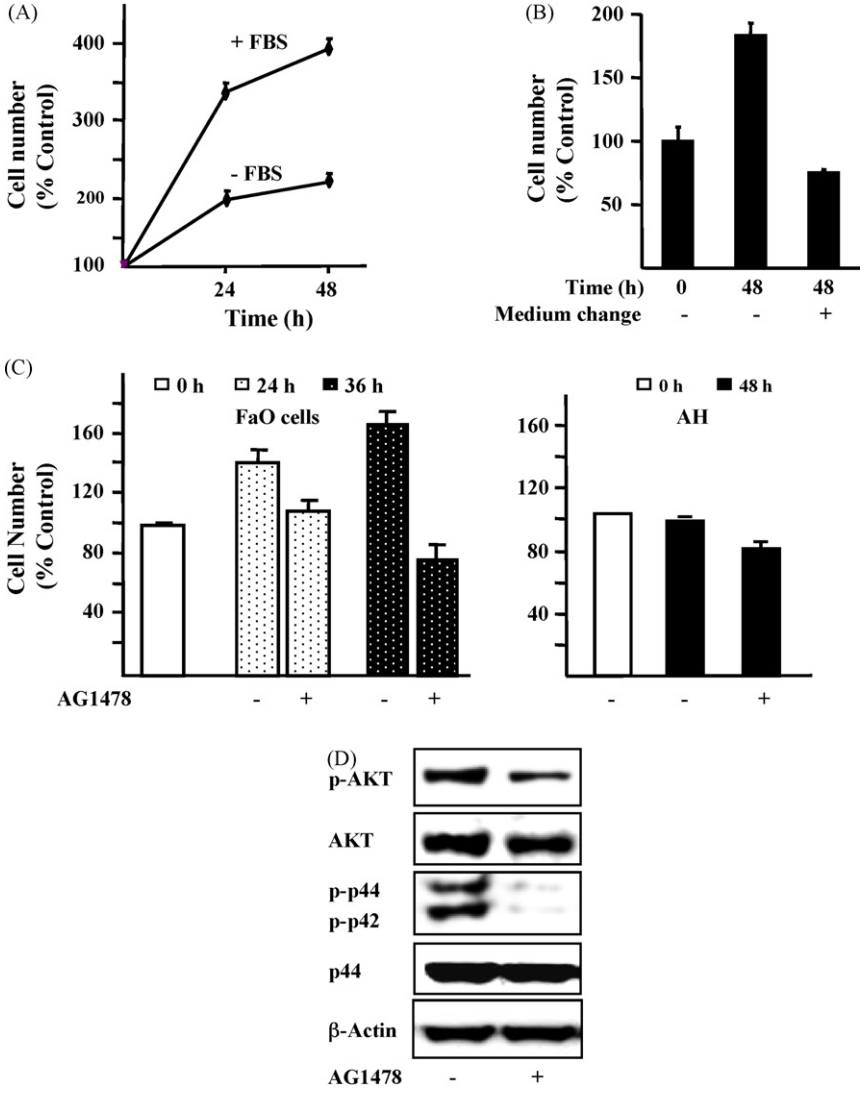

Fig. 2 – Autocrine growth of FaO rat hepatoma cells is dependent on the EGFR pathway. (A) Analysis of the increase in cell number when FaO rat hepatoma cells are cultured in the absence (−FBS) or in the presence (+FBS) of 10% FBS. (B) Effect of replacing medium every 6 h on the FBS-independent growth of FaO cells. (C) Effect of inhibiting the EGFR with AG1478 (20 μM) on cell number in FaO cells (Left) and adult hepatocytes (Right). (D) Western blot analysis of the AG1478 effect (20 μM, 24 h) on the basal activation of AKT and ERKs pathways in FaO rat hepatoma cells. β-actin was used as loading control. In A, B and C, results are expressed as percentage of the initial number of cells, and are the mean + S.E.M. ($n = 3$, in triplicate). In D, a representative experiment of three is shown.

Table 1 – Cell cycle analysis of FaO rat hepatoma cells after 24 h of treatment with AG1478 (20 μM), doxorubicin (5 μM) or both agents together

	G1/G0	S	G2/M
Control	83.63 ± 3.36	9.41 ± 1.86	6.96 ± 2.00
AG1478	92.27 ± 2.35*	2.01 ± 1.23**	5.54 ± 2.11
Doxo	72.98 ± 5.10	3.31 ± 1.27	23.72 ± 3.99
Doxo + AG	88.06 ± 2.88*	2.62 ± 1.65	9.33 ± 3.59*

Mean + S.E.M. of the percentage of cells in each cycle phase ($n = 5$). Student's t-test: AG1478 vs. Control and Doxo + AG vs. Doxo (*$p < 0.05$; **$p < 0.005$).

such as doxorubicin (used as antineoplastic drug in human HCC: [4]) in arresting cell growth. A detailed analysis of the effects on cell cycle indicated that the mechanism used by AG1478 and doxorubicin are completely different. AG1478 arrested cell cycle in G0/G1 phase. However, doxorubicin, in agreement with previous results [15], arrested FaO cells in G2/M phase (Table 1). Interestingly, when both the EGFR inhibitor and doxorubicin were present, cells arrested cycle in G0/G1. A specific analysis of DNA synthesis (incorporation of ^{3}H-Thymidine into DNA) indicated that both AG1478 and doxorubicin inhibited DNA synthesis, maximum effect being observed when both compounds were present together. Indeed, the combination of both agents completely blocked the autocrine synthesis of DNA (Fig. 3A).

Interestingly, we always observed an apparent increase in cell death when FaO rat hepatoma cells were treated with both AG1478 and doxorubicin. For this, we decided to analyse the effect of these agents on apoptosis. Caspase-3 was barely activated by doxorubicin, however, a great activation was observed when cells were treated with both AG1478 and doxorubicin (Fig. 3B). Caspase activation correlated with a great increase in the percentage of cells with a DNA content lower than 2C (hypodiploid cells), an index of apoptosis. FaO rat hepatoma cells were very resistant to doxorubicin-induced apoptosis, only showing slight effects at very high concentrations (20 μM). However, when the EGFR inhibitor was present, apoptosis was observed from the lowest concentrations used (Fig. 3C).

Next, we decided to check changes in the expression of different pro- and anti-apoptotic genes of the *bcl-2* family, which could justify the differences observed in apoptosis. Among a battery of genes tested, we found cooperation between AG1478 and doxorubicin in increasing Bax expression, both at the mRNA and protein level (Fig. 4A and B), which correlated with the presence of a higher percentage of cells showing translocation of Bax to the mitochondria, analysed by flow cytometry with specific antibodies (Fig. 4B). Furthermore, the mRNA and protein levels of the anti-apoptotic Bcl-x_L and Mcl-1 clearly decreased only when both AG1478 and doxorubicin were present (Fig. 4A and C). Taking all these results together, the apoptosis observed with AG1478 and doxorubicin together is coincident with high expression, and activation, of the pro-apoptotic Bax and decreased levels of the anti-apoptotic Mcl-1 and Bcl-x_L.

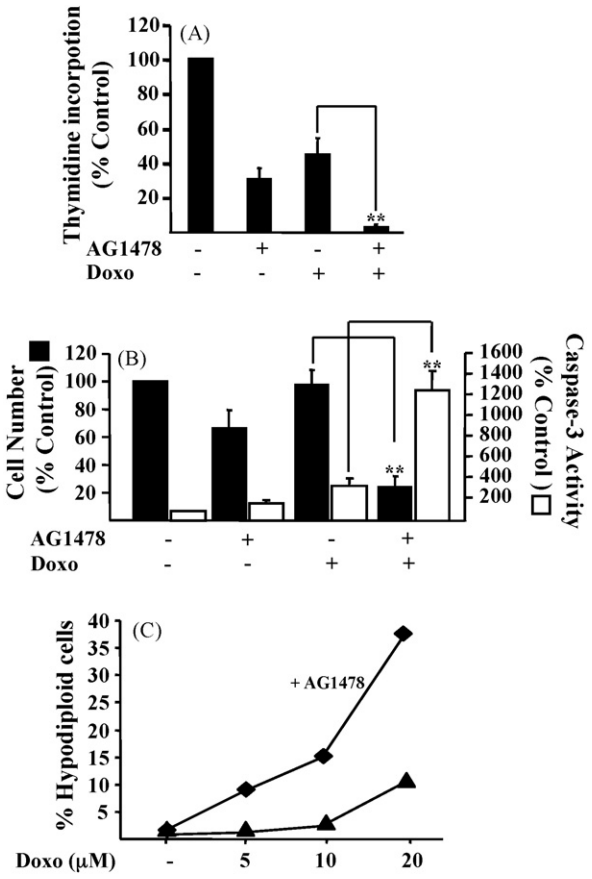

Fig. 3 – Inhibiting the EGFR pathway enhances the cytostatic and apoptotic effects of doxorubicin in FaO rat hepatoma cells. FaO cells were incubated during 24 h with or without AG1478 (20 μM) and/or doxorubicin (5 μM in A and B, or as indicated in C). (A) [^{3}H]-thymidine incorporation into DNA. Mean + S.E.M. ($n = 3$, in triplicate; Student's t-test: **$p < 0.005$). (B) Analysis of the number of viable cells (black bars) and caspase-3 activity (white bars). Results are expressed as percentage of the control (untreated cells) values and are the mean + S.E.M. ($n = 3$, in triplicate). (Student's t-test: **$p < 0.005$). (C) Percentage of cells with a DNA content lower than 2C, analysed by flow cytometry, after 24 h of treatment with doxorubicin at different concentrations, as indicated (rhombs). Effect of the additional treatment with AG1478 (20 μM) (triangles).

3.3. *Role of oxidative stress in the apoptosis induced by the combination of the EGFR inhibitor and doxorubicin in FaO rat hepatoma cells*

Considering previous data in the literature indicating the role of oxidative stress in doxorubicin toxicity [20] and the role of EGF in counteracting oxidative stress in hepatocytes [14], we wondered whether the apoptosis induced by the combination of AG1478 and doxorubicin correlated with ROS production and oxidative stress. Results shown in Fig. 5A illustrate that only the combination of both agents induced a significant increase in the intracellular content of peroxides, analysed with DCFH-DA as a fluorescent probe. The use of a specific probe for the analysis of mitochondrial ROS (Fig. 5B) indicated

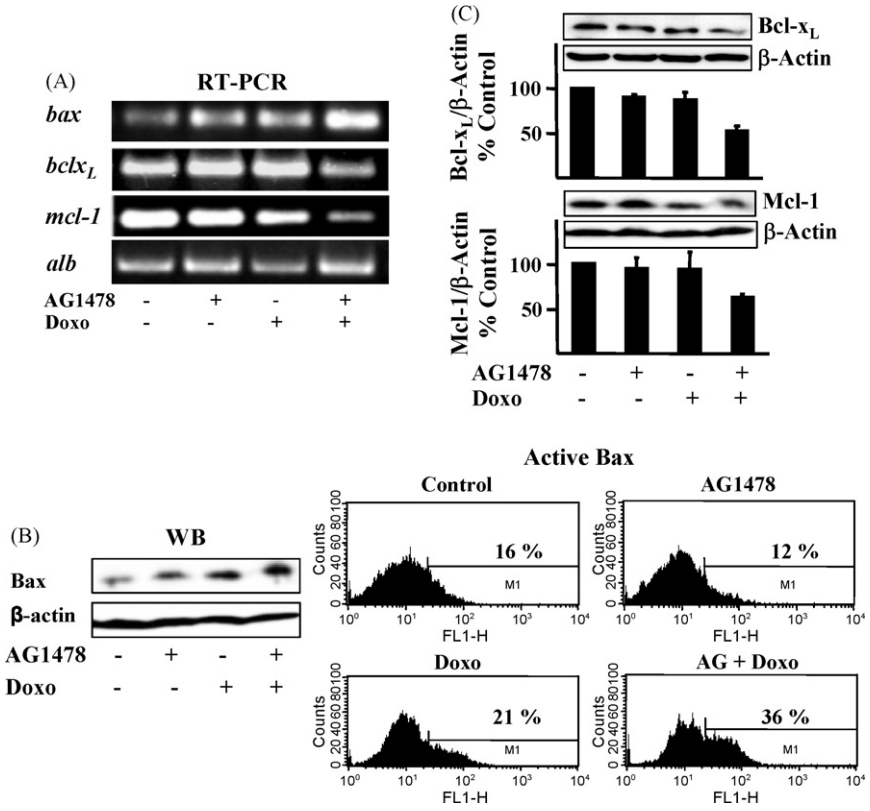

Fig. 4 – Combined treatment with doxorubicin and AG1478 induces down-regulation of Bcl-x_L and Mcl-1 and up-regulation of Bax. FaO cells were incubated during 24 h with or without AG1478 (20 μM) and/or doxorubicin (5 μM), as indicated. (A) Transcript levels of different members of the *bcl-2* family. Analysis by semiquantitative RT-PCR. Albumin (*alb*) was used as loading control. (B) Left panel: Bax protein levels analysed by Western blot. β-actin was used as loading control. A representative experiment of three is shown. Right panels: percentage of cells containing active Bax analysed by flow cytometry after 18 h of treatment. A representative experiment of two is shown. (C) Levels of the Bcl-x_L (upper panel) and Mcl-1 (lower panel) proteins. Analysis by Western blot. β-actin was used as loading control. Graphs represent the data from densitometric analysis of at least three different experiments. A representative experiment is shown at the top of each graph.

that not all the ROS were produced in the mitochondria (increase of mitochondrial ROS was lower than the increase in total ROS). One of the sources of extramitochondrial ROS in hepatocytes is the family of NADPH oxidases and we have recently found that the expression of *nox4*, an inducible member of the family, is low in hepatocytes, but responds to extracellular stimuli increasing its mRNA levels [14]. A detailed analysis of *nox4* transcripts by RT-PCR revealed that both AG1478 and doxorubicin, separately, increased its levels, maximum effect being observed when both agents acted together (Fig. 5C).

To explore the molecular mechanisms by which only the combination of AG1478 and doxorubicin produced a significant increase in ROS, we decided to analyse the glutathione intracellular levels (Fig. 6A). We observed slight, not significant, changes in its levels by cell treatment with AG1478 or doxorubicin alone, but a great decrease when both agents acted together. This decrease correlated with a down-regulatory effect on the levels of gamma-glutamylcysteine synthetase (γ-GCS), a key regulatory enzyme of the glutathione synthesis (Fig. 6B). All these results suggest that AG1478 and doxorubicin cooperate in inducing oxidative stress coincident with up-regulation of *nox4* and down-regulation of γ-GCS. Decrease in glutathione appears to be essential for the mechanism of apoptosis, since incubating cells with glutathione ethyl ester (GEE) significantly attenuated caspase activation (Fig. 6C). Furthermore, the cooperative effect of AG1478 and doxorubicin in inducing cell loss was not observed when cells were incubated in the presence of GEE (Fig. 6C). GEE was also able to attenuate the decrease in *bcl-*x_L and *mcl-1* transcript levels and the increase in the percentage of cells showing active Bax (Fig. 6D).

All these results together indicate that oxidative stress and decrease in glutathione levels are required for the apoptosis induced by the combination of the EGFR inhibitor AG1478 and doxorubicin in FaO rat hepatoma cells.

4. Discussion

In the recent years, antineoplastic pharmacology has focused the attention in the discovery of novel targeting strategies that

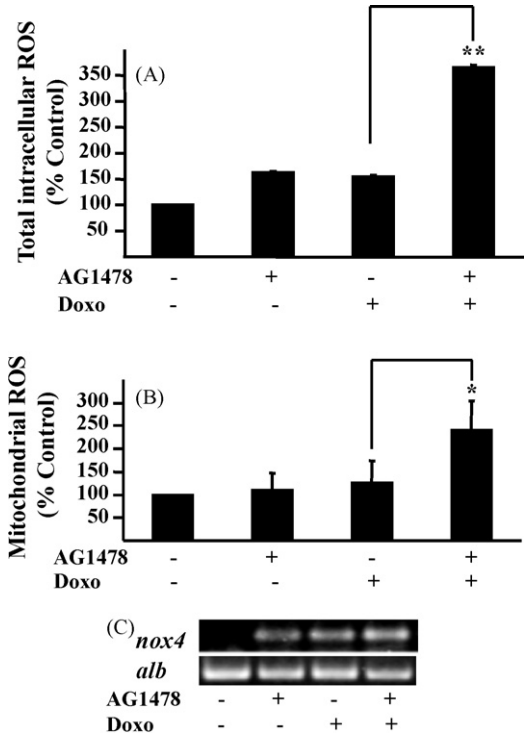

Fig. 5 – Combined treatment with doxorubicin and AG1478 induces an increase in ROS production. FaO cells were incubated with or without AG1478 (20 μM) and/or doxorubicin (5 μM). (A) Intracellular ROS production, analysed fluorimetrically (DCFH-DA as probe). (B) Mitochondrial ROS production, analysed fluorimetrically (MitoSOX as reagent). In both A and B the treatment lasted 24 h. Fluorescence is expressed as percentage of control (untreated cells) and results are the mean + S.E.M. ($n = 3$, in triplicate; Student's t-test: $^{*}p < 0.05$; $^{**}p < 0.005$). (C) Levels of *nox4* transcripts after 12 h of treatment, analysed by semiquantitave RT-PCR. Albumin (*alb*) was used as loading control. A representative experiment of two is shown.

inhibit specific intracellular pathways, overactivated in cancer cells, which govern the function of key molecules involved in tumor growth and progression. Among them, drugs that target tyrosine kinases, their ligands, or downstream signal transducers, have emerged as promising therapeutic tools. The EGFR is abnormally activated in many epithelial tumors. Several mechanisms are involved: receptor overexpression, gene amplification, activating mutations or increased levels of active receptor ligands [2]. Although great advances have been made in the knowledge of both the basic science of the EGFR network and the clinical activity of targeting EGFR agents, current research is focused on implementing the strategies to study the appropriate combinations both with conventional therapies, as well as with other anti-signaling agents [8,21–25].

Hepatoma/hepatocellular carcinoma is a major cause of cancer-related deaths. Current treatments are not effective and the identification of relevant pathways and novel therapeutic targets are much needed. In this work we have used a rat hepatoma cell model, the FaO cells, which show overactivation of the EGFR pathway (Fig. 1), to analyse: (1) the molecular mechanisms induced by the EGFR pathway that might contribute to autocrine cell growth and/or survival; and (2) the possibility that combined therapies of EGFR inhibitors and conventional antineoplastic agents, such as doxorubicin, may improve efficacy in blocking hepatoma cell proliferation and/or inducing cell death. Knowledge about the relevance of the EGFR pathway in liver cancer is quite limited. Here, we demonstrate that autocrine growth of FaO rat hepatoma cells (Fig. 2A) is mediated by cell release of extracellular factors (Fig. 2B) and correlates with overexpression of EGFR ligands and TACE/ADAM17, which provokes constitutive activation of the EGFR and higher intracellular levels of active Akt and ERKs (Figs. 1 and 2). Our results indicate that EGFR inhibition, which greatly attenuates constitutive Akt and ERKs activation (Fig. 2D), inhibits DNA synthesis (Fig. 3A), arrests cell cycle in G0/G1 phase (Table 1) and attenuates autocrine cell growth (Fig. 2C). Dysregulation of growth factor signaling, including EGF pathway, is also well established in human HCCs [26]. Overexpression of TGF-α or amphiregulin, ligands of the EGFR, is frequently observed in human HCCs [26,27]. Furthermore, the quantities of TACE/ADAM17 mRNA vary among different pathological types of HCC, but are significantly higher in poorly differentiated HCC than in well or moderately differentiated HCC [28]. Thus, the situation that we find in FaO cells might also occur in human HCC cells. Indeed, targeting the EGFR by inhibitors of the EGFR tyrosine kinase activity appears to arrest cell cycle in hepatocellular cancer cells [7,8].

Interestingly, when we have analysed the cell response to doxorubicin and AG1478, a clear cooperative effect on growth arrest and cell death is observed (Figs. 3 and 4). It is worthy to point out that when the EGFR inhibitor is present, doxorubicin is unable to arrest cell cycle in G2/M phase, which occurs when cells are treated only with doxorubicin (Table 1). Under these circumstances, cells die by apoptosis, effect that was not observed when cells are treated with the EGFR inhibitor or doxorubicin separately (Fig. 3). It has been suggested that doxorubicin might induce two distinct modes of cell death in hepatoma cell lines [29]. Low doses of this compound induces mitotic catastrophe, which eventually undergo cell death. In contrast, high doses may induce apoptosis, which more efficiently provokes loss in cell viability. Our results indicate that the combination of low doses of doxorubicin with an EGFR inhibitor may provoke a much more efficient mechanism of cell death by apoptosis, coincident with a complete inhibition of DNA synthesis, arrest of cells in Go/G1 phase of the cell cycle, up-regulation of Bax, which is translocated to the mitochondria, and decrease in the levels of Bcl-x_L and Mcl-1 (Fig. 4).

Efficiency of doxorubicin to induce cytoxicity has been related to the generation of ROS [20,30,31]. The administration of the H_2O_2 radical scavenger catalase attenuates the generation of apoptosis induced by this anti-tumor drug [32]. Results shown in this paper indicate that the combination of the EGFR inhibitor and doxorubicin greatly enhances ROS production and glutathione depletion in FaO cells (Figs. 5 and 6). Origin of ROS is not only mitochondrial, and the extramitochondrial ROS might be produced by the NADPH oxidase NOX4 (Fig. 5). It is interesting to point out that, as commented above, the EGFR

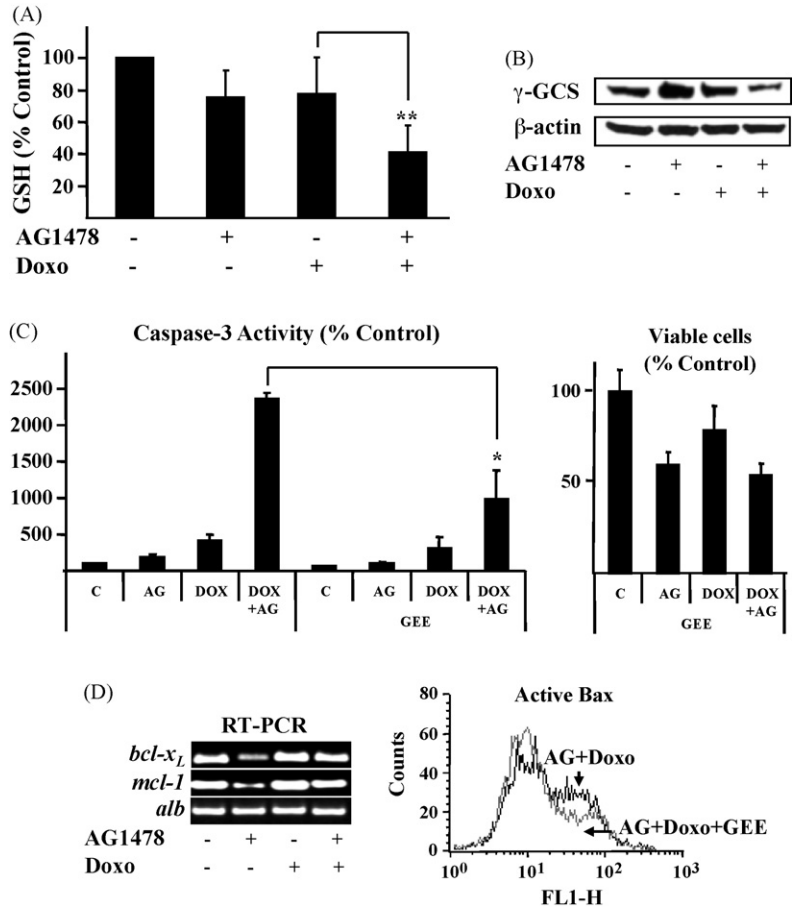

Fig. 6 – Combined treatment with doxorubicin and AG1478 induces a decrease in glutathione intracellular levels, coincident with down-regulation of gamma-glutamylcysteine synthetase. Protective effect of glutathione ethyl ester on the apoptosis. FaO cells were incubated for 24 h with or without AG1478 (20 μM) and/or doxorubicin (5 μM). In C and D, 2 mM of Glutathione ethyl ester (GEE) was added 30 min prior to treatments. (A) Glutathione intracellular levels were analysed fluorimetrically. Results are the mean + S.E.M. of three independent experiments. (Student's *t*-test: $^{**}p < 0.005$). (B) Western blot analysis of the gamma-glutamylcysteine synthetase (γ-GCS) protein. β-actin was used as loading control. A representative experiment of three is shown. (C) Protective effect of GEE on the apoptosis induced by the combined treatment of doxorubicin and AG1478. Effect on caspase-3 activation (left panel) and number of viable cells (right panel). Results are expressed as percentage of the control and are the mean + S.E.M. of three independent experiments. (Student's *t*-test: $^{*}p < 0.05$). (D) GEE attenuates the decrease in *bcl-x*$_L$ and *mcl-1* transcript levels (left panel, RT-PCR analysis) and Bax activation (right panel, analysed by flow cytometry) induced by the combined treatment of doxorubicin and AG1478. In both cases, a representative experiment of two is shown.

pathway counteracts *nox4* expression in hepatocytes [14]. Thus, the increase in *nox4* transcript levels found in FaO cells after treatment with the EGFR inhibitor corroborates this previous observation. However, it is the first time that a relationship between doxorubicin and NOX4 is proposed. In contrast to other NOX proteins, NOX4 produces large amounts of superoxide anion constitutively. NOX4 associates with the protein $p22^{phox}$ on internal membranes, where ROS generation occurs [33]. The regulation of NOX4 remains to be investigated in detail, but most of the available data indicate that modulation of the activity is only related to changes in gene transcription [34]. Thus, increase in NOX4 levels would allow cells to continuously produce intracellular superoxide. Furthermore, and not less important, combination of the EGFR inhibitor with doxorubicin induces down-regulation of γ-GCS, a key regulatory enzyme in the glutathione synthesis (Fig. 6B). When γ-GCS expression is enough to facilitate the synthesis of glutathione, cells might be protected from oxidative damage of cellular components. However, decrease in glutathione levels, caused by down-regulation of γ-GCS, might provoke an oxidative stress situation when cells are treated with the combination of the EGFR inhibitor and doxorubicin. Previous results indicate the relevance of the glutathione pathway in the resistance to doxorubicin-mediated apoptosis in tumor cells [35]. Indeed, glutathione peroxidase or glutathione S-transferase overexpression inhibits apoptosis in doxorubicin-treated human tumor cells [36,37] and depletion of glutathione by buthionine sulfoximine sensitizes rat hepatoma resistant cells to doxorubicin [38]. The oxidative stress, caused by the glutathione depletion, observed in cells treated with the EGFR

inhibitor and doxorubicin might mediate the mitochondrial-dependent apoptosis. In favour of this hypothesis, the presence of glutathione ethyl ester attenuates *bcl-x*$_L$ and *mcl-1* down-regulation and Bax activation in doxorubicin + AG1478-treated cells, which correlates with prevention of caspase activation and cell death (Fig. 6C and D). In the same line of evidence, previous results have indicated that extracellular peroxides induce mitochondrial permeability transition and apoptosis in hepatoma cells [39] and we have previously reported that Bcl-x$_L$ expression is regulated by oxidative stress in hepatocytes [40].

In summary, results presented in this paper indicate that overactivation of the EGFR pathway in hepatoma cells would induce basal growth and protection from pro-apoptotic agents, such as doxorubicin, generating drug resistance. The inhibition of the EGFR pathway in combination with another conventional cytotoxic drug, such as doxorubicin, has proved to be very effective in inhibiting growth and inducing apoptosis, coincident with an oxidative stress process. Indeed, targeting EGFR combined with other conventional pro-apoptotic drugs should potentially be effective in antineoplastic therapy towards liver cancer.

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