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**Inhibition of Apoptosis by Amphiregulin *via* an Insulin-like Growth Factor-1 receptor  
dependent pathway in non-small cell lung cancer cell lines<sup>f</sup>**

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Running title: Amphiregulin activates an IGF1-R dependent survival pathway

## SUMMARY

Several abnormalities in the insulin-like growth factor-1 (IGF1) and erbB receptors pathways stimulate the growth and survival of lung cancer cells but their mechanisms of action and cooperation are poorly understood. In this report, we have identified a new mechanism of apoptosis inhibition by amphiregulin through an IGF1-dependent survival pathway in Non-Small Cell Lung Cancer (NSCLC) cells: amphiregulin activates the IGF1 receptor that in turn induces the secretion of amphiregulin and IGF1.

In the absence of serum, the NSCLC cell line H358 resists apoptosis and secretes factors protecting the NSCLC cell line H322 from serum deprivation apoptosis. IGF1 receptor inhibitor AG1024 as well as epidermal growth factor receptor inhibitors AG556 and ZD1839 restore apoptosis in H322 cells cultured in H358 conditioned medium. Accordingly, the anti-apoptotic activity of H358 conditioned medium is completely abolished after incubation with anti-amphiregulin neutralizing antibody and only partially with anti-IGF1 neutralizing antibody. H358 conditioned medium and amphiregulin induce the IGF1 receptor phosphorylation in H322 cells, which is prevented by anti-amphiregulin neutralizing antibody. H358 cells secrete a high level of amphiregulin that, in combination with IGF1, prevents serum deprivation apoptosis. Finally, IGF1 receptor inhibitor blocks amphiregulin and IGF1 release by H358 cells.

## INTRODUCTION

In lung cancer, well characterized molecular abnormalities affect cell cycle control and apoptosis (1); in addition, a variety of peptide hormones and growth factors induce autocrine or paracrine stimulation of cell survival, proliferation, migration and angiogenesis. While tumor growth of small cell lung cancer is mainly mediated by neuropeptides such as bombesin-related peptides that bind to G protein-coupled receptor, the major autocrine/paracrine loops for non-small cell lung cancer (NSCLC)<sup>1</sup> cells involve growth factors that bind to tyrosine kinase receptors (2).

The erbB receptor family contains four distinct members: the epidermal growth factor receptor (EGFR, erbB-1/HER1), erbB-2/neu (HER2), erbB-3 (HER3) and erbB-4 (HER4). These receptors consist of an extracellular domain containing the ligand binding domain, a hydrophobic transmembrane domain and an intracellular domain conferring tyrosine kinase activity (3). Binding of specific ligands induces homo- or heterodimerization of the receptors within members of the erbB family (4) and activates distinct intracellular signal transduction pathways and biological responses, including cellular proliferation, differentiation, migration and survival. Five ligands of EGFR, the epidermal growth factor (EGF), transforming growth factor  $\alpha$  (TGF $\alpha$ ), amphiregulin (AR), heparin-binding EGF (HB-EGF) and betacellulin, can be produced by tumorigenic and, to a lesser extent, by non-tumorigenic human lung epithelial cells (5-7). As most cells of epithelial origin, NSCLC cells overexpress members of the erbB receptor family, in particular the EGFR and erbB-2/neu, but the prognostic impact of this overexpression is still controversial (5,6). Overexpression of TGF $\alpha$  and AR has been associated with shortened survival of patients with NSCLC (5,6,8,9).

Type 1 insulin like-growth factor receptor (IGF1-R) is also overexpressed in human lung tumors (10,11). The IGF1-R is composed of two extracellular  $\alpha$  subunits with the ligand-

binding domain and two  $\beta$  subunits that contain a single transmembrane domain and an intracellular domain with the tyrosine kinase activity. IGF1-R binds insulin-like growth factor-1 (IGF1) with high affinity and insulin-like growth factor-2 (IGF2) with much lower affinity. High levels of circulating IGF1 detected in patients with lung cancers (12,13) suggested that the IGF1/IGF1-R pathway could be involved in the pathogenesis of this disease. IGF1 is a potent stimulator of mitogenesis (14,15) and can promote cellular differentiation and transformation (16-18). Furthermore, IGF1 is a major survival factor and protects cells from apoptosis under a wide variety of circumstances (19,20), including cytotoxic agents (21-25), overexpression of *myc* (26), caspase activation (27), irradiation with UV-B (28), brain ischemia (29) and serum withdrawal (27,30-33).

Cross talk between the IGF1-R, the EGFR and their ligands has been reported. Stimulation of IGF1-R signaling interferes with the antitumoral activity of EGFR inhibitor (34) or of Trastuzumab, a monoclonal antibody used in clinic to inhibit the growth of erbB-2/neu expressing tumors (35). In addition, prolonged activation of the intracellular kinase ERK2 by EGF in mouse embryo fibroblasts requires a functional IGF1-R (36). Moreover, stimulation of the IGF1-R intracellular pathway was abolished by the blockade of EGFR in several cell systems (37-39). In keratinocytes, IGF1 induces the production and secretion of TGF $\alpha$  and AR (37) and in COS-7 cells, stimulation of the IGF1-R transactivates the EGFR, *via* a mechanism involving matrix metalloprotease-dependent release of HB-EGF (39). Thus IGF1/IGF1-R pathway transactivate the EGFR *via* an autocrine release of EGF-like factors (37,39).

Although the role of IGF1-R and EGFR pathways in growth and survival of lung cancer cells is well understood, their mechanism of action and cooperation are poorly understood. In this report, we analyzed the inhibition of serum deprivation apoptosis in two NSCLC cell lines H358 and H322 and showed that a reciprocal activation of AR and IGF1 pathways

induced cell survival. Indeed, we show here that AR phosphorylates the IGF1-R and that the subsequent activation of IGF1-R pathway induces the secretion of AR and IGF1. Subsequent cross talk between those growth factors and receptors activates a survival pathway in NSCLC cell lines.

## **EXPERIMENTAL PROCEDURES**

### ***Cell culture, serum withdrawal and H358 conditioned medium***

The human H358, H322, A549, HEL, K562 and Jurkat cell lines were cultured in RPMI 1640 medium; H69, H1417 and H2170 cells in RPMI medium supplemented with glucose 2.5  $\mu\text{g/l}$ , HEPES 10 mM, Na pyruvate 1 mM; CALU-1 and SAOS cells in McCoy's medium; MCF7 and HELA cells in DMEM medium; H810, H720 and H2106 cells in Hites medium; NHBE cells in SAGM medium. Media were supplemented with 10 or 5 % heat-inactivated fetal calf serum (FCS) and cells were cultured at 37 °C in a humidified atmosphere of 5 % CO<sub>2</sub>. All products were purchased from Gibco (Cergy Pontoise, France).

For serum deprivation, cells were rinsed once in PBS and cultured for at least 8 h in serum-free medium, which was then changed and culture continued in serum-free medium or in serum-free conditioned medium for the desired time. Conditioned media (CM) from cells were collected 72 h after serum-free culture, centrifuged (5000g) at 4 °C and stored at -80 °C until use.

### ***Drug treatment and immunoneutralization***

Tyrphotins AG556, AG1024 or AG1295 (Alexis Biochemical, Laufelfingen, Switzerland), quinazolin ZD1839 (Iressa, kindly provided by Dr R. Defrance, Astra-Zeneca, Rueil-Malmaison, France), CRM197 (Sigma-Aldrich, Saint Quentin Fallavier, France) were dissolved in DMSO; tyrphotin AG825 (Alexis Biochemical) in DMSO/methanol (v/v); 1,10-phenanthroline (Sigma-Aldrich) in ethanol and keep at -20 °C until use. AR and IGF1 recombinant proteins (Sigma-Aldrich) were dissolved in fresh medium just before use.

Anti-human EGF or AR mouse monoclonal, anti-human TGF $\alpha$ , betacellulin or IGF2 goat polyclonal (R&D Systems Europe, Ltd., Abingdon, UK) or anti-human IGF1 goat polyclonal (Oncogene Research Products, Fontenay sous Bois, France) neutralizing antibodies were

incubated overnight at 4 °C in H358 CM. The immunoneutralized H358 CM was then added to H322 cells for 96 h.

### ***Quantification of apoptotic cell death***

The morphological changes related to apoptosis were assessed by fluorescence microscopy after Hoechst 33342 (5 g/ml) staining of cells (Sigma- Aldrich) and the percent of apoptotic cells was scored after counting at least 200 cells.

The proteolytic activation of pro-caspase-3 was evaluated by Western Blotting: cells were washed twice in PBS and incubated in lysis buffer (Tris-HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 1 mM, Nonidet P-40 0.5 %, NaF 1 mM, Na<sub>3</sub>VO<sub>4</sub> 1 mM, phenylmethylsulfonyl fluoride (PMSF) 0.5 mM, leupeptin 10 µg/ml, aprotinin 10 µg/ml and pepstatin 10 µg/ml) for 30 min on ice. Protein content was assessed by the Bio-Rad D<sub>C</sub> Protein Assay kit (Bio-Rad Lab., Ivry sur Seine, France). Cell lysates (40 µg of protein) were subjected to polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane (PVDF, Millipore, Saint Quentin les Yvelines, France). After blocking nonspecific binding sites with 5 % nonfat milk in TPBS (PBS, Tween 20 0.1 %) for 30 min at room temperature, the membrane was incubated overnight at 4 °C with primary anti-human caspase-3 rabbit polyclonal antibody 1:1000 (Pharmingen, Becton Dickinson, Le Pont de Claix, France), diluted in TPBS. To ensure equal loading and transfer, membranes were also probed for actin using anti-actin rabbit polyclonal antibody 1:3000 (Sigma-Aldrich). The immunoreactive proteins were visualized using horseradish peroxidase conjugated goat anti-mouse antibody diluted 1:10000 in TPBS and enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Orsay, France).

In some experiments, active caspase-3 was detected by flow cytometry using phycoerythrin (PE) -conjugated monoclonal active caspase-3 antibody kit (Becton Dickinson

PharMingen) following the manufacturer's instructions. Analysis was performed on a Becton Dickinson FACScan flow cytometer. Red fluorescence (PE, FL-2) was detected at 575 nm.

Cellular DNA was extracted by a salting-out procedure and detected after agarose gel electrophoresis.

### ***IGF1-R immunoprecipitation***

Cells were washed twice in PBS and incubated in lysis buffer (Tris-HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 1 mM, Nonidet P-40 0.5 %, NaF 1 mM, Na<sub>3</sub>VO<sub>4</sub> 1 mM, PMSF 0.5 mM, leupeptin 10 µg/ml, aprotinin 10 µg/ml and pepstatin 10 µg/ml for H322 cells; Tris-HCl 50 mM pH 7.4, NaCl 150 mM, Nonidet P-40 1 %, deoxycholate sodium 0.5 %, SDS 0.1 % NaF 1 mM, Na<sub>3</sub>VO<sub>4</sub> 1 mM, PMSF 0.5 mM, leupeptin 10 µg/ml, aprotinin 10 µg/ml and pepstatin 10 µg/ml for H358 cells) for 30 min on ice. Immunoprecipitation of IGF1-R was performed using 1 µg anti-IGF1-R β subunit rabbit polyclonal antibody (Santa Cruz Biotechnology, Tebu, Le Perray en Yvelines, France), overnight at 4 °C with continuous agitation. Immune complexes were collected using 80 µl of protein G-agarose (Sigma Aldrich) following continuous agitation for 1 h at 4 °C. Immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membranes for protein immunoblotting. Nonspecific binding sites were blocked with 3 % BSA in TTBS (TBS, Tween 20 0.1 %) for 30 min at room temperature. Protein tyrosine phosphorylation was detected by 1:1000 dilution of anti-phosphotyrosine mouse monoclonal antibody (Upstate Biotechnology, Euromedex, Mundosheim, France). IGF1-R was detected by 1:1000 dilution of the anti-IGF1-R β subunit rabbit polyclonal antibody. The immunoreactive proteins were visualized using horseradish

peroxidase-conjugated goat anti-mouse 1:20000 or anti-rabbit 1:10000 antibodies in TTBS and ECL (Amersham Pharmacia Biotech.).

### ***AR and IGF1 dosage***

Conditioned media were obtained as previously described from serum-deprived H358 or H322 cells. Cells were plated at a density of  $5 \times 10^6$  cells/flask ( $75 \text{ cm}^2$ ) or  $8 \times 10^5$  cells/well (6 wells plate). AR in the medium was analyzed using specific ELISA. Primary monoclonal anti-human AR antibody (2  $\mu\text{g/ml}$ , R&D Systems) was coated to the bottom of 96-well plates and incubated overnight at room temperature. The wells were washed with TPBS (PBS, Tween 20 0.05 %), blocked by the addition of PBS containing 1 % BSA and 5 % sucrose for 1 h at room temperature and washed again. Solutions containing varying concentrations of biologically active recombinant amphiregulin or assay samples were diluted in buffer (Tris 20 mM pH 7.3, NaCl 150 mM, BSA 0.1 %, Tween 20 0.05 %) and incubated 2 h at room temperature. The wells were washed prior to incubation with a mixture of the secondary biotinylated anti-human amphiregulin antibody (25 ng/ml, R&D Systems) and horseradish peroxidase-conjugated avidin 1:250 in buffer. Following incubation for 2 h at room temperature, the wells were incubated with substrate reagents A (cat. 2606KC) and B (cat. 2607KC) (1:1 ratio, Becton, Dickinson, PharMingen) 30 min in the dark. Adding  $\text{H}_2\text{SO}_4$  1M stopped the reaction and optical density was measured at 450 nm with an ELISA plate reader. IGF1 level in culture medium was analyzed by radioimmunoassay using the IGF1 extraction kit (Nichols Institute Diagnostics) following the manufacturer's instructions.

## RESULTS

### **H358 NSCLC cells are resistant to serum deprivation apoptosis and secrete anti-apoptotic factors**

After 96 h of culture in serum-free medium, H322 cells underwent apoptosis as revealed by morphological change (figure 1A). These changes coincided with DNA fragmentation visualized after agarose gel electrophoresis (figure 1B) and proteolytic cleavage of pro-caspase-3 in its p12 active fragment (figure 1C). Conversely, no evidence of cell death was detected in H358 cells, even after 120 hours of serum deprivation (figure 1A-C).

In order to determine whether the resistance of H358 cells was due to the constitutive activation of a survival pathway or whether those cells secrete anti-apoptotic factors, serum-free conditioned medium from H358 cells (H358 CM) was collected and used for culturing H322 cells. H358 CM inhibited apoptosis of H322 cells, evaluated by the absence of morphological changes or cleavage of caspase-3, after 96 h of culture (figure 1D). This effect was dose-dependent (data not shown).

### **Inhibition of serum deprivation apoptosis in H322 and H358 NSCLC cell lines by EGF and IGF1 receptors**

Human lung cancer cells are known to express EGFR, erbB-2/neu, IGF1-R and platelet-derived growth factor (PDGFR) receptors. We first considered the possible involvement of these receptors in the survival of H322 cells cultured in H358 CM. Serum-deprived H322 cells were incubated in medium supplemented with 10 % serum, in serum-free medium or in H358 CM in the presence of several highly specific inhibitors (figure 2). Tyrphostin AG825 and AG1295, which inhibited respectively the erbB-2/neu and the PDGFR, did not modify the survival of H322 cells cultured in presence of serum, in serum-free medium or in H358 CM

(figure 2A, B). The IGF1-R inhibitor tyrphostin AG1024 (1  $\mu$ M) did not modify apoptosis in H322 cells cultured in the presence or in the absence of serum, but restored, in a dose dependent manner, apoptosis when cells are cultured in H358 CM (figure 2C). Both EGFR inhibitors tyrphostin AG556 (20  $\mu$ M) and quinazolin ZD1839 (500 nM) did not modify apoptosis of the cells in the presence of serum, increased the level of apoptosis of the cells when cultured in serum-free medium and restored apoptosis in H322 cells cultured in H358 CM (figure 2D, E).

We then analyzed the survival of H358 cells cultured in serum-free medium in presence or absence of the same inhibitors (Table 1). In presence of serum none of the inhibitors induced apoptosis of H358 cells. In contrast, the EGFR inhibitor tyrphostin AG556 (10  $\mu$ M) as well as the IGF1-R inhibitor tyrphostin AG1024 (10  $\mu$ M) restored H358 cells apoptosis when cultured in serum-free medium (Table 1). Conversely, concentration of up to 20 or 40  $\mu$ M of the erbB-2/Neu inhibitor tyrphostin AG825 and the PDGFR inhibitor tyrphostin AG1295 had no effect on H358 cell survival (Table 1). These results were confirmed by a western analysis of proteolytic activation of pro-caspase-3 in its p12 active fragment (figure 3).

Altogether, these data highly suggested that H358 cells secreted autocrine/paracrine factors activating the IGF1-R and EGFR in H358 and H322 cells.

### **AR and IGF1 inhibit serum deprivation apoptosis of H358 and H322 cells**

In order to identify factors of the EGF and IGF families involved in the anti-apoptotic activity of H358 CM, we used neutralizing antibodies. The anti-apoptotic activity of H358 CM was completely abolished by a pre-incubation with the anti-AR neutralizing antibody and to a lower extent (about 50 %) by the anti-IGF1 antibody (Table 2). In contrast, pre-

incubation of H358 CM with anti-EGF, anti-TGF $\alpha$ , anti-betacellulin, anti-IGF2 antibodies, or with CRM197, which neutralized the HB-EGF, did not alter anti-apoptotic activity of H358 CM (Table 2). These results were confirmed by treatment of H358 cells with an antisense oligonucleotide directed against the AR mRNA, which significantly restored apoptosis of H322 cells cultured in H358 CM (not shown).

We then quantified AR and IGF1 secreted by H358 cells cultured in the absence of serum (Table 3). The level of IGF1 secreted by H358 cells was not significantly different than those secreted by other cancer cell lines from lung or other origins, but the level of AR was very high. Furthermore, H322 cells secreted IGF1 but AR was below detectable levels (Table 3).

We then used AR or IGF1 recombinant proteins to analyze inhibition of H322 cells serum deprivation apoptosis. When AR or IGF1 were used as single agent, the apoptosis inhibition was dose-dependant and observed only at high concentrations (figure 4). In contrast, when AR and IGF1 proteins were used in combination, the maximal apoptosis inhibition observed in H322 cells cultured in serum-free medium was reached with 5 ng/ml of AR and 1 ng/ml of IGF1, the concentrations present in H358 CM (figure 4).

These data confirmed that both AR and IGF1 are involved in the anti-apoptotic activity of H358 CM and suggested a cooperation between the growth factors to inhibit serum deprivation apoptosis in the two cell lines.

### **Secretions of AR and IGF1 are induced by the IGF1-R**

The release of soluble EGF-like growth factors involves proteolysis mediated by matrix metalloproteases (40,41) and the IGF1-R (37,39). In order to determine the mechanism by which a large quantity of AR is released by H358 cells, we assessed whether treatment with the IGF1-R inhibitor AG1024 or the metalloproteases inhibitor 1,10-phenanthroline affected the AR release by serum-deprived H358 cells. As shown in figure 5, incubation with AG1024

or 1,10-phenanthroline both markedly decreased AR level detected in the serum-free culture medium. In contrast, incubation of H358 cells with 1,10-phenanthroline had no effect on IGF1 level, but AG1024 decreased IGF1 release in the serum-free culture medium (figure 5).

The data suggested that the IGF1-R pathway regulated the AR secretion through metalloproteases activation. Furthermore, the IGF1-R was also involved in IGF1 release.

### **AR activates the IGF1-R in H322 cells**

We then analyzed the activation of the IGF1-R in H322 cells. The IGF1-R was unphosphorylated in H322 cells cultured in serum-free medium (figure 6A, B). As expected, IGF1 induced the tyrosine phosphorylation of its receptor. Moreover, we observed the phosphorylation of the IGF1-R on H322 cells after 30 min of incubation in H358 CM (figure 6A). This phosphorylation was prevented when H358 CM was pre-incubated with the anti-AR neutralizing antibody (figure 6B). Moreover, incubation of H322 cells for 30 min with AR recombinant protein in serum-free medium induced the phosphorylation of the IGF1-R (figure 6B). Thus, in H322 cells, AR activated the IGF1-R. Accordingly, H358 cells cultured in serum-free medium, which secreted IGF1 and high amount of AR, presented a constitutive phosphorylation of the IGF1-R (figure 6C).

## DISCUSSION

We have shown here that the reciprocal activation of AR and IGF1 pathways induced inhibition of serum deprivation apoptosis in NSCLC cell lines H358 and H322. We demonstrated, for the first time to our knowledge, that AR activated the IGF1-R, which in turn induced the secretion of AR and IGF1. Thus, AR can inhibit apoptosis in NSCLC cells through an IGF1-R dependent pathway.

AR, an EGF-related growth factor produced by epithelial cells, functions as an autocrine/paracrine cell proliferation factor (42,43). Several lines of evidence suggest that AR is an autocrine growth factor in human carcinoma cells (44-51) and tumorigenicity of a human breast epithelial cell line is abolished by treatment with AR antisense (49). A protective role of the EGF-like growth factors against apoptosis has been demonstrated in several non-transformed and tumor cell types (52-56). Accordingly, anti-apoptotic activity of AR has been suggested by a study in human breast carcinoma cells (48). AR is overexpressed in a significant percentage of tumorigenic human lung epithelial cells (7). Moreover, an extensive analysis of the expression of EGF-related peptides and receptors in a large group of 195 stage I-IIIa NSCLC patients showed that enhanced AR expression was the only variable to be significantly correlated with a reduced overall survival (6). We confirm here that AR could be a potent inducer of NSCLC cells autonomous survival.

IGF1 and its receptor the IGF1-R contribute to carcinogenesis. The IGF1-R effects several growth-promoting functions: it stimulates mitogenesis (14,15), promotes cellular transformation (16,18) and protects cells from apoptosis (19-33); a dominant-negative IGF1-R mutant induced extensive apoptosis *in vivo* and inhibited growth *in vitro* of rat glioblastoma C6 cells (57). We confirmed in this study that the activation of the IGF1/IGF1-R pathway inhibits NSCLC cell apoptosis.

Known EGFR ligands, including AR, are synthesized as cell surface precursor proteins and soluble growth factors are produced by proteolysis (40,41). Activation of the IGF1-R pathway induce the secretion of soluble EGF-related peptide such as AR and TGF $\alpha$  in keratinocytes and HB-EGF in COS-7 cells that in turn activate the EGFR (37,39,58). We showed here that inhibition of the IGF1-R or the metalloproteases on H358 cell line decreased the level of secreted AR, suggesting that the IGF1-R increased the shedding of pro-AR from H358 cell surface by metalloprotease activation, as described for HB-EGF (39). Whether or not this mechanism is sufficient to explain the very high level of AR secreted by H358 cells is under investigation. Our results showed also here that AR stimulated the IGF1-R which in turn induced the release of AR, an amplification phenomenon which could also explain the high levels of AR secreted by H358 cells. Furthermore, H358 cells express a mutated constitutively active Ras protein (59) and Ras activation has been shown to increase the production of EGF-related peptides (60,61). Accordingly, H322 cells, which did not secrete AR, express a wild-type Ras protein (59).

AR is known to elicit its biological activity through binding and activation of the EGFR. We demonstrated that AR can also induce the phosphorylation of IGF1-R. Furthermore, in H322 cells, the anti-apoptotic activity of H358 CM was strongly inhibited by both an IGF1-R inhibitor and anti-AR neutralizing antibody, although EGFR inhibitor and anti-IGF1 neutralizing antibody were less effective. Altogether, these data suggested that in presence of the two growth factors and their receptors, the anti-apoptotic effect is preferentially mediated by AR and the IGF1-R pathway. The activation of IGF1-R by EGF-related peptides has never been demonstrated although it was suggested in results from Swantek *et al.* in mouse embryos fibroblastic cells. These authors showed that EGF induced a prolonged activation of ERK2 intracellular kinase in cell containing functional IGF1-R, but only a weaker and transitory

activity of the kinase in cells that do not express IGF1-R or that express a mutated form of the receptor. Transfection of cells with IGF1-R restored the capacity of EGF to induce prolonged activation of ERK2 (36), thus suggesting that the IGF1-R was required to transduce the EGF-mediated signal.

Whether AR can activate the IGF1-R by a direct or an indirect interaction is under investigation. Interaction of AR with the IGF1-R could be direct or due to oligomerization of AR and IGF1 before binding to the receptor. AR could also transactivate indirectly the IGF1-R after binding on its specific receptor, the EGFR. Although transactivation of IGF1-R by EGFR activation has never been described before, cases of transactivation of erbB family receptors by IGF1-R have been observed (37-39). Moreover, in glioblastoma, IGF1-R signaling is involved in resistance mechanisms to anti-EGFR therapy (34) and, in breast cancer cell models that overexpress erbB-2/neu, an increased level of IGF1-R signaling appears to interfere with the action of Trastuzumab, an anti-erbB2/neu antibody (35). By using combinations of antisense oligonucleotides directed against erbB-2 mRNA and IGF1-R, it has also been shown that IGF1-R directs erbB2 phosphorylation (62); this interaction involves physical association of both receptors and the formation of an heteromeric complex. Similar interaction between EGFR and IGF1-R has never been described.

Emergence of a tumoral clone resulted from accumulation of abnormalities resulting to a prolonged survival and a stimulation of growth and mobility. We previously showed that H358 NSCLC cells presented an increased resistance to p53 and BAX-mediated apoptosis (63). Resistance to apoptosis in H358 cell line is linked to conformational abnormalities of the BAX protein and an inhibition of its translocation from the cytosol to the mitochondria; this phenomenon is reversed by treatment with caffeine. In this report, we characterized another original mechanism of apoptosis inhibition in NSCLC cells. It remains to be determined if

there is a link between both these observations and, in particular, if the activation of the IGF1-R pathway by AR can modify the phosphorylation of the BCL2 family members and inactivate BAX.

In conclusion, secretion of growth factors, in particular EGF-related peptides, by human lung epithelial cells is well established, but the consequences of the coexpression of EGFR and its ligands by malignant cells on lung tumor progression are still controversial as it is for IGF1-R expression. Our study, which demonstrates for the first time the activation of IGF1-R by AR, in addition to the stimulation of AR release by IGF1-R activation, is a very important step in the elucidating the involvement of AR and IGF1 in lung tumor development and progression. Further studies on the relevance of those growth factors *in vivo* on fresh tumor samples will allow development of a new therapeutic strategy.

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## FOOTNOTES

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<sup>1</sup> The abbreviations used are: AR, amphiregulin; CM, conditioned medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding epidermal growth factor; IGF1, insulin-like growth factor-1; IGF1-R, insulin-like growth factor-1 receptor; IGF2, insulin-like growth factor-2; NSCLC, non-small cell lung carcinoma; PDGFR, platelet-derived growth factor receptor; TGF $\alpha$ , transforming growth factor  $\alpha$ .

## FIGURE LEGENDS

### **Figure 1: Effect of serum withdrawal on apoptosis in H358 and H322 NSCLC cells.**

Serum deprivation induces apoptosis in H322 but not in H358 NSCLC lines. **A:** Kinetics of apoptosis induced by serum withdrawal in H322 (open circles) or H358 cells (filled circles).

Percentage of apoptosis was assessed by fluorescence microscopy after Hoechst staining of the cells. Results are expressed as the mean  $\pm$  SD of three independent experiments. **B:**

Agarose gel electrophoresis of DNA extracted from H322 or H358 cells cultured in RPMI-serum 10 % (10) or in serum-free medium (0). DNA from H358 cells treated for 24 hours with 100 nM staurosporine was used as positive control of apoptotic DNA fragmentation (C+). **C:** Western blot analysis of the p32 pro-caspase-3 and p12 fragment of active caspase-3 in H322 or H358 cells cultured in RPMI-serum 10 % (10) or in serum-free medium (0).

**D:** Conditioned medium from H358 cells protects H322 cells from serum deprivation apoptosis. Apoptosis in H322 cells was detected by two methods: detection of the p12 active fragment of caspase-3 by immunoblotting or after counting Hoechst stained cells cultured in RPMI-serum 10 % (10) or serum-free medium (0) or in serum-free conditioned medium from H358 cells (CM).

Percentages of apoptosis are expressed as the mean  $\pm$  SD of three independent experiments.

\*\*\*: Statistically more than H358 CM;  $p < 0.01$ .

### **Figure 2: Effect of tyrosine kinase receptors inhibition on serum deprivation apoptosis in H322 cells.**

H322 cells were cultured in RPMI medium supplemented with 10 % serum (10), serum-free medium (0) or conditioned medium from H358 cells (CM). The activity of indicated concentrations of AG825 (**A**), AG1295 (**B**), AG1024 (**C**), AG556 (**D**), and ZD1839 (**E**) was

analyzed. Apoptosis was analyzed by fluorescence microscopy after Hoechst staining of cells and by detection of the p12 active fragment of caspase-3 by immunoblotting. Percentage of apoptosis detected in serum-free medium condition (0) was normalized at 100. Apoptosis was expressed as a rate of apoptosis in serum-free medium and as the mean  $\pm$  SD of three independent experiments.

**Figure 3: Effect of tyrosine kinase receptor inhibition on serum deprivation apoptosis in H358 cells.**

Western blotting analysis of pro-caspase-3 proteolytic activation in its p12 active fragment in H358 cells cultured in serum-free medium in the presence of indicated concentrations in  $\mu$ M of tyrosinostats AG556, AG825, AG1024 or AG1295.

**Figure 4: AR and IGF1 recombinant proteins inhibit serum deprived apoptosis in H322 cells.**

H322 cells were cultured in medium supplemented with 10 % serum (10), in serum-free medium (0), in conditioned medium from H358 cells (CM) or in serum-free medium supplemented with the indicated concentrations of AR or IGF1 recombinant proteins. Apoptosis was analyzed by immunolabelling of active caspase-3 and flow cytometry detection. Percentage of apoptosis was expressed as a rate of apoptosis detected in serum-free medium (0), normalized at 100. Data represent the mean  $\pm$  SD of at least three independent experiments. \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; statistically less than serum-free medium condition (0).

**Figure 5: Role of IGF1-R and metalloproteases in AR and IGF1 secretions by serum-deprived H358 cells.**

Kinetics of AR (left panel) or IGF1 (right panel) release were measured in H358 conditioned medium using ELISA detection.  $8 \times 10^5$  cells were incubated for the times indicated in serum-free medium (0 % serum), in serum-free medium supplemented with AG1024 1  $\mu$ M or 1,10-phenanthroline 100  $\mu$ M. Data were expressed as the mean  $\pm$  SD of three independent experiments.

**Figure 6: Effect of H358 conditioned medium, AR and IGF1 on IGF1-R tyrosine phosphorylation.**

Serum-deprived H322 cells were incubated: **A**, for 30 minutes in serum-free medium (-), in serum-free medium with IGF1 50 ng/ml (IGF1), or for indicated times in serum-free H358 CM (CM), **B**, for 30 minutes in serum-free medium (-), in H358 CM (CM), in H358 CM pre-incubated with anti-AR neutralizing antibody (CM + Ac  $\alpha$ AR), or in serum-free medium with 50 ng/ml of IGF1 (IGF1) or AR (AR), prior to detergent lysis and immunoprecipitation of the IGF1-R  $\beta$  subunit. **C**, serum-deprived H358 cells were incubated for 30 minutes in serum-free medium (-), in H358 CM (CM), or in serum-free medium with 50 ng/ml of IGF1 (IGF1) or AR (AR), prior to detergent lysis and immunoprecipitation of the IGF1-R  $\beta$  subunit.

Tyrosine phosphorylation was visualized by protein immunoblotting with monoclonal anti-phosphotyrosine IgG as described (upper lane). Equal loading of immunoprecipitated proteins was confirmed by reprobing immunoblots with polyclonal anti-IGF1-R  $\beta$  subunit antibody (lower lane). IgG: immunoglobulin control for immunoprecipitation. Data are representative of at least three separate experiments.

**Table 1: Effect of tyrosine kinase receptors inhibition on serum deprivation apoptosis in H358 cells.**

The activity of tyrosine kinase inhibitors AG556, AG1024, AG825 or AG1295 on apoptosis in H358 cells cultured in medium supplemented (10 % serum) or not (0 % serum) with serum was analyzed. Apoptosis was detected by fluorescence microscopy after Hoechst staining of cells. Percentage of apoptosis was expressed as the mean  $\pm$  SD of at least three independent experiments.

**Table 2: Effect of neutralization of the members of the EGF and IGF families on the anti-apoptotic activity of H358 conditioned medium.**

H358 conditioned medium (CM) was pre-incubated with neutralizing antibodies, before being added to serum-deprived H322 cells for 96 h. Apoptosis was detected by fluorescence microscopy after Hoechst staining of cells. Percentage of apoptosis was expressed as a rate of serum deprivation apoptosis in H322 cells. Data represent the mean  $\pm$  SD of at least three independent experiments.

ND50: neutralizing dose 50 given by the manufacturer, Ig: immunoglobulin used as control, BTC: betacellulin.

**Table 3: Detection of AR and IGF1 in cell culture mediums.**

AR and IGF1 concentrations were measured in H358, H322 or other cell lines conditioned media using ELISA detection. Five millions of cells were incubated during 72 h in serum-free medium. Data were expressed as the mean  $\pm$  SD of at least three independent experiments.

NSCLC: non small cell lung cells, SCLC: small cell lung cells, LCNEC: large cell neuroendocrine lung cells.

**Table 1**

<b>Receptor</b>	<b>Inhibitor</b>	<b>Concentration (<math>\mu</math>M)</b>	<b>Apoptosis 10 % serum</b>	<b>Apoptosis 0 % serum</b>
	Control		0.9 $\pm$ 0,1	1.9 $\pm$ 1,3
<b>EGFR</b>	AG 556	2		4.1 $\pm$ 0,0
		10	2.2 $\pm$ 1.3	<b>46.5 <math>\pm</math> 14.0</b>
<b>IGF1-R</b>	AG 1024	5		11.2 $\pm$ 3.3
		10	1.7 $\pm$ 1.2	<b>29.3 <math>\pm</math> 0.2</b>
<b>Erb-B2/Neu</b>	AG 825	10		4.0 $\pm$ 0.0
		20	2.3 $\pm$ 1.2	4.4 $\pm$ 0.6
<b>PDGFR</b>	AG 1295	40	0.9 $\pm$ 0.8	4.7 $\pm$ 1.2

**Table 2**

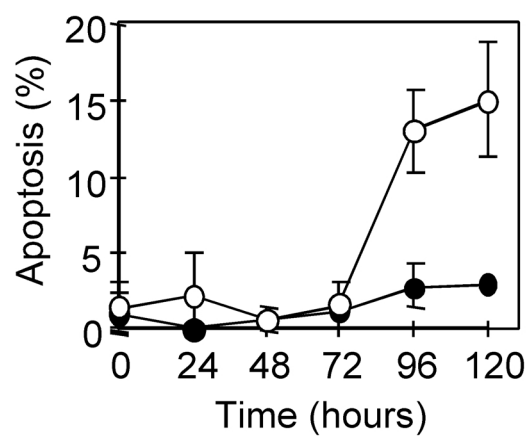
	<b>ND50 (<math>\mu\text{g/ml}</math>)</b>	<b>Used concentrations (<math>\mu\text{g/ml}</math>)</b>	<b>Apoptosis (% / Control)</b>
<b>0 CM</b>			100 7.1 $\pm$ 8.8
<b>CM + Ig</b>			3.4 $\pm$ 6.6
<b>CM +</b>			
<b>anti-EGF Ab</b>	0.04-0.08	up to 5	4.4 $\pm$ 6.2
<b>anti-TGF<math>\alpha</math> Ab</b>	0.2-0.4	up to 5	11.3 $\pm$ 10.8
<b>anti-AR Ab</b>	5-15	10 15	<b>45.9 <math>\pm</math> 8.6</b> <b>104.2 <math>\pm</math> 16.7</b>
<b>CRM197 (HB-EGF)</b>	10	10 20	16.9 $\pm$ 15.0 22.1 $\pm$ 19.7
<b>anti-BTC Ab</b>	0.04-0.08	up to 5	17.3 $\pm$ 14.5
<b>anti-IGF1 Ab</b>	2-5	5 10	<b>47.7 <math>\pm</math> 10.0</b> <b>55.1 <math>\pm</math> 19.3</b>
<b>anti-IGFII Ab</b>	5-15	up to 30	12.2 $\pm$ 10.6

**Table 3**

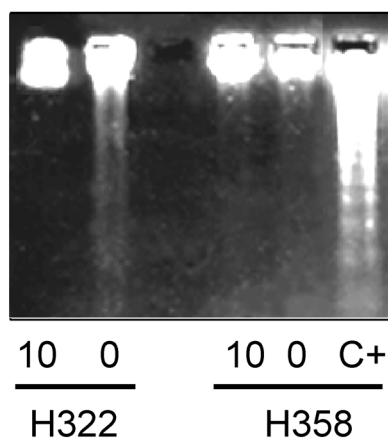
<b>Cell lines</b>		<b>AR pg/ml</b>	<b>IGF1 pg/ml</b>
<b>H358</b>	NSCLC	5603 ± 1635	920 ± 113
<b>H322</b>	NSCLC	Not detectable	345 ± 361
<b>CALU-1</b>	NSCLC	100 ± 11	810 ± 14
<b>A549</b>	NSCLC	118 ± 17	810 ± 127
<b>H2170</b>	NSCLC	129 ± 13	487 ± 217
<b>H69</b>	SCLC	105 ± 18	690
<b>H1417</b>	SCLC	133 ± 7	70 ± 14
<b>H810</b>	LCNEC	152 ± 10	49 ± 2
<b>H2106</b>	LCNEC		
<b>H720</b>	Human lung atypical carcinoid	195 ± 60	150
<b>NHBE</b>	Normal human bronchial epithelial cells	813 ± 46	60
<b>SAOS</b>	Human osteosarcoma	145 ± 25	245 ± 78
<b>MCF7</b>	Human breast adenocarcinoma	85 ± 12	775 ± 78
<b>HELA</b>	Human cervix adenocarcinoma	178 ± 8	745 ± 149
<b>HEL</b>	Human erythroleukemia	100 ± 12	440 ± 170
<b>K562</b>	Human chronic myelogenous leukemia	82 ± 20	365 ± 21
<b>JURKAT</b>	Human T cell leukemia	124 ± 18	215 ± 92
<b>CHO</b>	Chinese hamster ovary	175 ± 118	805 ± 35

Hurbin A. *et al.*, Figure 1

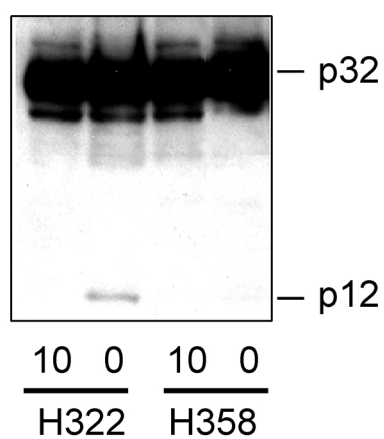
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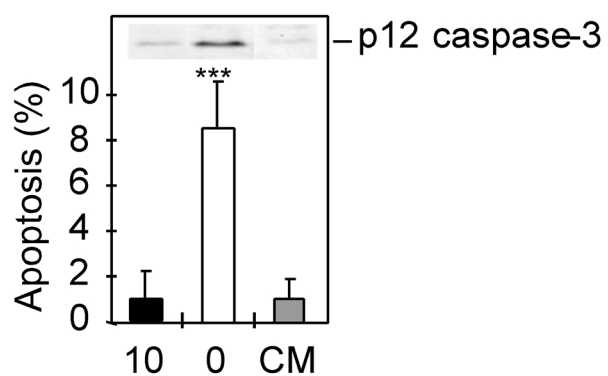
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**C**

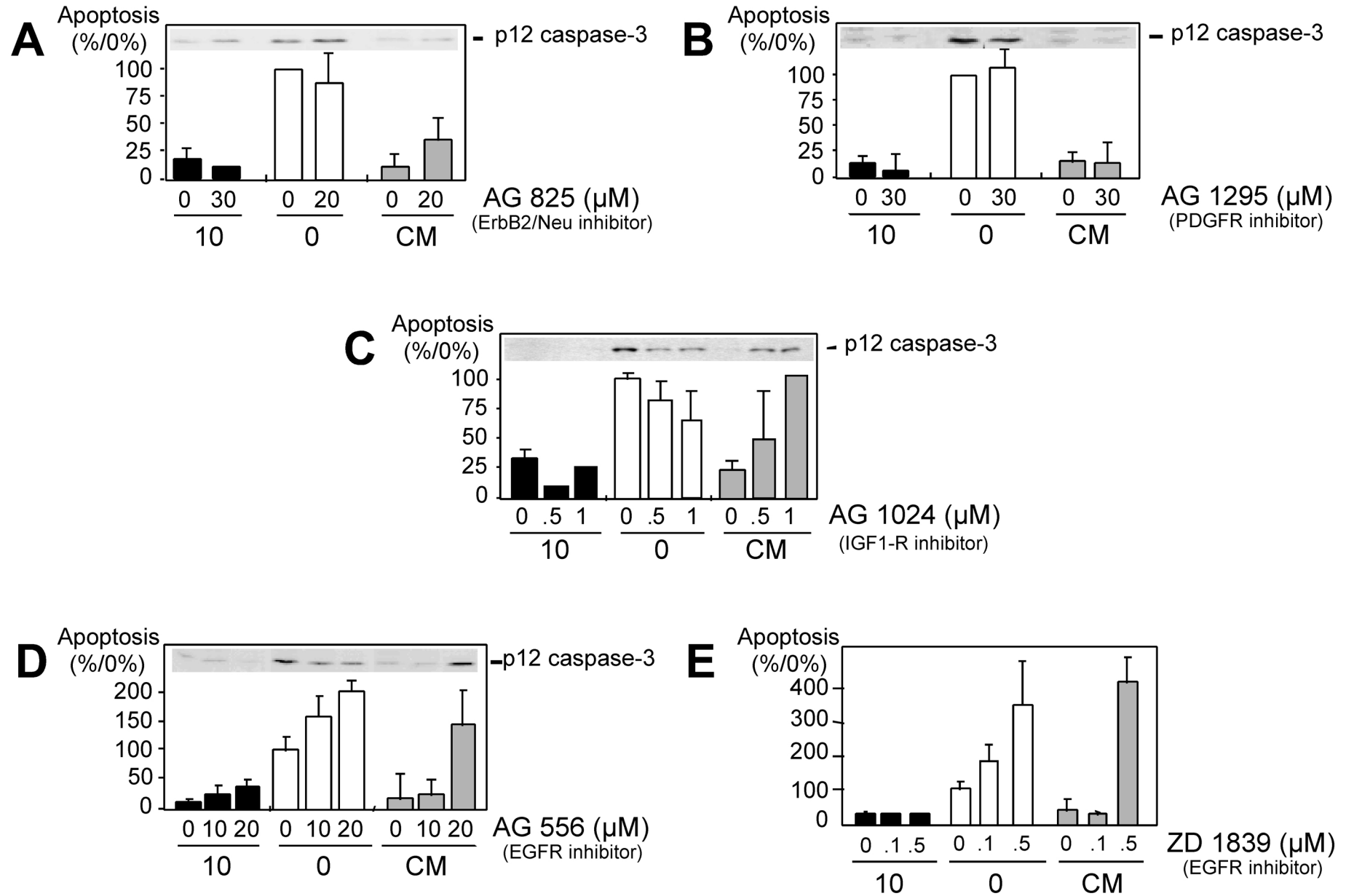


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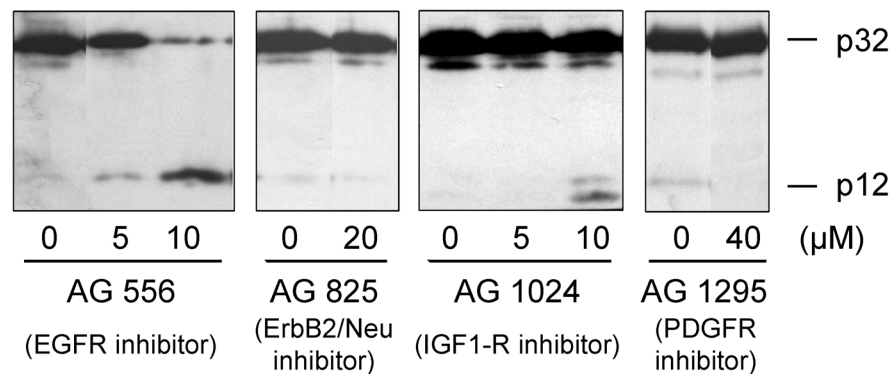
# Hurbin A. *et al.*, Figure 2

H322

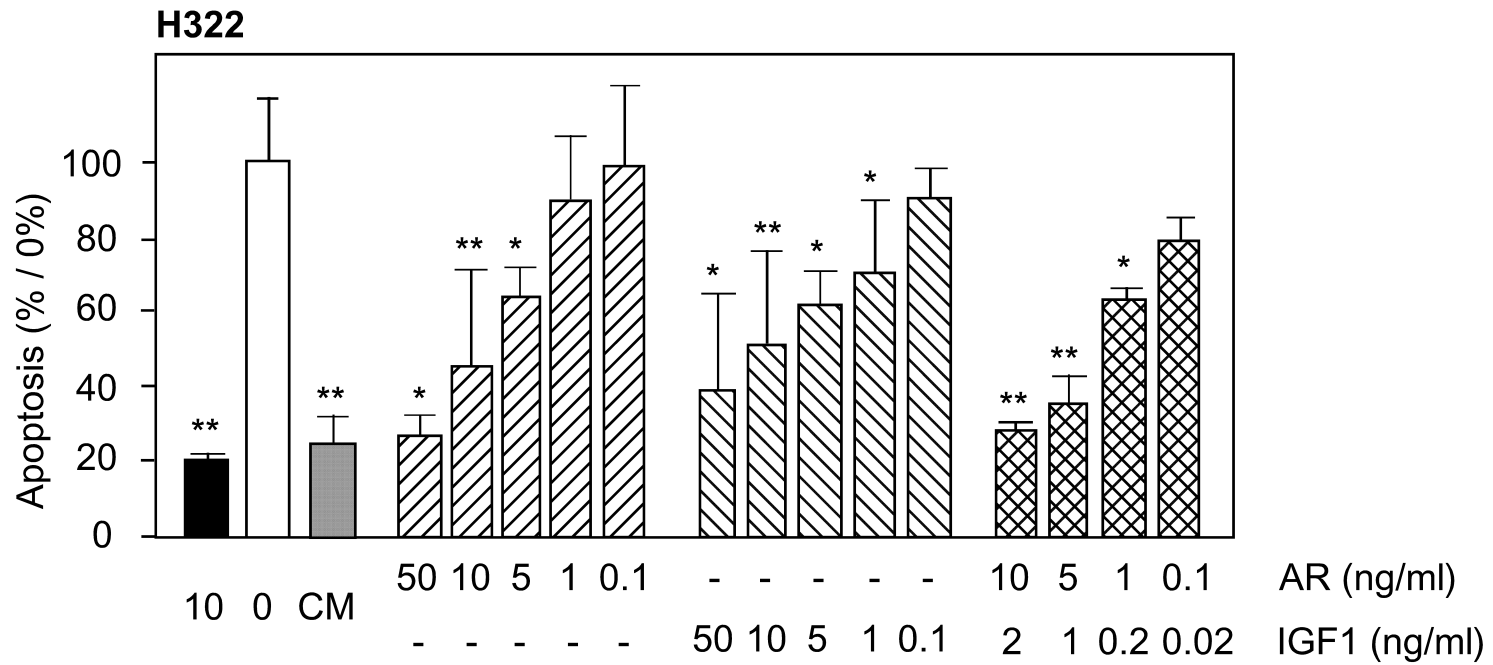


## Hurbin A. *et al.*, Figure 3

### H358

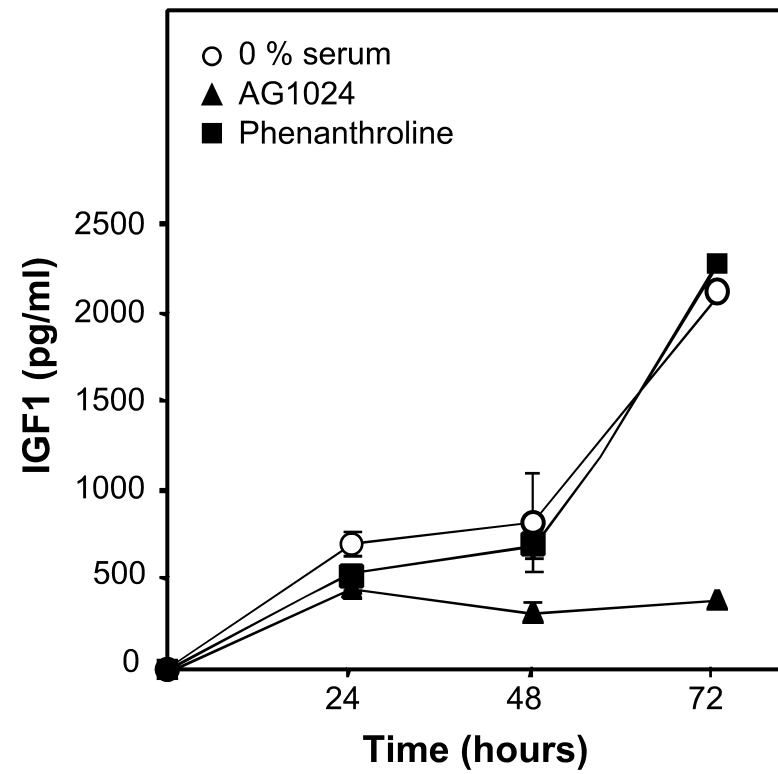
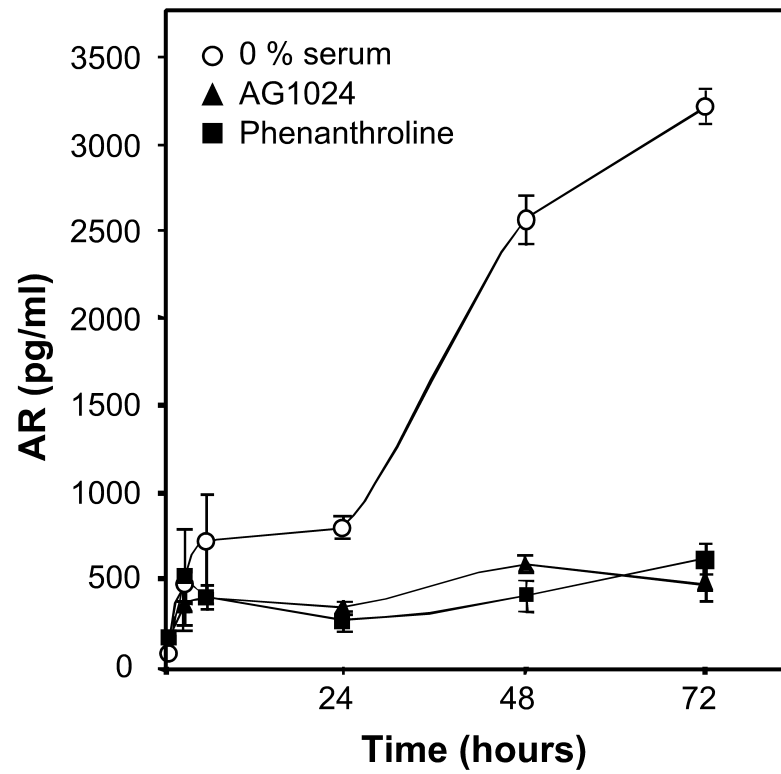


Hurbin A. *et al.*, Figure 4



Hurbin A. *et al.*, Figure 5

H358



Hurbin A. *et al.*, Figure 6

