Abstract. Background: The therapeutic role of EGFR inhibitors in thyroid malignancies is still controversial even though the full activation of EGF signaling has recently been proposed as involved in the dedifferentiation of human thyroid cancers. Materials and Methods: Agents which target EGFR signaling (erlotinib, cetuximab and panitumumab) were evaluated at preclinical level in a panel of thyroid tumor cell lines. Results: Erlotinib induced a dose-dependent inhibition of cell proliferation together with inhibition of EGF-induced AKT and ERK1/2 signaling only in poorly-differentiated thyroid carcinoma FRO cells. By contrast, anti-EGFR monoclonal antibodies were inactive. Of note, erlotinib enhanced the proapoptotic activity of doxorubicin and paclitaxel and exhibited synergy with paclitaxel in poorly-differentiated thyroid carcinoma cells. Conclusion: EGFR signaling may represent a molecular target only in poorly-differentiated thyroid carcinoma cells, and agents that inhibit EGFR tyrosine kinase may be more effective than monoclonal antibodies which target the extracellular domain of the receptor.

Malignant thyroid tumors of follicular cell origin are traditionally classified as either well-differentiated (WDTC) or anaplastic thyroid carcinoma (ATC). The vast majority of patients with WDTC have an excellent prognosis, whereas patients with ATC have uniformly poor prognosis (1-2). There is growing evidence for the existence of a group of tumors that fall between WDTC and ATC in terms of both morphologic phenotype and biological behavior. These tumors, classified as poorly-differentiated thyroid carcinoma (PDTC), may represent intermediate entities in the progression from WDTC to ATC (2-4). In this case, clinical, epidemiological, and pathological evidence supports the concept of stepwise progression as a process of loss of cell differentiation (5). This process is accompanied by loss of thyroid-specific functions and properties, such as thyroid-stimulative hormone (TSH) receptor signaling and iodine uptake, which eventually makes the tumors inaccessible to conventional therapy (i.e., radiiodine and/or TSH suppression therapy). Finally, extensive local tumor growth and/or distant metastases preclude any further surgical intervention (2, 6). PDTCs and ATCs represent 3-5% of all thyroid cancer cases and lead to death within a few months after diagnosis. The increase in our understanding of the mechanisms involved in thyroid tumor progression will lead to improved treatment of patients with this disease (2, 5-6).

The epidermal growth factor receptor (EGFR) is a member of the ErbB receptor tyrosine kinase (TK) superfamily (7). It is a transmembrane receptor whose activation, through binding by epidermal growth factor (EGF) or transforming growth factor-α, leads to activation of the intrinsic TK and the cascade of intracellular signaling events that regulate cell proliferation and migration, angiogenesis, metastases and cell survival (8). Thus, the inhibition of the EGFR signal transduction pathway is a potential target for anticancer therapy. Over the last decade, several new agents which target EGFR signaling have exhibited promising clinical activity in several human malignancies (9). Indeed, the inhibition of EGFR signaling can be achieved by means of monoclonal antibodies (i.e., cetuximab and panitumumab) which bind to the ectodomain of the receptor with an affinity higher than endogenous ligands, preventing the receptor activation, or by means of small inhibitors of the EGFR TK (e.g., erlotinib and gefitinib) (9, 10). Monoclonal antibodies as well as small inhibitors block the proliferation of a wide range of human tumor cell lines, induce cell-cycle arrest and/or increase in
apoptosis and inhibit angiogenesis by blocking the production of VEGF-A (11-16). Both cetuximab and panitumumab showed significant clinical activity in patients with epithelial tumors, including head and neck squamous-cell carcinoma (HNSSC), colorectal cancer and non-small cell lung cancer (NSCLC) as single agents as well as in combination with either chemotherapy or radiation (9-10, 17). Erlotinib showed activity against human HNSSC, NSCLC and pancreatic tumors both in vitro and in vivo studies (9-10, 18).

The clinical role of inhibitors of EGF signaling in thyroid malignancies is still controversial. A number of reports have demonstrated the up-regulation of EGFR gene expression in human PDTCs and linked overexpression of EGFR with early disease progression, poor survival and resistance to chemotherapy (19-20). Our group recently reported that EGF signaling is poorly activated in WDTC cells, whereas the full activation of the EGF signaling favors the transition toward a less-differentiated, TSH-independent, angiogenic and invasive phenotype in human thyroid carcinoma cells (21). By contrast, while a significant inhibition of EGF-dependent proliferation in ATC cell lines has been observed in response to anti-EGFR agents (22), the EGFR TK inhibitor gefitinib produced unsatisfactory results in a phase II trial in human iodine-refractory PDTCs and ATCs (23). While these results would suggest that the inhibition of EGF signaling may represent a potential therapeutic target in PDTC, they raise questions about the appropriate agent which should be evaluated in clinical trials, and the possibility of combining anti-EGFR agents with other molecular-targeted drugs or traditional cytotoxicities. Hence, in the present study, we evaluated the antiproliferative activity of erlotinib, cetuximab and panitumumab in PDTC and WDTC cell lines and their ability to synergize with conventional cytotoxic agents.

Materials and Methods

Cell cultures. Human PDTC FRO, moderately-differentiated follicular thyroid carcinoma WRO and WDTC PTC-1 cells were cultured in DMEM containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. WRO cells were purchased from ECACC (Salisbury, UK), FRO cells were kindly provided by Dr. Fusco (University of Naples Federico II, Naples, Italy) with the permission of Dr. Fagin (Memorial Sloan Kettering Cancer Center, New York, NY, USA) and PTC-1 cells were kindly provided by Dr. Pontecorvi (Catholic University, Rome, Italy). Indeed, according to Schweppe et al. (24), PTC-1, WRO and FRO cells are unique thyroid carcinoma cell lines and WRO cells consist of the follicular origin of these cells (24). Human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. Prudovsky (Maine Medical Center Research Institute, Scarborough, ME, USA), cultured as previously reported (25) and were used as positive control for RT-PCR analysis. All reagents were purchased from Sigma-Aldrich, (Milan, Italy), unless otherwise specified. Erlotinib was obtained from Roche (Milan, Italy), cetuximab from Merck KgaA (Milan, Italy), and panitumumab from Amgen (Milan, Italy). To evaluate cell growth rate, cells were plated in 6-well plates, incubated in the presence and absence of the specified inhibitor at concentrations reported in the Results, harvested and counted in a Bürker chamber (three countings per sample). Incubation with drugs was carried out continuously and drug-containing fresh medium was changed at 48 h intervals. Results represent the average of three experiments.

Immunoblot analysis. Immunoblot analysis was performed as previously reported (26). Specific bands were revealed by using rabbit anti-phosphoERK1/2 (pERK1/2) (Upstate Cell Signaling, Milan, Italy), rabbit anti-ERK1/2 (Calbiochem, Milan, Italy), rabbit anti phosphoAKT (pSer473) (pAKT) (Upstate Cell Signaling, Milan, Italy) and a rabbit anti-AKT (Upstate Cell Signaling, Milan, Italy) polyclonal antibodies.

Cytotoxicity assay. MTT assay was used as an in vitro cell viability test to evaluate drug sensitivity (27). Briefly, thyroid carcinoma cells were pretreated with 750 nM erlotinib, 200 nM cetuximab or 500 nM panitumumab for 72 h, seeded into 24-well plates (2x10^4 cells/well) and incubated in the presence of increasing concentrations of doxorubicin, cisplatin or paclitaxel for 48 h (0.0001-100 μM). Control cells were treated with increasing concentrations of the same antibiotic drug without preincubation with anti-EGFR agents. After the removal of the drugs, cells were incubated in a drug-free medium for 24 h, 600 μM MTT solution was added to each well and MTT incorporation was measured 3 h later using a Bio-Tek microplate reader (model EL-340; BioMetallics, Princeton, NJ, USA). Three independent cytotoxicity assays (each using four replicates for each drug concentration) were performed to calculate IC_{50} of doxorubicin, cisplatin and paclitaxel, defined as the drug concentration which induced cell death in 50% of the cell population.

Apoptosis was evaluated by cytofluorimetric analysis of annexin V- and 7-aminoactinomycin D (7-AAD)-positive cells by using the FITC-Annexin V/7-AAD Kit (Beckman Coulter, Cassina De’ Pecchi, Milan, Italy). Stained cells were analyzed by EPICS XL Flow Cytometer (Beckman Coulter). Ten thousand events were collected per sample. Positive staining for annexin V as well as double staining for annexin V and 7-AAD were interpreted as signs of apoptosis (28).

Analysis of combination effects. Combination analysis was performed using the method described by Chou et al. (29-30). The influence of the sequence on the cytotoxic activity of the combination of ERFR inhibitors and antiblastic agents was evaluated by comparing the sequential assay with assays involving the two drugs simultaneously or alone. The combination effect was evaluated from isoeffect analysis of combination indices (CIs), calculated as follows

\[ CI=C_{A}/C_{X_{A}} + C_{B}/C_{X_{B}} \]

where C_{A} and C_{B} are the concentrations of the two drugs alone respectively, needed to achieve a given effect (x%) and C_{X_{A}} and C_{X_{B}} are the concentrations of the two drugs needed to obtain the same effect when combined. The CIs were calculated under the assumption of a mutually exclusive drug interaction. The combination was considered as positive (synergistic) when the CI was <1 and negative (antagonistic) when it was >1.
RNA extraction and RT-PCR analysis. Total RNA was extracted using the TRIzol Reagent according to the manufacturer’s procedures (Invitrogen, San Diego, CA, USA). For the first-strand synthesis of cDNA, 5 μg of RNA were used in a 20 μl reaction mixture utilizing a cDNA Superscript II (Invitrogen, San Diego, CA, USA) according to the supplier’s instructions. One μl from cDNA mixture was amplified using the Taq Gold DNA Polymerase kit (Applied Biosystems, Monza, Italy) in a Gene AMP PCR System 9700 Thermal Cycler (Applied Biosystems). Reaction conditions were 95˚C for 10 min, followed by 35 cycles of 30 s at 95˚C, 30 s at 56˚C (65˚C for β-actin), 5 min at 70˚C and 7 min at 72˚C. The following primers were used: EGFR forward 5’-TCCCTCAGCCACCCATATGTAC-3’, reverse 5’-GTCTCGGGCGATTTTGGAGAATTC-3’ (PCR product 125 bp); β-actin, forward 5’-GGCATCGTGATGGACTCCG-3’, reverse 5’-GCTGGAAGGTGGACAGCGA-3’ (PCR product 819 bp).

Statistical analysis. Unpaired Student’s t-test was used to establish the statistical significance between different rates of cell proliferation or apoptosis in treated cells and the respective untreated controls. Statistically significant values (p<0.05) are reported in Figure legends.

Results

Erlotinib exhibits a dose-dependent antiproliferative activity in PDTC FRO cells. In order to evaluate the sensitivity of thyroid carcinoma cells with different degrees of cell differentiation to anti-EGFR signaling agents, papillary WDTC PTC-1, follicular moderately-differentiated WRO and PDTC FRO cells were tested. In preliminary experiments, the expression of EGFR gene in the three thyroid carcinoma cell lines was evaluated and it was observed that while WRO and FRO cells express high levels of EGFR mRNA (Figure 1A) and protein (21), WDTC PTC-1 cells are characterized by low expression of the EGFR gene (Figure 1A). Thus, cells were cultured in the presence and the absence of 10 μM erlotinib, 200 nM cetuximab and 500 nM panitumumab for 4 days and evaluated for the rate of cell proliferation. Interestingly, the growth of PTC-1 and WRO cells was not affected by exposure to the three agents. By contrast, PDTC FRO cells exhibited a minimal inhibition of cell proliferation.
in response to cetuximab and a 60% inhibition in response to erlotinib (Figure 1B). Consistently, FRO cells showed a dose-dependent inhibition of cell proliferation after exposure to increasing concentrations of erlotinib for 4 days with maximal activity at 10 μM (Figure 1C) and confirmed the higher sensitivity to erlotinib after exposure to the three anti-EGFR agents for up to 12 days (Figure 1D). By contrast, increasing concentrations of cetuximab and panitumumab did not reveal any dose-dependent antiproliferative activity in the three thyroid carcinoma cell lines (data not shown).

In order to explore the mechanism responsible at molecular level for this divergent sensitivity of WDTC and PDTC cells to anti-EGFR agents, the ability of the three agents to inhibit ERK1/2 and AKT signaling in response to EGF in PDTC FRO cells was evaluated. Thus, based on our previous observation that only PDTC cells exhibit the full activation of signaling pathways downstream of EGFR (21), FRO cells were serum-starved for 48 h, exposed to 500 nM erlotinib, 200 nM cetuximab or 500 nM panitumumab for 48 h, stimulated with EGF in the presence and the absence of the three anti-EGFR agents and evaluated for the phosphorylation of ERK1/2 and AKT by immunoblot analysis. Interestingly, while cetuximab and panitumumab failed to inhibit the EGF-dependent activation of ERK1/2 and AKT pathways (data not shown), erlotinib inhibited the ability of EGF to up-regulate ERK1/2 and AKT phosphorylation (Figure 2). These results suggest that erlotinib inhibits the signaling pathways downstream of EGFR at concentrations lower than those responsible for its antiproliferative activity.

The inhibition of the EGFR signaling results in a more chemosensitive phenotype. It is well established that human undifferentiated thyroid carcinomas are aggressive malignancies resistant to chemotherapeutic agents. Thus, based on the hypothesis that EGF signaling is responsible for the induction of antiapoptotic pathways in tumor cells (31), we tested the ability of erlotinib, cetuximab and panitumumab to enhance the sensitivity of PDTC FRO cells to the proapoptotic activity of doxorubicin, cisplatin and paclitaxel. FRO cells were exposed to subtoxic concentrations of cisplatin, doxorubicin and paclitaxel as single-agents or after pretreatment with 750 nM erlotinib, 200 nM cetuximab or 500 nM panitumumab for 72 h and evaluated for apoptotic cell death by annexin V and 7-AAD staining (Figure 3A). Interestingly, pretreatment with erlotinib significantly enhanced the ability of antiblastic agents to induce apoptotic cell death with maximal activity on doxorubicin and paclitaxel cytotoxicity, whereas cetuximab and panitumumab pretreatment produced minimal effect on the cytotoxic activity of the three antiblastic drugs. In parallel experiments, FRO cells were cultured in the presence and in absence of the three anti-EGFR agents for 72 h, and incubated in the presence of increasing concentrations of doxorubicin, cisplatin or paclitaxel. Control cells received the treatment with the antiblastic agent alone. The ability of these antiblastic agents to induce cell death in 50% of the cell population (IC50) was calculated by MTT incorporation assay curves. Interestingly, the pretreatment with erlotinib favored a statistically significant decrease in the IC50 of doxorubicin and paclitaxel, whereas cetuximab and panitumumab did not enhance the cytotoxic activity of the same agents (Table I). Furthermore, the cytotoxic activity of cisplatin was not affected by pretreatment with any anti-EGFR signaling agents (Table I).
The interaction between erlotinib and paclitaxel or doxorubicin was further evaluated by combination index analysis in FRO cells. Cells were exposed to i) erlotinib alone for 72 h, ii) doxorubicin or paclitaxel alone for 48 h, iii) the sequential or simultaneous combination of the two drugs (erlotinib for 72 h followed by doxorubicin or paclitaxel for 48 h or the simultaneous combination of erlotinib and doxorubicin or paclitaxel for 48 h). The cytotoxic effect of these combinations was evaluated by MTT incorporation analysis and the CI isobologram equation was used to evaluate the interaction between these agents (29-30). Interestingly, the sequential combination of erlotinib and paclitaxel showed the maximal synergism, whereas only an additive effect was exhibited by sequential exposure to erlotinib and doxorubicin (Figure 3B). These positive interactions were not observed in FRO cells exposed to the sequence of erlotinib and cisplatin or to the simultaneous combination of erlotinib with any antiblastic agent (data not shown). These results suggest that the inhibition of EGFR signaling in PDTC FRO cells results in the down-regulation of antiapoptotic mechanisms and in the induction of a phenotype more sensitive to specific antiblastic agents.

Figure 3. The synergism between erlotinib and traditional cytotoxics. A: FRO cells were cultured in the presence and absence of 750 nM erlotinib for 3 days and further treated with increasing concentrations of doxorubicin, paclitaxel and cisplatin for 2 days. The rate of apoptotic cell death was evaluated by staining with annexin V and 7-AAD. P-values indicate the statistical significance of the rates of apoptosis in treated cells compared to the respective untreated control (*p=0.01; **p=0.04; °p=0.027; °°p<0.022). B: Plot of the combination indices versus the cytotoxicity calculated from MTT assay data of thyroid carcinoma cells exposed sequentially to erlotinib for 3 days followed by doxorubicin or paclitaxel for 2 days.
Discussion

PDTCs and ATCs are unresponsive to standard treatments and are characterized by a poor prognosis (2). Thus, novel therapeutic approaches are needed in order to improve patient outcome (32). Based on our previous finding that the full activation of EGF signaling in thyroid carcinoma cells is responsible for the transition toward a less-differentiated, more invasive and angiogenic phenotype (21), the hypothesis that EGF signaling may represent a molecular target in PDTCs was tested at preclinical level. To address this issue, the activity of agents which target EGFR signaling in a panel of thyroid carcinoma cell lines at different degrees of cell differentiation were evaluated. Indeed, the EGFR pathway is known to exert strong stimulatory effects on cell proliferation, migration, angiogenesis, metastases formation and cell survival (9, 11-16), and specific anti-EGFR agents inhibit the growth of a variety of human cancer cells and enhance the cytotoxic activity of several antiblastic agents (9). Our results provide evidence that erlotinib might deserve to be further evaluated in PDTCs, since it enhances the cytotoxic activity of antiblastic agents and synergizes with paclitaxel. By contrast, agents which target the extracellular domain of EGFR are inactive either in WDTC or in PDTC cell lines.

These results may be relevant for future investigation of the anticancer activity of anti-EGFR agents in human PDTCs, since they address several preclinical unsolved issues about these drugs in human thyroid carcinomas. It is noteworthy that, in spite of a similar expression of EGF gene in differentiated and undifferentiated thyroid carcinoma cell lines, only undifferentiated thyroid carcinoma cells are sensitive to agents which target EGFR signaling, and, among them, only erlotinib induces a dose-dependent inhibition of cell proliferation. This finding is consistent with our observation that, in spite of a significant expression of EGF at mRNA and protein levels, follicular thyroid carcinoma WRO cells are not able to activate AKT signaling in response to EGF, whereas PDTC FRO cells exhibit the full activation of ERK1/2 and AKT signaling after EGF stimulation (21). These observations suggest that i) EGF signaling may represent a molecular target only in PDTCs, and ii) the full activation of the signaling pathways downstream of EGF is a prerequisite for a positive response to erlotinib in thyroid carcinoma cells and may be evaluated as a surrogate marker of sensitivity to anti-EGFR agents in thyroid malignancies.

An issue that remains controversial is whether single-agent anti-EGFR therapy deserves to be tested in thyroid carcinoma trials. While previous preclinical studies suggested a potential sensitivity of thyroid tumor cells to the EGFR TK inhibitor, gefitinib (22), the treatment of human iodine-refractory or anaplastic thyroid carcinomas with this agent resulted in a disease stability only in 48% of patients in a phase II study, raising concerns about the clinical activity of gefitinib in this malignancy (23). In such a perspective, it is important to recall that different EGFR TK inhibitors demonstrated divergent clinical activities in the same human neoplasm. For instance, in NSCLC, gefitinib failed to demonstrate a significant anticancer activity in the phase III ISEL trial, whereas erlotinib showed an overall survival benefit in the BR21 trial (10). However, it is important to underline that, in our study, erlotinib exhibited a single-agent antiproliferative activity at concentrations that cannot be achieved in patients with standard non-toxic dose regimens (33). Moreover, cetuximab or panitumumab were ineffective in WDTC and PDTC cells, raising the question about the specificity of action of the EGFR TK inhibitor erlotinib. Indeed, while monoclonal antibodies are highly specific toward their ligands, since they specifically target the extracellular domain of the receptor, erlotinib inhibits the intracellular receptor-TK domain of the EGFR with high affinity but it is also known not to be absolute in its specificity for the EGFR TK (33) and to have off-target effects (34). Thus, even though in our study erlotinib is the only anti-EGFR agent responsible for a dose-dependent antiproliferative activity, and low doses of erlotinib prevent the EGF-induced activation of ERK1/2 and AKT pathways, we cannot rule out the hypothesis that erlotinib activity may depend on the simultaneous inhibition of other signaling pathways. Consistently, the lack of activity of anti-EGFR antibodies is in agreement with previous findings showing ineffectiveness of cetuximab in a panel of differentiated and anaplastic thyroid carcinoma cells (35). Taken together, this evidence suggest that single-agent anti-EGFR therapy may be not worthy of further evaluation in human thyroid malignancies.

The last issue that deserves to be discussed is the potential use of erlotinib in combination with other anticancer agents. Indeed, to expand the concept of an EGFR TK inhibitor-based therapeutic approach to thyroid carcinomas, the present study focused on possible synergistic activities between erlotinib and chemotherapeutics such as doxorubicin, cisplatin and paclitaxel. The finding that a low concentration of erlotinib enhances the ability of doxorubicin and paclitaxel to induce apoptotic cell death, and, acts in synergism with paclitaxel is of clinical interest. While anthracyclines have been used for many years as standard therapy for radioiodine-refractory thyroid cancer with unsatisfactory results (36), paclitaxel has been proposed as a novel chemotherapeutic agent for this malignancy (37). It is well known that thyroid cancers are chemoresistant tumors and that the stimulation of EGFR signaling represents a mechanism responsible for the activation of several survival pathways (e.g., PI3K/AKT, BCL2), favoring the resistance to anticancer agents in several human tumor cell models (38).
Furthermore, our observation is consistent with recent evidence showing that cetuximab enhances the cytotoxic activity of irinotecan in ARO cells (39), and that gefitinib potentiates the activity of radiation in ARO and WRO cells (40). Thus, even though some of these findings need to be reconsidered since ARO cells have been recently questioned as a valuable ATC cell model (24), it is reasonable to speculate that the pharmacological blockade of EGF signaling may represent a tool to down-regulate antiapoptotic mechanisms and enhance the sensitivity of PDTC cells to traditional cytotoxics. Thus, these findings suggest that the synergism between erlotinib and paclitaxel may be clinically relevant and, thus, deserves to be further evaluated in animal thyroid tumor models and, eventually, at clinical level in human PDTCs.

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References


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