Synergistic antitumor activity of cetuximab and namitecan in human squamous cell carcinoma models relies on cooperative inhibition of EGFR expression and depends on high EGFR gene copy number

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Abbreviations: CI, combination index; EGFR, epidermal growth factor receptor; HIF-1alpha, hypoxia-inducible factor-1 alpha; MAPK, mitogen-activated protein kinase; moAbs, monoclonal antibodies; TKIs, tyrosine kinase inhibitors; PBS, phosphate-buffered saline; SCC, squamous cell carcinoma; TPT, topotecan; TV, tumor volume; TVI, tumor volume inhibition.

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Abstract

Purpose: In spite of a frequent overexpression of epidermal growth factor (EGFR) in squamous cell carcinoma (SCC), the efficacy of cetuximab alone is limited. Given the marked activity of namitecan, a hydrophilic camptothecin, against SCC models, the present study was performed to explore the efficacy of the cetuximab-namitecan combination in a panel of SCC models.

Experimental design: We examined the antiproliferative and antitumor activities of the cetuximab/namitecan combination in four SCC models characterized by a different EGFR gene copy number/EGFR protein level. We also assessed the effects of the combination on EGFR expression at both mRNA and protein levels and investigated the molecular basis of the interaction between the two agents.

Results: Cetuximab and namitecan exhibited synergistic effects, resulting in potentiation of cell growth inhibition and, most importantly, enhanced therapeutic efficacy, with high cure rates in three SCC models characterized by high EGFR gene copy number, without increasing toxicity. The synergistic antitumor effect was also observed with the cetuximab-irinotecan combination. At the molecular level, the two agents produced a cooperative effect resulting in complete down-regulation of EGFR. Interestingly, when singly administered, the camptothecin was able to strongly decrease EGFR expression mainly by transcriptional inhibition.

Conclusions: Our results i) demonstrate a marked efficacy of the cetuximab/namitecan combination, which reflects a complete abrogation of EGFR expression as a critical determinant of the therapeutic improvement, in SCC preclinical models, and ii) suggest EGFR gene copy number as a possible marker to be used for patient selection in the clinical setting.
**Translational Relevance**

The epidermal growth factor receptor (EGFR) is a validated therapeutic target in many human cancers. In spite of the frequent EGFR overexpression in squamous cell carcinoma (SCC), the anti-EGFR antibody, cetuximab, as a single agent exhibits marginal efficacy. Our study shows that a combination including cetuximab and a topoisomerase I inhibitor, namitecan or irinotecan, produced synergistic effects, resulting in complete regression of SCC preclinical models characterized by high *EGFR* gene copy number. The result was achieved at well-tolerated doses, thus indicating a good therapeutic index. The study also provides a molecular basis for a rational combination to be exploited for possible therapeutic applications in the clinical setting, and suggests *EGFR* gene copy number as a possible marker for patient selection.
Introduction

The epidermal growth factor receptor (EGFR), a member of the ErbB receptor tyrosine kinase family, plays an essential role in the regulation of important cellular processes such as proliferation, survival, adhesion, migration and differentiation (1-2) as well as in DNA damage response (3-4). Abnormal activation of EGFR through different mechanisms, including overexpression, mutations and autocrine ligand production (5), has been implicated as a crucial regulator of the pathogenesis process and progression in a variety of human cancers (2, 6-8). There appear to be distinct mechanisms for EGFR activation in different tumor types. Specifically, in squamous cell carcinoma (SCC) of the cervix, head and neck, and lung, EGFR mutations are absent or detectable in small percentages of cases (<5%) (9-12). In such tumors, a more frequent mechanism of EGFR activation is the amplification/increased copy number of the gene (13), leading to protein overexpression. Genomic gain in the EGFR gene was consistently associated to adverse clinical outcome in patients with cervical (9), lung (14) and head and neck (15) SCC.

EGFR is a validated therapeutic target in many human cancers. Currently, there are two therapeutic approaches to inhibit EGFR: tyrosine kinase inhibitors (TKIs) and anti-EGFR monoclonal antibodies (moAbs). TKIs target the intracellular tyrosine kinase domain of the receptor by competing for the ATP binding pocket, thus inhibiting phosphorylation of the receptor and its downstream targets. MoAbs, such as cetuximab, disrupt the EGFR signaling pathway through inhibition of ligand binding and induction of receptor internalization followed by degradation in lysosomes (16). In addition, cetuximab binding to the extracellular EGFR domain promotes the activation of antibody-dependent cellular cytotoxicity (17). Despite of the
role of EGFR in the malignant phenotype, therapy with EGFR targeting agents only exhibited limited efficacy. Indeed, no clinical responses were reported following cetuximab monotherapy in cervical SCC (18) and a marginal efficacy of the MoAb was observed in head and neck SCC patients with metastatic disease (13% response rate) (19). However, cetuximab is the first targeted therapeutic agent to show a significant improvement in the overall survival for patients with locally advanced head and neck SCC when used in combination with radiation (20) or for recurrent or metastatic disease when used in combination with chemotherapy (21).

We recently reported that namitecan, a novel hydophilic camptothecin analogue, exhibited antitumor activity in a large panel of human tumor models (22-23) and an excellent activity, superior to that of irinotecan, in the treatment of SCC models (23). Since EGFR expression has been implicated as a determinant of response to radiation and to chemotherapy (24-26), the present study was performed to explore the therapeutic potential of cetuximab/namitecan combination against four SCC models characterized by different levels of EGFR expression and to elucidate the role of EGFR in response to the namitecan-containing therapy. For comparative purpose, we also assessed the effect of cetuximab/irinotecan combination. The results provided evidence of a synergistic antitumor activity of cetuximab/topoisomerase I inhibitor (namitecan or irinotecan) combination, with high cure rates, which was related to the level of EGFR protein expression/EGFR gene copy number of the tumor model. Ex-vivo and cellular studies demonstrated that the main mechanism of such a synergistic interaction was the cooperative inhibition of EGFR expression induced by the two agents. Specifically, the EGFR down-regulation induced by namitecan and SN-38 seems to rely primarily on a marked and persistent transcriptional down-regulation of gene expression. The early activation of p38 MAPK, which in turn phosphorylates EGFR at Ser1046/1047 thereby inducing its degradation also contributed to topoisomerase I inhibitor-induced EGFR inhibition.
Materials and methods

Cell lines

Three human SCC lines derived from skin (A431) and uterine cervix (Caski and SiHa) and a topotecan (TPT)-resistant subline of A431 (A431/TPT (27)) were used in the study. All the cell lines were cultured in RPMI-1640 media (Lonza, Verviers, Belgium) supplemented with 10% FBS and grown in a humidified incubator with 5% CO₂ at 37°C. Cell lines are periodically monitored for DNA profile of short tandem repeats analysis by the AmpFISTR Identifiler PCR amplification kit (Applied Biosystems, Foster City, CA).

Determination of c-MET and EGFR gene copy number variations

Genomic DNA was extracted using the iPrep™ Purification Instrument with the iPrep™ ChargeSwitch® Forensic Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol, and relative concentration was quantified using the Infinite Infinite® 200 NanoQuant Spectrophotometer (Tecan, Männedorf, Switzerland).

MET and EGFR gene copy number variations were assessed using real-time PCR TaqMan Copy Number Assays (Applied Biosystems), using RNase P gene as endogenous control. Furthermore, MAD1L1 and CFTR genes, being located respectively on the p and q-arm of chromosome 7, were also used to exclude the presence of chromosome 7 polysomy. The assays were performed using the Applied Biosystems ViiA™ 7 Real-Time PCR System (Applied Biosystems) according to the manufacturer’s protocol. Copy number variations of the target genes were determined as relative quantification (RQ) based on the ΔΔCt method and using control samples as calibrators with the ABI SDS software 1.1 (Applied Biosystems).
### Mutational status analysis

Mutation status of *KRAS, BRAF, KRAS, NRAS, PIK3CA* and *EGFR* genes was determined as detailed in Supplementary Methods.

### Drugs

For in vitro studies, namitecan (Sigma-Tau, Pomezia, Rome, Italy) and SN-38 and SB203580 and SB202190 (Sigma Chemical Company, St. Louis, MO) were initially dissolved in dimethyl sulfoxide and then diluted in sterile saline before use. Cetuximab (Erbitux®, Merck Serono, Darmstadt, Germany) was diluted in sterile saline before use. For in vivo studies, namitecan was dissolved employing a magnetic stirrer in sodium lactate buffer (50 mM) adjusted to pH 4.0 with the addition of hydrochloric acid. Irinotecan was dissolved in sterile distilled water. Both camptothecins were administered i.v. in a volume of 10 ml/kg. Cetuximab was ready to use and was delivered i.p. at the dose of 0.2 ml/mouse.

### Growth inhibition study

The antiproliferative activity was evaluated after 72 h of drug exposure by cell counting (27). Drug concentrations able to inhibit cell proliferation by 50% (*IC*$_{50}$) and 20% (*IC*$_{20}$) were calculated from dose-response curves.

### Antitumor activity study

To generate tumor xenografts, exponentially growing cells (A431 and A431/TPT, $10^7$ cells/mouse; SiHa $2.5 \times 10^7$ cells/mouse, Caski $10^7$ cells/mouse) were s.c. injected into the mice flanks. For antitumor activity studies, groups of four/five mice bearing tumor implanted in both
flanks were employed. Tumor fragments were implanted on day 0, and tumor growth was followed by biweekly measurements of tumor diameters with a Vernier caliper. Tumor volume (TV) was calculated according to the formula: TV (mm$^3$) = $d^2D/2$, where $d$ and $D$ are the shortest and the longest diameter, respectively. Treatment started 5-13 days after implant, when the tumors were just palpable, but established (TV=80-90mm$^3$). Namitecan, irinotecan and cetuximab were administered every fourth day for four times (q4dx4). Cetuximab was given 1 h after each administration of the camptothecin.

The efficacy of the drug treatment was assessed as a) tumor volume inhibition percentage (TVI%) in treated versus control mice, calculated as: TVI% = 100-[(mean TV treated/mean TV control) × 100]; b) complete responses (CR), i.e. complete disappearance of the tumors for at least 10 days. The toxicity of the drug treatment was determined as body weight loss and lethal toxicity. Deaths occurring in treated mice before the death of the first control mouse were ascribed to toxic effects.

Antibodies and western blot analysis

The antibodies used in the study were: anti-EGFR (Upstate Biotechnology, Lake Placid, NY); anti-vinculin (Sigma); anti-phospho EGFR (Ser1046/47), anti-phospho EGFR (Tyr1045), anti-phospho p38 MAPK (Thr180/Tyr182) (Cell Signaling Technology, Danvers, MA); anti-p38 MAPK and anti-c-Cbl (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot analysis was carried out as described previously (27) and as detailed in Supplementary Methods.

Quantitative reverse-transcription PCR

Total RNA was isolated from SCC cell lines using the RNAqueous®-4PCR Kit (Ambion Europe Ltd., Huntingdon, UK), according to the manufacturer's instructions. EGFR mRNA
expression was assessed by quantitative reverse transcription-PCR (qRT-PCR) as detailed in Supplementary Methods.

**Immunofluorescence staining**

Immunofluorescence staining of EGFR and c-Cbl were carried out as detailed in Supplementary Methods.

**Data analyses**

In in vitro growth inhibition studies, the type of drug interaction was evaluated by the Chou and Talalay's (28) method using the CalcuSyn software (Biosoft, Cambridge, UK). Accordingly to it, combination index (CI) value. CI<1, =1 and >1 indicated synergism, additive effect and antagonism, respectively. Synergism is further refined by CalcuSyn as synergism (CI=0.3-0.7) and strong synergism (CI=0.1-0.3). In antitumor activity studies, Student’s *t* and Fisher’s exact test (two-tailed) were used for statistical comparison of tumor volumes and complete responses, respectively, in mice.

**Results**

The panel of gynecological SCC cell lines used in the study was characterized by different levels of EGFR expression at both protein (Fig. 1A) and mRNA (Fig. 1B) level. Specifically, strong EGFR overexpression was found in the A431 cell line and, although to a lesser extent, in its TPT-resistant subline A431/TPT, which was paralleled by a marked gain in *EGFR* gene copy number (Fig. 1B). Conversely, moderate or almost negligible EGFR protein levels were present
in Caski and SiHa cell lines, respectively, in agreement with the small, although different increases in EGFR gene copy number (Fig. 1C). In addition, mutational analysis indicated that all cell lines harbored wild type EGFR, KRAS, NRAS, BRAF and PI3KCA genes, with the only exception of Caski cells in which a PI3KCA exon 9-activating mutation (p.E545K) (29) was present (Fig. 1C). Finally, a similar MET copy number was observed in the different cell lines (Fig. 1C).

The namitecan-cetuximab combination induced synergistic antitumor effects in SCC models as a function of EGFR gene copy number

Given the hypersensitivity of SCC to namitecan (23), in the study we used a suboptimal dose of the drug (10 mg/kg, i.e., 1/3 of the maximum tolerated dose) to allow a comparison of single-drug treatment and combination of namitecan with the anti-EGFR antibody, cetuximab. Irinotecan was used as reference compound at 17 mg/kg (1/3 of the maximum tolerated dose). Under such treatment conditions, in the A431 model namitecan still produced a significant tumor growth inhibition (84%) with an appreciable number of complete tumor regressions (3/8). Cetuximab (1 mg/mouse) produced a good antitumor effect without evidence of complete tumor regression. The efficacy of the combination was impressive, because all animals exhibited complete tumor response with no evidence of disease at the end of the experiment (90 days after the last treatment) (Fig. 2, Table 1). In an independent experiment, single-agent therapy with irinotecan resulted in a significant tumor growth inhibition (78%) with evidence of complete response in one tumor. Cetuximab showed a good antitumor activity and induced 3/8 complete response. Combined cetuximab/irinotecan treatment resulted in complete tumor regression in all treated animals without any evidence of tumor regrowth until the end of experiment (90 days after the last treatment) (Table 1, Supplementary figure S1).
The A431/TPT subline, which was highly resistant to TPT (27), was still responsive to namitecan at the low dose level, producing appreciable tumor growth inhibition but without complete tumor regression. Surprisingly, in spite of a reduced expression of the target EGFR, cetuximab was very effective in the control of tumor growth, resulting in 99% TVI and 6/8 complete responses. The addition of cetuximab to treatment with namitecan resulted in complete regression of all tumors, with no evidence of tumor regrowth in 8/8 animals at the end of the experiment (Fig. 2, Table 1). Irinotecan produced a 72% TVI without evidence of complete tumor regression. 8/8 animals treated with the combination irinotecan and cetuximab experienced complete tumor regression (Table 1 and Supplementary Figure S1). Complete responses were observed in all animals treated with cetuximab alone or in combination with irinotecan without disease manifestation until the end of treatment (Table 1 and Supplementary Figure S1).

The Caski model, which expresses a lower level of EGFR, was still responsive to both namitecan and cetuximab. Again, the combination of the two agents resulted in increased efficacy, as evidenced by complete regression of all treated tumors (Fig. 2, Table 1). The combination of irinotecan with cetuximab was less effective than the combination containing namitecan, at least in terms of complete response rate (Table 1 and Supplementary Figure S1). This finding would suggest that the level of EGFR expression is more critical for irinotecan to achieve synergistic interaction.

The SiHa model, which is characterized by a very low level of EGFR expression, exhibited a lower responsiveness to both namitecan and cetuximab than other models. When animals were treated with the combination, only a slight not statistically significant increase in efficacy was observed, and no animal experienced tumor regression (Fig. 2, Table 1). It is important to emphasize that the curative efficacy of the cetuximab/namitecan (or irinotecan) combination was achieved at well-tolerated doses of each agent without evidence of appreciable
toxicity (Table 1).

In vitro growth inhibition studies on the same SCC models showed a variable cellular sensitivity to cetuximab, with IC$_{50}$ values ranging from 1.47 to 931 µg/ml (Supplementary Fig. S1A), which was directly correlated to EGFR protein expression/EGFR gene copy number of the tumor cell line. Conversely, no appreciable differences were observed in the sensitivity of the four cell lines to namitecan (Supplementary Fig. S2A). In combination studies, a synergistic interaction between the effects of cetuximab and namitecan, as determined by the Chou and Talalay method (28), was observed in A431, A431/TPT and Caski cells (Supplementary Fig. S2B). The extent of the synergistic effects was related to the level of EGFR protein expression/EGFR gene copy number of the cell line. Consistent with in vivo data, only an additive effect of the two agents in combination was found in the SiHa cells expressing the lowest EGFR level (Supplementary Fig. S2B).

Cetuximab and namitecan cooperate in inhibiting EGFR expression

In the search for possible molecular determinants of the cetuximab/namitecan synergistic interaction, we investigated therapy-induced changes in the expression levels of EGFR in A431, A431/TPT and SiHa xenografts. Western blot results showed that not only cetuximab but also namitecan, when singly administered, induced a marked reduction of EGFR levels, and that an almost complete abrogation of the protein expression was observed in all tumors exposed to the combined treatment (Fig. 3A).

Cellular studies were carried out to elucidate the mechanism through which namitecan inhibited EGFR expression. Consistent with in vivo findings, a 24 h exposure to namitecan induced a dose-dependent decrease in EGFR expression in the different cell lines. In addition, results obtained in cells exposed to the drug combination confirmed a cooperative effect of
namitecan and cetuximab in suppressing EGFR protein expression (Fig. 3B). Similar results were obtained when cells were treated with equimolar concentrations of SN-38 alone or in combination with cetuximab (Supplementary figure S3A).

Since it has been recently reported that topoisomerase I inhibition can trigger a transcriptional stress and consequently interfere with translation of specific genes, such as HIF-1alpha, in human cancer cells (30), we assessed the ability of namitecan to modulate EGFR mRNA levels in treated cells. Quantitative RT-PCR results showed that, in all cell lines, EGFR mRNA levels were decreased at 24-h after treatment with namitecan. Conversely, no appreciable interference with EGFR mRNA levels was observed in cells exposed to cetuximab alone. In addition, the extent of the namitecan-induced reduction of EGFR mRNA levels was not significantly modified in the combination with cetuximab (Fig. 3C). As shown in Supplementary figure S3B, SN-38 was able to inhibit EGFR mRNA expression at a comparable extent. Overall such data indicate that namitecan (or SN-38) -mediated topoisomerase I inhibition leads to EGFR mRNA down-regulation.

Since the namitecan-induced effect on EGFR mRNA expression levels could not justify the almost complete abrogation of EGFR protein observed in treated SCC cells at 24 h, we assessed the treatment-induced interference with EGFR cellular localization in A431 cells to define whether the decline of EGFR protein abundance was paralleled by an increased internalization and consequent ubiquitination of EGFR (Fig. 4A). As expected, EGFR was internalized after treatment with cetuximab (31). Interestingly, also namitecan showed the ability to induce EGFR internalization (Fig. 4A). Since it is known that the process of EGFR degradation is dependent on the ability of the E3 ubiquitin ligase c-Cbl to bind the receptor to its phosphorylated Tyr1045 residue (32), we examined whether, following drug-induced internalization, EGFR co-localizes with the c-Cbl protein in A431 cells. Unlike the
well-characterized EGF-induced formation of EGFR-c-Cbl complexes (Supplementary Fig. S4), cetuximab and namitecan induced translocation of EGFR into intracellular vesicles which did not co-localize with the c-Cbl protein (Fig. 4B), in accordance with recent evidence indicating that cetuximab induces internalization and subsequent ubiquitination of EGFR, recruiting an E3 ligase distinct from c-Cbl (31, 33). Our fluorescence microscopy findings were further corroborated by immune-coprecipitation assay results showing that namitecan and cetuximab did not induce EGFR binding to c-Cbl in spite of an increased EGFR ubiquitination (data not shown).

Since recent reports showed that some anticancer drugs cause EGFR degradation via phosphorylation at Ser1046/1047 residues by p38 MAPK in different tumor cell lines (32, 34, 35), and taking into account that irinotecan has been shown to activate p38 MAPK in HCT cell lines (36), we examined the effects induced by namitecan on the expression of the active phosphorylated form of p38 MAPK in A431 cells (Fig. 5). The activation of p38 MAPK was appreciable starting from a 1 h exposure to namitecan (alone or in association with cetuximab) and still present, at comparable levels, at 24 h. In parallel, an increased EGFR phosphorylation at Ser1046/1047 was found in cells exposed to namitecan, which reached its maximum at 4 h (Fig. 5A). In contrast, a negligible effect on EGFR phosphorylation at Tyr1045 was observed following namitecan exposure (data not shown), according to the lack of colocalization of internalized EGFR and c-Cbl (Fig. 4B). Comparable effects on p38 MAPK activation and EGFR phosphorylation at ser 1046/47 were observed when A431 cells were exposed to SN-38 (Supplementary fig. S5A).

To better understand the role of activated p38 MAPK in namitecan-induced EGFR down-modulation, we used two specific inhibitors SB203580 (37) and SB202190 (36) in A431 and Caski cells. A 2 h treatment of cells with SB203580 (10 μM) or with SB202190 (5μM) before a
24 h exposure to namitecan, alone or in association with cetuximab, strongly reduced EGFR phosphorylation at Ser1046/47 in A431 cells (Fig. 5B and Supplementary Fig. S5B and S5C). In addition, pretreatment with SB203580 or SB202190, which did not affect by itself EGFR expression levels, partially restored EGFR expression in namitecan-treated cells (Fig. 5B and Supplementary Fig. S5B and S5C). Similarly, pretreatment with p38 MAPK inhibitors was able to partially restore EGFR protein expression in A431 cells exposed to SN-38 for 24 h (Supplementary Fig. S5B and S5C). Such a protective effect against namitecan-induced EGFR degradation was even more pronounced in Caski cells (Fig. 5C). Conversely, no appreciable effect of SB203580 and SB202190 pretreatment on cetuximab-induced EGFR down-modulation was found in either cell line (Fig. 5B, C and Supplementary Fig. S5B and S5C). Taken together, our findings support that namitecan and SN-38 promote the early activation of p38 MAPK, which in turn phosphorylates EGFR at Ser1046/47, thus inducing its degradation.

However, the contribution of drug-induced p38 MAPK activation to the overall inhibition of EGFR expression and consequently to the cetuximab/topoisomerase I inhibitor synergistic interaction seems to be limited. In fact, in A431 cells, a 48 h of exposure to namitecan (or SN-38) alone or in association with cetuximab, was able to induce a decrease in EGFR mRNA levels of about 90% (Fig. 5D), which was accompanied by a complete abrogation of EGFR protein expression (Fig. 5E). Due to the lack of substrate available to be phosphorylated by p38 MAPK, pre-treatment with the inhibitor SB203580 failed to rescue EGFR protein expression in camptothecin-treated cells (Fig. 5E). In addition, the pretreatment with p38 inhibitor SB203580 failed to impair namitecan/cetuximab synergistic interaction (Supplementary Fig.6S).

**Discussion**
Although single-agent therapy with cetuximab shows a limited efficacy in the treatment of SCC (38), an improved benefit is expected when it is combined with conventional cytotoxic agents (21, 39). In this context, novel rational strategies to improve the efficacy of combination treatments and to reduce drug-induced side effects are needed.

Our study provides evidence that cetuximab, when used in combination with namitecan induced a synergistic effect that resulted in potentiation of cell growth inhibition in 3 of the 4 SCC cell lines used in the study. The most interesting observation was the enhanced therapeutic efficacy seen in the treatment of SCC xenografts, where the combination produced a curative effect in most treated animals. Consistent with in vitro findings, a synergistic effect of the combination was appreciable in tumor models characterized by a high EGFR gene copy number. In fact, only the SiHa tumor, which carries the lowest number of EGFR gene copies, did not exhibit a significant therapeutic benefit by the combination treatment, thus suggesting a marginal impact of the receptor tyrosine kinase in the growth of the tumor. A synergistic antitumor effect was also observed when cetuximab was combined with irinotecan only in the two highly EGFR expressing tumor models. The substantial improvement with the combined therapy was achieved without an increase in toxicity, because well-tolerated doses of each agent were used. SCC is known to be responsive to namitecan, but curative efficacy requires treatment with maximum tolerated doses, which are associated with significant toxicity (22, 23).

The present study also provides valuable information on the molecular/cellular bases of the synergistic drug interaction. Specifically, the combination of cetuximab and namitecan produced a cooperative effect resulting in a complete down-regulation of EGFR. Although the effect of cetuximab on EGFR protein levels has been already described (31), the ability of topoisomerase I inhibitors to down-regulate EGFR expression is somewhat unexpected. In a previous study Liu et al. reported the ability of irinotecan to upregulate the EGFR pathway (40).
However, the authors showed an increase in the phosphorylation of EGFR (at Tyr 1068) in the absence of significant changes in total EGFR protein expression in gastric cancer cells after 16 h of treatment with irinotecan in presence of EGF stimulation. Since no time-course experiments were carried out, we do not know whether the increase of EGFR phosphorylation was followed by a reduced expression of EGFR protein at later time points, as observed in our study in A431 cells (Fig.5, Supplementary Fig.S5). Indeed, namitecan- or SN-38-treated cells showed an appreciable increase of EGFR phosphorylation at ser1046/47 after 4 h, which was accompanied by EGFR down-regulation at 24 h. Due to different times of analysis and culture conditions (±EGF), results from the two studies are not directly comparable.

We herein demonstrate that namitecan (or SN-38)-induced EGFR down-regulation was mainly the result of transcriptional inhibition of gene expression. Indeed, drug treatment appreciably decreased EGFR mRNA levels in SCC cells in a time dependent manner with the maximum inhibition (about 90%) being observed at 48 h. The effects of camptothecin-mediated topoisomerase I inhibition at the transcription level have recently been recently investigated by Baranello L et al. (30) in human cancer cells for another gene (e.g., HIF1-alpha). The study showed that topoisomerase I inhibition can trigger a transcriptional stress resulting in an impaired balance of mRNAs and antisense transcripts that consequently affect HIF1-alpha expression (30).

Since, as expected, cetuximab did not affect EGFR mRNA levels, and the partial abrogation of EGFR mRNA levels by namitecan, or SN-38, we observed at early time points could not explain the complete degradation of EGFR protein observed in combination-treated cells, additional mechanisms had to be considered.

There is accumulating evidence that EGFR down-regulation involves internalization and subsequent degradation of the activated receptor in lysosomes (32, 41, 42). In the present study, we found that down-regulation of EGFR induced by namitecan as well as by cetuximab treatment
correlated with the internalization and increased ubiquitination of EGFR, which occurred independently of c-Cbl, an ubiquitin ligase responsible for EGFR degradation after EGF binding (5, 31, 33). It has previously been reported that other drugs, induce down-regulation of EGFR without the requirement of c-Cbl binding (34, 35, 42-45). In particular, epigallocatechin gallate and 17-N-allylamino-17-demethoxygeldanamycin cause EGFR down-regulation via phosphorylation at Ser1046/1047 by p38 MAPK in different tumor cell lines (43). Although phosphorylation of serine residues and of Tyr1045 are essential for EGF-induced receptor ubiquitination (32, 42), Oksvold MP et al. (46) found that only the serine residues are critical for EGFR internalization and degradation. Different agents such as UV radiation (47) and cisplatin (48), as well as oxidative stress (49), can induce internalization of the EGFR but not degradation. On the contrary, gemcitabine was found to cause ligand-independent internalization and degradation of EGFR (50).

Here we report that both namitecan and SN-38 induced an early p38 MAPK activation and EGFR phosphorylation at ser 1046/47 in SCC cells, consistent with a recent observation indicating that irinotecan treatment activates p38 MAPK in HCT 116 cell lines (36). The role of p38 MAPK as an early event in EGFR down-regulation was confirmed through inhibition of p38 MAPK with the specific inhibitor SB203580 and SB202190, which by themselves did not affect the EGFR levels but partially or almost completely restored EGFR expression in namitecan- and SN-38-treated SSC cells at 24 h, without affecting cetuximab-induced EGFR down-modulation. At later time points when a complete abrogation of EGFR protein expression, as a consequence of the marked transcriptional down-regulation of the gene, was observed, the relevance of p38 MAPK appeared negligible. Indeed, the pre-treatment with p38 MAPK inhibitor SB203580 failed to rescue EGFR protein expression in namitecan- and SN-38-treated cells. In addition, the marginal role of p38 MAPK in the drug synergistic interaction was confirmed by inability of
SB203580 to impair the synergism. Conversely cell exposure to SB203580 enhanced namitecan cytotoxic activity, in keeping with previous evidence reported by Paillas et al. (36), demonstrating a synergistic interaction between irinotecan and p38 MAPK inhibition in colon adenocarcinoma cell lines.

Overall, results from the present study clearly demonstrate a synergistic antitumor activity of the namitecan-cetuximab combination. It is likely that the therapeutic potential of this combination could be a common feature of other camptothecins, as observed with irinotecan. However, although in cellular studies the synergistic effect was directly related to the level of EGFR protein expression/EGFR gene copy number, in vivo the contribution of EGFR appeared to be more complex, because no close correlation was found between the efficacy of the combination and the level of gene expression. The low efficacy of namitecan plus cetuximab against the SiHa model was consistent with a critical role of modulation of EGFR function in tumor response.

Given the complexity of tumor biology, the single-agent therapy with a drug targeting a single alteration is unlikely to achieve an effective and persistent control of tumor growth. To overcome the limitation of single-agent therapy, combination strategies have widely been used. A rational selection of the drug combination is critical to optimize treatment efficacy. As shown in our study, knowledge of the biological context of various tumor types and of the mechanism of drug action provides the basis for identifying synergistic interactions to be exploited in the clinical setting. Taking into account the good therapeutic index and the impressive efficacy of the cetuximab/camptothecin combination, our results may have obvious clinical implications. The combination therapy including cetuximab and a camptothecin may be a promising approach for the therapy of SCC, with special reference to patients with tumor characterized by a high EGFR gene copy number.
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References


Figure 1. Biochemical and molecular characteristics of squamous cell carcinoma (SCC) cell lines. **A) EGFR protein expression Levels.** Western-blot analysis was performed on lysates from SSC cells. Cropped blots are reported. Full-length gels are included in Figure S7. **B) EGFR mRNA levels of expression.** Real time PCR analysis of levels of EGFR in SSC cells. **C) Summary of EGFR, MET gene amplification and of EGFR, KRAS, BRAF, NRAS, PI3KCA mutational status.** Determination of relative EGFR or MET gene copy number by quantitative Real-Time PCR was performed as described in Materials and methods. DNA sequence analyses were carried out as described in Materials and methods and Supplementary methods.

Figure 2. Antitumor activity of namitecan, cetuximab alone or in combination against four SSC xenografts. Namitecan (10 mg/kg) and cetuximab (1 mg/mouse) were administrated i.v. or i.p., respectively, with an intermittent treatment schedule (q4dx4). The indicated ratios refer to the number of regrowing tumors/total treated tumors. (○) control untreated tumors; (▲) cetuximab; (△) namitecan; (●) namitecan plus cetuximab. Results of three experiments are shown.

Figure 3. **A) EGFR protein expression in tumor xenografts treated with cetuximab and namitecan alone or in combination.** A431, A431/TPT and SiHa tumor samples were frozen and then extracted 24 h after the second administration of namitecan (30 or 10 mg/kg) and cetuximab (1 mg/mouse) alone or in combination. **B) EGFR protein expression levels in cell lines.** SCC cells were lysed after 24 h of treatment with different concentrations of namitecan (µM) and with a cetuximab concentration corresponding to IC20 values for each cell line (A431, 0.5 µg/ml; A431/TPT, 50 µg/ml, Caski and SiHa, 100 µg/ml). Exposure period of membrane to film has been increased in SiHa to highlight the differences in EGFR expression levels between control and treated samples. Cropped blots are reported. Full-length gels are included in Supplementary
data (Supplementary Figure S7). To improve the clarity of the SiHa blots, membrane film has been downsized and so uncropped blots could not be presented in Supplementary Figure S7. C) **EGFR mRNA of expression levels in SCC cell lines.** Real time PCR analysis of levels of EGFR in cells treated with cetuximab or ST1968 alone or in combination for 24 h harvested as in A).

**Figure 4.** A) **Immunofluorescence analysis of EGFR localization.** A431 cells were treated with cetuximab (0.5 µg/ml) and namitecan (0.01 µM) for 4 h and then fixed by paraformaldehyde. Cells were stained with EGFR (green signal), and nuclei were counterstained with Heochst 33342 (blue signal). Bar=10 µM. Representative data from two to three independent experiments are shown. B) **Immunofluorescence analysis of EGFR and c-Cbl distribution** in control and cetuximab-, namitecan-, or namitecan plus cetuximab-treated A431 cells. Cells were exposed to cetuximab (0.5 µg/ml) and namitecan (0.01 µM) for 4 h. After fixation in paraformaldehyde, cells were stained for EGFR (green signal) and c-Cbl (red signal). Bar = 10 µm. Representative data from two to three independent experiments are shown.

**Figure 5.** A) **Levels of EGFR phosphorylation at Ser1046/47 and of p38 MAPK phosphorylation.** A431 cells were exposed to cetuximab (0.5 µg/ml) or namitecan (0.01 µM) for 1, 2, 4 and 24 h, and protein extracts were then harvested and examined. Vinculin was used as loading control. Cropped blots are reported. Full-length gels are included in Supplementary Figure S7. B) and C) **Effect of p38 MAPK inhibition on EGFR phosphorylation.** A431 (B) and Caski (C) cells were pretreated with 10 µM SB203580 for 2-h and then treated with cetuximab (0.5 µg/ml, A431 cells; 100 µg/ml, Caski cells) or namitecan (0.01 µM) alone or in combination for 24 h. An antibody to vinculin was used to control for protein loading. For phosphorylated EGFR (Ser1046/47), a higher film exposure compared to Fig. 4A was shown to
highlight the differences in expression levels between SB203580-pretreated and not-pretreated samples. Cropped blots are reported. Full-length gels are included in Supplementary Figure S7.

**D) EGFR mRNA of expression levels in A431 cells.** Real time PCR analysis of EGFR mRNA levels in A431 cells treated with cetuximab (0.5 μg/ml) or namitecan (0.01μM) or SN-38 (0.01μM) alone or in combination for 48 h. **E) Effects of p38 MAPK inhibition on levels of EGFR phosphorylation and on EGFR protein expression.** A431 cells were pretreated with SB203580 for 2 h and then treated with cetuximab (0.5μg/ml) or namitecan (0.01μM) or SN-38 (0.01μM) alone or in combination for 48 h. Cells were lysed and protein extracts were then examined. Vinculin was used as loading control. For phosphorylated EGFR (ser1046/47) and EGFR blots, exposure period of membrane to film has been increased to highlight the complete abrogation of EGFR protein expression. Consequently, the higher film exposure caused the drop of the EGFR protein modulation in cetuximab-treated compared to untreated cells. Cropped blots are reported. Full-length gels are included in Supplementary Figure S7.
Figure 1
Figure 2
Figure 3

A

A431

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Table 1. Activity of i.v. namitecan, 10 mg/kg, or irinotecan, 17 mg/kg and i.p. cetuximab, 1 mg/mouse, q4dx4 on human squamous cell carcinomas xenografted in nude mice.

Tumor fragments were implanted on both flanks at day 0. Treatment started when mean tumor volume was 80-90 mm³.

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<sup>a</sup>Tumor volume inhibition % in treated over control mice, determined one week after the last treatment.

<sup>b</sup>Complete responses, i.e. disappearance of tumor lasting at least 10 days.

<sup>c</sup>Body weight loss percentage induced by treatment; the highest change is reported. No toxic death was observed.

*P<0.05, **P<0.01 by Fisher’s exact test vs namitecan-treated mice.

***P<0.01, ****P<0.001 by Fisher’s exact test vs irinotecan-treated mice.
Synergistic antitumor activity of cetuximab and namitecan in human squamous cell carcinoma models relies on cooperative inhibition of EGFR expression and depends on high EGFR gene copy number

Valentina Zuco, Michelandrea De Cesare, Calogero Lauricella, et al.

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