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Synergic antiproliferative and antiangiogenic effects of EGFR and mTor inhibitors on pancreatic cancer cells

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ABSTRACT

The in vitro efficacy of both EGFR inhibitor gefitinib and mTor inhibitor rapamycin, either administrated alone or in different combination schedules, was analysed in four pancreas cancer cell lines. Both drugs were found to induce cell growth inhibition, apoptosis as well as a slight but stable accumulation of cells in the G0/G1 phase. In all cell lines, neither gefitinib nor rapamycin affected EGFR and the expression of its downstream effectors. By contrast, gefitinib inhibited in a fast and completely way p-EGFR and partially p-Akt while a 3 days-rapamycin exposure resulted in the inhibition of the expression of both mTor and p70S6K. Moreover, after early stimulation, the mTor inhibitor produced a progressive, and almost complete inhibition of p-Akt. The analysis of combined gefitinib and rapamycin administration showed a clear schedule-dependent activity which turned out to be synergic only when gefitinib was given before rapamycin. This synergism seemed to depend on increase of both p-Akt and p70S6K inhibition, the greater the induction of apoptosis, the higher the decrease in cell cycle rate. Moreover, the antiangiogenic activity of the two drugs given in combination was demonstrated by a strong reduction of VEGF release which turned out to be more pronounced in the synergic schedule, and HIF-1α inhibition-independent. Our results suggest that the schedule of gefitinib followed by rapamycin, acting at different levels of the EGFR cellular pathway, could induce antitumor and antiangiogenic effects of clinical interest in the pancreas cancer model.

1. Introduction

Pancreatic cancer is a disease with a very poor prognosis also because conventional chemotherapy with gemcitabine, either administrated alone or in association with other cytotoxic drugs, only modestly improves both response and survival rates [1]. As a consequence, improving our understanding of the molecular events involved in the genesis and progression of pancreatic cancer has become urgent also in a view to identifying potential biomolecular targets for new therapeutic strategies.

Multiple factors have recently been shown to be possible causes of pancreatic tumor genesis and aggressiveness: (i) activating mutations in the small GTPase Ki-ras and/or in various growth factors [2], (ii) high expression of EGFR and of its ligands EGF or TGF-α (detected in more than 90% of human pancreatic cancer cases) [3], (iii) constitutively phosphorylated/active PI3K/Akt/FRAP/mTor/p70s6K pathway often associated with the aberrant expression of PTEN [4–6], (iv) insufficient blood supply, inducing HIF-1 stabilization and activation to transcript several genes such as glucose transport, angiogenic factors [7], etc.

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Several of these biomolecular steps have already been considered as potential pharmaceutical targets. More specifically, a role as potential targets of anticancer therapies has been suggested for both the epidermal growth factor receptor (EGFR) and its downstream effector, mTor, which regulates the translation of some mRNA encoding for proteins driving cell growth, proliferation, and angiogenesis [8].

EGFR, one of the four ErbB (or HER) receptor tyrosine kinases, is responsible for activation of several membrane-nuclear pathway, such as (GTPase)-mediated signal transduction to mitogen-activated protein kinase (MAPK) cascade, G protein-coupled receptor (GPCR)-mediated EGFR transactivation via intracellular Ca^{2+} signaling, phosphatidylinositol polyphosphate (PIP or PI3K/Akt) signaling, etc. In particular, PI3K/Akt signalling has been shown to be involved in many basic cellular functions and there is now strong evidence that the serine/threonine-specific kinase mTor, placed downstream of the PI3K/Akt pathway, is phosphorylated in response to mitogens [9]. mTor acts as a gatekeeper for cell-cycle progression from G1 to S phase by mTor-dependent phosphorylation of p70 and 4E-BP1 [9–11].

In spite of this biological background, EGFR inhibitors have so far shown only modest clinical activity against pancreatic cancer [12,13], thus suggesting that other factors are likely to play a role in this partial failure, including compensatory activation of either downstream pathway effectors or alternative survival pathway [14]. Against this background, the fact that some oral anti-mTor molecules, including rapamycin, everolimus (RAD001), Ariad (AP23573) and temsirolimus (CCI-779), present in phase II/III trials, are giving the first encouraging signs of an antitumour activity [15], has generated an intense interest in these molecules.

In addition, EGFR inhibitor capability to reduce the phenomenon of the paradoxical activation of IGF-I signalling, due to the exposure to mTor inhibitors [16,17], accounts for an additional evidence supporting the need to explore the effects of the interaction between these two drugs.

The possibility that a double inhibition of the EGFR pathway could stress antiangiogenic efficacy, as already reported for gefitinib and rapamycin, should also be taken into account [18,19]. In this prospective the role of HIF-1α modulation could be crucial since it is highly expressed in hypoxic condition and, perhaps more interestingly, in most pancreatic cancer cell lines in normoxic condition [7].

In line with the assumption that combined therapy with more target-oriented drugs, which inhibit different steps of a signal transduction pathway commonly altered in cancer diseases, could have a particularly clinical efficacy, we hypothesized that combining an EGFR inhibitor with a mTor inhibitor is likely to give better clinical results. Preliminary in vitro experiences [20] seem to support our hypothesis and some phase II clinical trials on non-small cell lung cancer, progressive glioblastoma, prostate or breast cancer (http://www.clinicaltrials.gov, [21–23]) are trying to validate in clinical settings.

In this paper and for the first time in pancreatic cancer, we provide preclinical evidence on the in vitro efficacy of combination of an EGFR inhibitor, gefitinib [24,25], with a mTor one, rapamycin [26,27], also stressing the crucial relevance that different schedules of administration of the two drugs could have on downstream effectors.

2. Materials and methods

2.1. Drugs and chemicals

Gefitinib (ZD1839/Iressa) was provided by AstraZeneca Pharmaceuticals (London—U.K.). Stock solutions were prepared at 20 mM in DMSO and stored in aliquots at −20 °C. Rapamycin was provided by Sigma–Aldrich (USA) and dissolved at 200 μg/ml in DMSO, and aliquots were kept at −20 °C. Further dilutions were made in medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 50,000 UL^{-1} penicillin and 80 μM streptomycin.

2.2. Cell lines

Four pancreas cancer cell lines of human origin were used, MiaPaCa-2 and Panc-1 (from pancreas adenocarcinoma), and Capan-1 and AsPC-1 (from metastatic liver and ascites sites). Cells were routinely cultured in DMEM (Capan-1, AsPC-1 and Panc-1) and 20 mM Hepes-RPMI (MiaPaca-2) supplemented with 10% foetal bovine serum, 2 mM glutamine, 50,000 UL^{-1} penicillin and 80 μM streptomycin in a humidified incubator at 37 °C with an atmosphere containing 5% CO_2.

Cells were trypsinized once a week with trypsin/EDTA (0.25%/0.02%) and medium was changed twice a week. Doubling time of MiaPaCA-2 and Panc-1 was 18 ± 1; of Capan-1 was 48 ± 1 and of AsPC-1 was 24 ± 1 h.

2.3. Evaluation of cytotoxicity

Determination of the IC_{50} was performed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay. On day 1, 10,000 cells/well in a volume of 200 μl were plated in 96-wells plates. In each plate, one column contained cells not exposed to drugs (control), and 5 columns contained cells exposed to increasing concentrations of drugs. Each drug or drug combination was repeated in 6 identical wells. On day 2, gefitinib (0.01, 0.1, 1, 10 and 100 μM) or rapamycin (5, 10, 50, 100, and 500 ng/ml) were added with different times of drug exposure (1 and 3 days). For each drug, results were expressed as dose-effect curves with a plot of the fraction of unaffected (surviving) cells versus drug concentration. The IC_{50} was defined as the drug concentration yielding a fraction of affected (no surviving) cells = 0.5, compared with untreated controls and was calculated utilising CalcuSyn ver.1.1.4 software (Biosoft, UK). Each experiment was done in triplicate.

2.4. Cell cycle perturbation

Cells were exposed to gefitinib (1, 3, 5, 10 and 20 μM) or rapamycin (10, 30, 50, 100 and 300 ng/ml) for 1–3 days. Cells were harvested, washed twice in ice-cold PBS (pH 7.4), fixed in 4.5 ml of 70% ethanol at −20 °C, and washed once in ice-cold PBS. The pellet was resuspended in PBS containing 1 mg/ml RNase, 0.01% NP40 and the cellular DNA was stained with 50 μg/ml propidium iodide (Sigma). Cells were stored in ice for 30 min prior to analysis. Cell cycle determinations were performed using a FACScan flow cytometer (Becton Dickinson), and data were interpreted using the CellQuest software, provided by the manufacturer.

2.5. Drugs experimental condition

In the following experiments and when not clearly reported, gefitinib and rapamycin have been utilized at the following concentrations:

Cell lines				
Drug	Panc-1	Capan-1	MiaPaCa-2	AsPC-1
Gefitinib (μM)	7.6	2.1	5.5	6
Rapamycin (ng/ml)	30	100	100	100

2.6. Drug combination studies

To define the best schedule for the combination, either simultaneous or sequential utilisation of the two drugs were tested. Median Drug Effect Analysis [28] was used to determine the interaction between gefitinib and rapamycin, using a fix exposure time of 3 days for each drug. The cells were exposed to the 2 drugs combination using 3 different modalities:

Simultaneous drugs-exposure: gefitinib and rapamycin were given simultaneously for 3 days;
Gefitinib after: rapamycin was given for 3 days, then, after 2 wash steps performed with the medium, gefitinib for 3 days;
Gefitinib before: gefitinib was given for 3 days, then, after 2 wash steps with the medium, rapamycin for 3 days.

Each combination of drugs was tested in 5 scalar drug concentrations, using a constant ratio of 1:1 of the above mentioned concentrations.

Growth inhibition was assayed by the MTT test and the cytotoxicity of the combination was compared with the cytotoxicity of each drug alone in every experiment, and each experiment was performed at least 3 times.

Dose–response interactions (antagonism, additivity and synergism) were expressed as a non-exclusive case combination index (CI) for every fraction affected (FA), using the software CalcuSyn ver 1.1.4 (Biosoft), based on the methods of Chou and Talalay [28].

2.7. Western blot

Proteins were extracted from 3×10^6 cells by sonication in RIPA buffer (0.5 M NaCl, 1% Triton X100, 0.5% NP40, 1% deoxycolic acid, 3.5 mM SDS, 8.3 mM Tris–HCl pH 7.4, 1.6 mM Tris base) and treated with 1 mM PMSF or 20% protease inhibitor cocktail (Sigma, Missouri, USA). Total proteins were measured by Bradford method and 25–50 μg were electrophoretically separated on 10–12.5% acrylamide gel (SDS-PAGE by Laemli). Signal was detected by chemoluminescence assay (ECL-Plus, Amersham Life Science, UK). Expression level was evaluated by densitometric analysis using Quantity One software (Biorad, Hercules, CA) and β-actin expression level was used to normalize the sample values.

2.8. Antibodies

The monoclonal antibody anti-EGFR and the anti-HIF-1α were provided by BD transduction (USA); anti-phosphotyrosine antibody, PY99 (Santa Cruz Biotechnology, USA) was used for the immunoprecipitation assay; anti-AKT, anti-phosphoAKT, anti-ERK1/2 and anti-phospho-ERK1/2 were provided by Cell Signalling, USA. A mouse-HRP and a rabbit-HRP (Amersham Pharmacia Biotech, Upsala, Sweden) were used as secondary antibody.

2.9. Immunoprecipitation assay

Cells were lysed in RIPA-IP buffer (140 mM NaCl, 20 mM Tris/HCl, 10 mM EDTA pH 8, 10% glycerol, 1% NP40, 1 mM Na-deoxycolic acid, 1 mM PMSF) passed through a 22 gauge syringe and cleared by centrifugation at 10,000 × *g* at 4 °C for 10 min. Proteins were immunoprecipitated by incubating 0.1–0.3 mg of total cell lysate with 0.2 μg of anti-phosphotyrosine antibody or EGFR antibody for 1 h at 4 °C. 2–5 μl of protein A/G agarose (Santa Cruz Biotechnology, USA) were incubated overnight at 4 °C. The cell suspension was centrifuged at 2.600 rpm and the pellet was washed three times with PBS and then resuspended in 10 μl of Laemli buffer. Each sample was separated on 10% acrylamide gel and Western blot was performed as described above.

2.10. Analysis of apoptosis

Apoptosis detection was further investigated by the Cell Death ELISA^{PLUS} kit (Roche Molecular Biochemicals, Milan, Italy). The test is based on the detection of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by biotinylated antihistone-coupled antibodies, and their enrichment in the cytoplasm is calculated as the absorbance of sample cells/absorbance of control cells. The enrichment factor was used as a parameter of apoptosis and shown on the *Y*-axis as mean ± S.E. Experiments were performed according to the manufacturer's instructions.

2.11. Analysis of VEGF release

Cells, plated in 24 multiwells plates, were incubated with gefitinib and or rapamycin at the same concentration utilised in Western blot analysis. 200 μl of cell surnatant from each sample (only those from MiaPaCa-2 cells were diluted 1:10) was utilised for VEGF release by the Quantikine—Human VEGF immunoassay (R&D Systems – Minneapolis, USA), following manufacturer's instruction.

3. Results

The activity of gefitinib and rapamycin, either administrated alone or in combination, was analysed in a panel of pancreas cancer cell lines from primary tumour (MiaPaCa-2 and Panc-1) and metastatic sites (Capan-1 and AsPC-1). Starting from the analysis of the phenotypic effects of the drugs, which were assessed in terms of cell growth and apoptosis modulation, the study was further focused on the exploration of the molecular pathways involved through the investigation of drugs-induced cell cycle and EGFR pathway modulation. Moreover, the effects that the combined administration of gefitinib and rapamycin have on VEGF release and HIF-1α

Table 1 – Three days Rapamycin and gefitinib activity evaluation in living cell lines

Cell line	IC50	
	Rapamycin (μg/ml)[a]	Gefitinib (μM)
Miapaca 2	291 ± 2	5.5 ± 0.6
Aspc1	196 ± 4	6 ± 0.3
Panc1	179 ± 5	7.6 ± 0.5
Capan-1	184 ± 6	2.1 ± 0.1

Means ± S.E.M. of three independent experiments.
[a] Extrapolation calculated by CalcuSyn software analysis.

modulation of Panc-1, AsPC-1 and MiaPaCa-2 cells under normoxic condition were also been analysed.

3.1. *Single drug administration*

3.1.1. *Gefitinib*

In order to measure gefitinib capability to inhibit cell growth, all cell lines were incubated for 1 and 3 days with the drug at various concentrations (range 0.01–100 μM); the cell growth inhibition was analysed by MTT assay and the IC_{50} was determined. After 1-day gefitinib exposure, IC_{50} values were higher than 100 μM and, after 3 days of continuous drug exposure, they ranged between 2 and 8 μM (Table 1).

The analysis of gefitinib efficacy in inducing apoptosis showed that, after a 3-day administration, the drug increased the ''apoptosis enrichment factor'' by about 1.5 fold compared to drug-untreated cells (Fig. 1). The analysis of cell cycle modulation by gefitinib, carried out as a function of drug concentrations and of exposure times, showed a slight but progressive cells accumulation in the G0/G1 phase, in all cell lines. This phenomenon, which was drug concentration-dependent, exhibited a peak of about 10% at IC_{50} concentration in each cell lines (Fig. 2), but was observed to be time-independent until day three of treatment.

In each cell line, the gefitinib concentration capable of inducing either cell growth inhibition or cell cycle decrease was lower than the plasma concentration, reported to reach even 10 μM in some clinical studies [29].

In order to determine if gefitinib activity on EGF receptor occurs through the survival and/or the proliferation pathways, it was decided to explore the effect of the drug modulation on Akt and Erk1/2. In all cell lines, the drug did not modulate the expression level of EGFR, Akt and Erk1/2 (data not shown); by contrast, it induced a fast (from 30 min to 8 h) and complete p-EGFR inhibition. This phenomenon was reversible and completely suppressed after 3 days of continuous drug exposure. Moreover, the gefitinib-dependent p-EGFR inhibition caused a progressive and stable-over-time p-Akt inhibition down to 50% after 3 days exposure, whereas the p-Erk1/2 pathway did not seem to be involved. Fig. 3 summarised the main results obtained in MiaPaCa-2 cells, on p-EGFR and p-Akt, representative of those obtained in all cell lines (max variability 10%).

Moreover, in AsPC-1, Panc-1, as well as MiaPaCa-2, gefitinib confirmed its well-known capability of reducing VEGF (already shown in other cancer models [18]) with a 30–50% reduction of the angiogenic factor release after 3 days drug exposure. This latter effect was further increased after 3 days of drug wash out (Fig. 7), while HIF-1α expression resulted to be not modulated by the drug in normoxic conditions (data not shown).

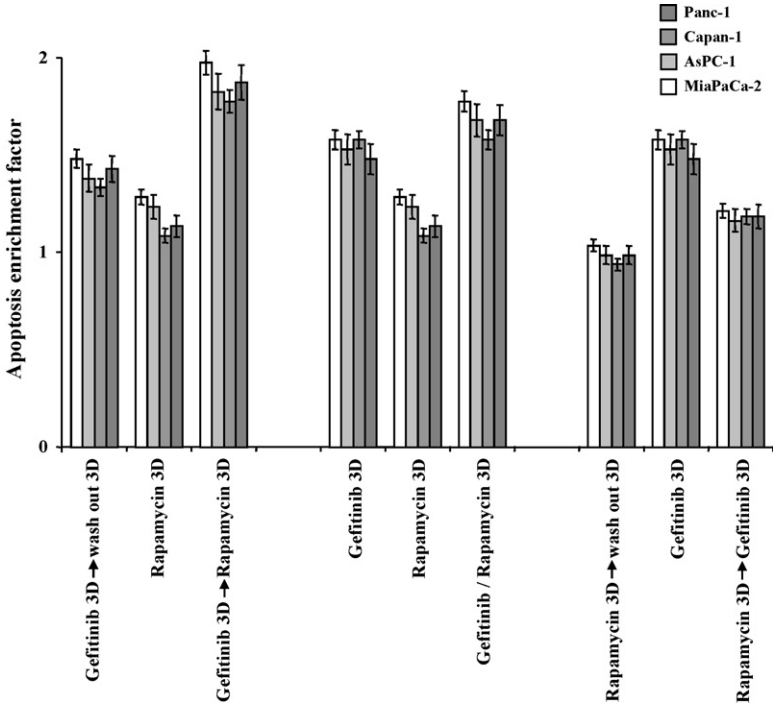

Fig. 1 – Apoptotic effect of gefitinib and/or rapamycin. Cells were treated with each drug alone or in combination for 3 days. Cytoplasmic DNA-histone complex was measured by ELISA. Experiments were performed in triplicate and results are expressed as means ± standard deviation from three experiments.

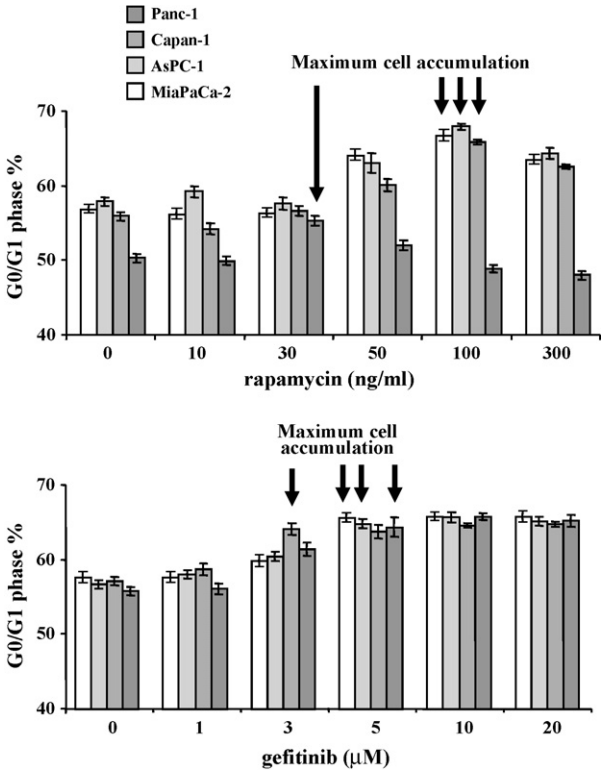

Fig. 2 – Cell cycle modulation. Cells were incubated, for 3 days, with gefitinib (1–20 μM) or rapamycin (10–300 ng/ml) and the cell cycle was analysed by flow cytometry analysis as described in Section 2. Experiments were performed in triplicate and results are expressed as means ± standard deviation from three experiments.

3.1.2. *Rapamycin*

Cells were incubated with rapamycin (from 5 to 500 ng/ml) for 1 and 3 days. After 3 days exposure, IC_{50} values in all cell lines were found to exceed 500 ng/ml and to range between 180 and 300 μg/ml as theoretically assessed by software extrapolation (Table 1).

In all cell lines, rapamycin was capable of inducing apoptosis with an about 1.2 fold increase of the ''apoptosis enrichment factor'' compared to drug-untreated cells (Fig. 1).

The cell cycle analysis showed that the perturbation was rapamycin concentration-dependent with the most pronounced accumulation (+15% compared to control) occurring in the G0/G1 phase at 30 ng/ml in Panc-1 cells and at 100 ng/ml in the other cell lines (Fig. 2). This phenomenon was found not to be time exposure-dependent in the first 3 days of drug-exposure.

These preliminary data have shown 3-days IC_{50} values higher than the rapamycin plasma concentration levels reported in treated patients (about 147 ng/ml) [30]. In order to avoid rapamycin-dependent toxic effects resulting from excessive drug concentration, it was decided to use the max cell cycle perturbation-inducing drug concentration in the subsequent experiments described below.

The analysis of the targets related to intracellular rapamycin's activity (Akt, mTor and p70S6K) showed that the drug was unable to modulate the baseline level of Akt, mTor and p70S6K. Conversely, a partial inhibition (about 50%) of the phosphorylated form of mTor, was observed to occur fast (within 30 min to 1 h) and be followed by a complete recovery of the baseline value after 3 days drug exposure (data not shown); the inhibition of the downstream effector p-p70S6K was observed to be more stable and progressive with a 70% decreased expression after 3 days of drug exposure. To our surprise rapamycin was also able to influence the

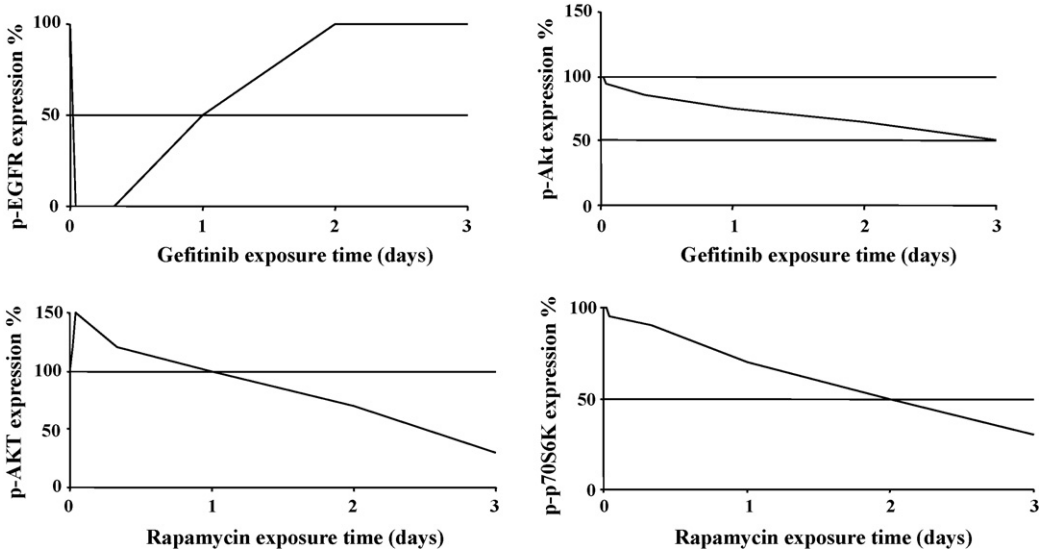

Fig. 3 – EGFR pathway modulation by gefitinib or rapamycin. MiaPaCA-2 cells were incubated with gefitinib 5.5 μM or rapamycin 100 ng/ml and the protein extracts were analysed by immunoprecipitation and/or Western blot. In Western blot, the amount of the different targets was determined using monoclonal or polyclonal specific antibodies and the β-actin was used to normalize the values. The results are shown as plots of p-EGFR/gefitinib dose, p-Akt/gefitinib dose, p-Akt/rapamycin dose, p-p70S6K/rapamycin dose. Experiments were performed in triplicate.

Table 2 – Rapamycin and gefitinib activity in different pancreatic cancer cell lines

Cell line	Gefitinib → Rapamycin		Rapamycin → Gefitinib		Gefitinib + Rapamycin	
	CI	Effect	CI	Effect	CI	Effect
MiaPaCa-2	0.31 ± 0.05	Syn	8.2 ± 0.75	Strong Ant	4.6 ± 1.2	Strong Ant
Panc-1	0.74 ± 0.04	Moderate Syn	>10	Very strong Ant	3.0 ± 1.25	Ant
AsPC-1	0.81 ± 0.95	Moderate Syn	7.6 ± 0.6	Strong Ant	1.05 ± 0.07	Nearly additive
Capan-1	0.77 ± 0.1	Moderate Syn	8.6 ± 0.85	Strong Ant	0.95 ± 0.07	Nearly additive

Means ± S.E.M. of three independent experiments. Syn: synergism and Ant: antagonism.

phosphorylation status of Akt with an early stimulation within 5–15 min (already reported by 13) followed by a progressive inhibition (about 80%) after 3 days of continuous drug exposure. Fig. 3 shows rapamycin-dependent modulation of p-Akt and p-p70S6K in MiaPaCa-2 cells, representative of results obtained in the other cell lines (max variability of 15%).

Moreover, 3-day rapamycin exposure strongly reduced VEGF release (Fig. 7), as already reported by other authors [19], but did not modulate HIF-1α expression (data not shown), in AsPC-1, Panc-1 and MiaPaCa-2.

3.2. *Combined drugs administration*

The next step of the study was the analysis of the effects of gefitinib and rapamycin combination as a function of the administration schedule utilised.

The efficacy of the two drugs in combination was determined adopting three different schedules of administration (''simultaneous'', ''gefitinib before'' and ''gefitinib after''), as described in Section 2. The CI showed the ''gefitinib before'' schedule to be more effective (synergism) than the other two schedules in all cell lines (with only slight differences) while both the ''simultaneous'' and the ''gefitinib after'' schedules resulted in effects ranging from nearly additive or antagonism (Table 2). An example of dose/cell growth inhibition plots obtained in MiaPaCa-2 cells is given in Fig. 4.

The analysis of the ''apoptosis enrichment factors'' in the three drugs combination schedules showed apoptosis to increase in the synergic schedule more than in the additive or antagonist ones (Fig. 1).

Consistently with the results obtained in terms of cell growth inhibition, the analysis of the cell cycle modulation by the two drugs in the various combinatorial schedules showed that only in the synergistic one (''gefitinib before''), an increased accumulation of cells in the G0/G1 phase was evident (ranging between 20 and 35%). By contrast, the other two schedules resulted in a less evident (below 10%) block of cell cycle. Fig. 5 shows the histograms of the MiaPaCa-2 cell cycle modulation obtained by the synergic schedule (''gefitinib before'') and by the two drugs administrated alone.

To explain the kinetic results obtained with the combination of gefitinib and rapamycin, the modulation of the phosphorylated forms of specific targets was analysed, too. In all cell lines and after a fast and complete inhibition, p-EGFR was observed to recover the baseline level with up to 2-day drug exposure; conversely, p-Akt and p-p70S6K were found to be more remarkably reduced in the synergic schedule (50–70% and 40–60%, respectively) compared to the additive or antagonist schedules. Fig. 6 shows the modulation of p-EGFR, p-Akt and p-p70S6K in MiaPaCa-2 cells incubated with the two drugs in the three different schedules.

Finally, the analysis of the antiangiogenic effectiveness of the combined administration of gefitinib and rapamycin showed (i) that the highest decrease in VEGF release was achieved when gefitinib was given before rapamycin (Fig. 7) and (ii) confirmed the failure of the two drugs to modulate HIF-1α expression in pancreatic cancer cell lines (data not shown).

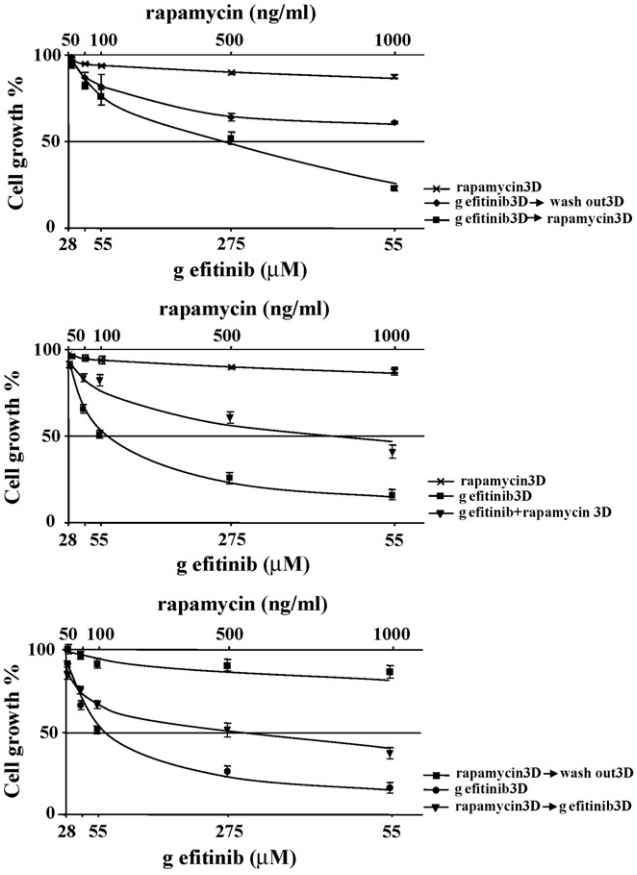

Fig. 4 – Cytotoxicity of gefitinib plus rapamycin. MiaPaCa-2 cells were incubated with gefitinib plus rapamycin alone or in combination, utilising three different schedules, and the survival of cells was determined using MTT assay. The results elaborated by CalcuSyn software are showed as dose/cell growth inhibition plots of the mean of three different experiments.

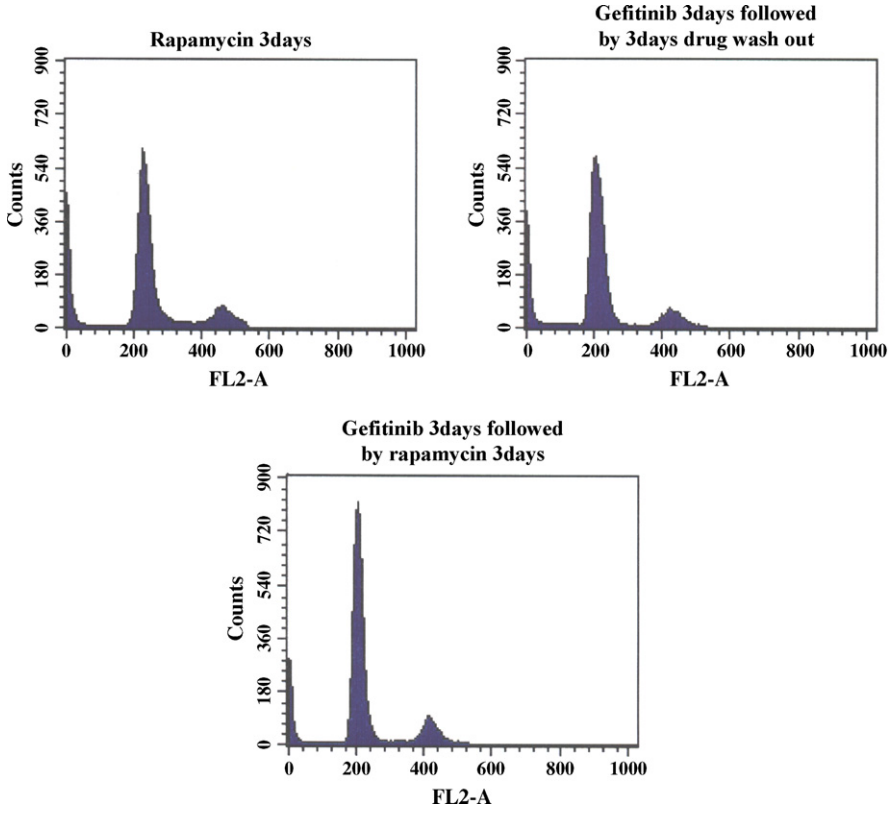

Fig. 5 – Cell cycle modulation by gefitinib followed by rapamycin. MiaPaCa-2 cells were incubated with 3 days-gefitinib (5.5 μM), 3 days-rapamycin (100 ng/ml) and utilising the synergic schedule, 3 days-gefitinib followed by 3 days-rapamycin. Cell cycle was determined by flow cytometry analysis as described in Section 2.

4. Discussion

The EGFR alterations frequently observed in pancreatic cancer have suggested the use of EGFR inhibitors as new promising anticancer agents.

The low pharmacological response of pancreatic cancer patients to TK inhibitors [31] has, however, highlighted the need to analyse the efficacy of combinatorial approaches based on conventional chemotherapeutics and/or other biological drugs. In principle, TK inhibitors and mTor inhibitors account for potential candidates of the above combinatorial approaches since they inhibit both the upstream and downstream mediators of the same signal transduction pathways; furthermore, this combination has already been suggested for clinical treatment in other cancer diseases [32].

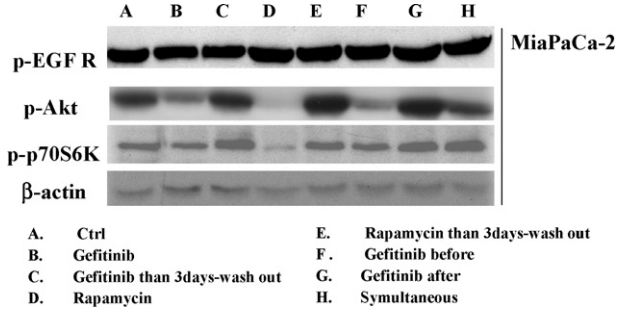

Fig. 6 – EGFR pathway modulation by gefitinib plus rapamycin. MiaPaCa-2 cells were incubated with gefitinib 5.5 μM and/or rapamycin 100 ng/ml for 3 days and the protein extracts analysed by immunoprecipitation and/or Western blot. β-actin was utilised to normalise each target value.

A preliminary assessment of gefitinib activity was carried out in all the pancreas cell lines under study which confirmed the role of gefitinib as a cytostatic drug (mediated by cells accumulation in the G0/G1 phase), rather than as a cytotoxic drug. Gefitinib activity turned out to be mainly mediated by survival pathway inhibition. p-Akt was, in fact, progressively inhibited for the first 3 days of drug exposure even though gefitinib effect on p-EGFR was only transient thus allowing for a recovery of the baseline value after 2 days exposure [33,34].

Our results are consistent with the literature data on gefitinib effectiveness in pancreas cell lines as they have confirmed (i) a slight accumulation of cells in the G0/G1 phase, (ii) a slight apoptosis after 1 day drug exposure and (iii) a parallel p-EGFR and p-Akt modulation [35].

Rapamycin activity was then characterised in all the pancreatic cancer cell lines under consideration which have confirmed the role of rapamycin as a cytostatic drug, with accumulation of cells in the G0/G1 phase and inhibition of the phosphorylated mTor, followed by inhibition of p70S6K. Also these data are in line with the data present in the literature in terms of both therapy targets and cell cycle modulation. What is more, a very small cell growth inhibition in the first 3 days of

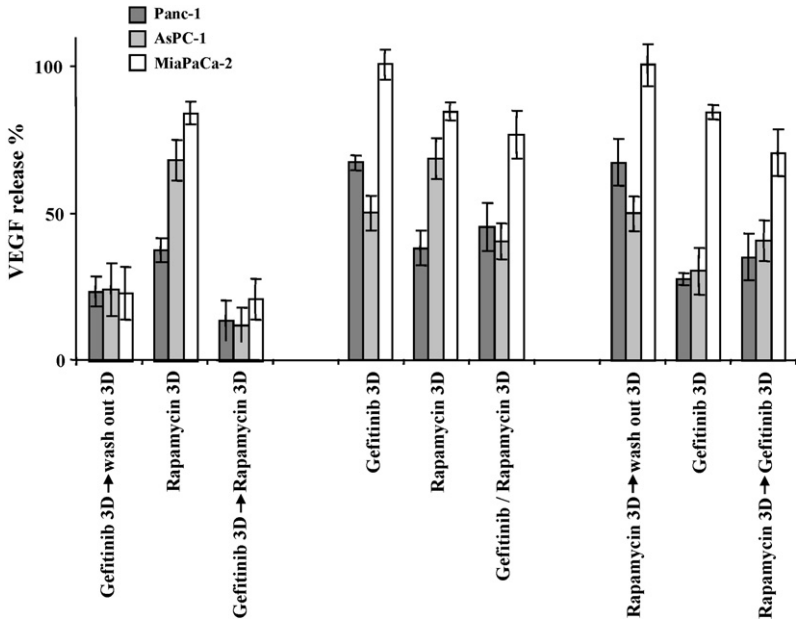

Fig. 7 – VEGF release by gefitinib plus rapamycin. VEGF release was analysed in surnatants from MiaPaCa-2, Panc-1 and AsPC-1, treated with gefitinib at IC_{50}, and/or rapamycin at 100 ng/ml (with the exception of Panc-1 given at 30 ng/ml) for 3 days. Results are expressed as percentage of control (drug untreated cells).

exposure at a concentration of rapamycin lower than the plasma concentration [4,36], has also been confirmed.

To determine the potential advantages likely to emerge from combined administration of gefitinib and rapamycin in an in vitro model of pancreatic cancer, the biomolecular effects of the different schedules [34,37–39], were analysed. This analysis has highlighted a clear synergism of the two drugs (in terms of both cell gowth inhibition and apoptosis induction) only when gefitinib was given before rapamycin. It is our belief that the above synergism results from the increased accumulation of cells in the G0/G1 phase, the inhibiting effects on both p-EGFR and the survival pathway, the modulation of p-Akt and of phosphorylated mTor and p-p70S6K, of the ''gefitinib given before'' schedule.

From a theoretical standpoint, this synergism seems to be substantiated by some recent studies focused on the activation of Akt phosphorylated form by rapamycin-dependent mTor inhibition [16].

Our experimental results overlap those obtained, in terms of proliferation, apoptosis and target modulation, when combining EGFR inhibitor, AEE788, with mTor inhibitor, RAD001 [32]. The differences observed in the amount of inhibited proteins, are clearly related to the different experimental conditions (i.e. in vitro model, time of exposure ranging from 3 days of the present study to 1 h [32]; absence of EGFR pathway stimulation with EGF).

Our study was also focused on the possibility of improving the already well-established antiangiogenic effects of gefitinib and rapamycin [18,19]. Such antiangiogenic effect is usually assessed by determining VEGF release and correlating it with HIF-1α modulation [7] in vitro studies. We confirmed that, in pancreatic cancer model, gefitinib and rapamycin also act as antiangiogenic drugs capable of reducing VEGF release; this VEGF release reduction became stable after 3 days of drug exposure and tended to increase after additional days of drug wash out. Furthermore, we proposed to use the combination of the two drugs also in a view to enhancing their antiangiogenic activity. In conclusion, our study has been able to show the efficacy of two drugs, EGFR and mTor inhibitors, for the first time in a pancreatic cancer model. This efficacy has also been shown to depend heavily on the schedule utilised to combine the EGFR inhibitor with the mTor inhibitor.

As additional comment, we think that the involvement of ABCG2, the drug efflux pump usually involved in camptothecins resistance [40], should be explored in more details. Actually, rapamycin seems to inhibit ABCG2 [41] while gefitinib interacts with it as a function of the time of exposure [42–44]. The synergism of the ''gefitinib before'' schedule could also be accounted for by rapamycin's capability to reduce previously gefitinib-stimulated ABCG2 activity, thus inducing prolonged accumulation of the EGFR inhibitor into cells. This hypothesis is presently being explored by our research group.

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