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Combined Targeting of Epidermal Growth Factor Receptor and MDM2 by Gefitinib and Antisense MDM2 Cooperatively Inhibit Hormone-Independent Prostate Cancer

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ABSTRACT

Purpose: **The epidermal growth factor receptor (EGFR) may play a relevant role in the progression, hormone therapy resistance, and prognosis of prostate cancer patients. Also MDM2, a negative p53 regulator that interacts with retinoblastoma (Rb), E2F, $p19^{arf}$ and the ras-mitogen-activated protein kinase(MAPK) cascade plays an important role in prostate cancer progression and prognosis. On the basis of the EGFR and MDM2 role in integrating signaling pathways critical for prostate cancer progression, we investigated whether their selective combined blockade may have a cooperative antitumor effect in prostate cancer. For this purpose, we have used the EGFR tyrosine kinase inhibitor gefitinib (ZD1839, Iressa) and a second generation hybrid oligonucleotide antisense MDM2 (AS-MDM2), respectively.**

Experimental Design: **Gefitinib and AS-MDM2 were administered to hormone-refractory and hormone-dependent human prostate cancer cells *in vitro* and to mice bearing tumor xenografts, evaluating the effects on growth, apoptosis, and protein expression, *in vitro* and *in vivo*.**

Results: **We demonstrated that the combination of gefitinib and AS-MDM2 synergistically inhibits the growth of hormone-independent prostate cancer cells *in vitro*. This effect is accompanied by the inhibition of MDM2, phosphorylated Akt (pAkt), phosphorylated MAPK (pMAPK), and vascular endothelial growth factor (VEGF) expression and by Rb hypophosphorylation. The combination of the two agents in nude mice bearing the same hormone-independent tumors caused a potent cooperative antitumor effect. Tumor samples analysis confirmed the inhibition of MDM2, pAkt, pMAPK, VEGF, and basic fibroblast growth factor expression.**

Conclusions: **This study shows that EGFR and MDM2 play a critical role in the growth of prostate cancer, especially hormone-dependent, and that their combined blockade by gefitinib and AS-MDM2 causes a cooperative antitumor effect, supporting the clinical development of this therapeutic strategy.**

INTRODUCTION

The development of prostate cancer from androgen-dependent to hormone-refractory disease is a complex multistep process involving a network of signaling molecules. Among them, a key role is played by the activated epidermal growth factor receptor (EGFR), a major transducer of mitogenic signals and inducer of angiogenic growth factors and neoangiogenesis, which is involved in pathogenesis and progression of several human cancers (1). It has been demonstrated that EGFR expression increases during the natural history of prostate cancer and the progression from hormone-dependence to hormone-refractory disease (2, 3). We have recently shown that EGFR has a potent independent prognostic effect on disease-free survival, when evaluated by a Cox multivariate analysis (4). On this basis, EGFR-targeted drugs could be of therapeutic relevance in prostate cancer (3). Gefitinib (ZD1839, Iressa) is an orally active EGFR tyrosine kinase inhibitor that has shown antitumor activity in a variety of human cancer types, including prostate cancer, alone and in combination with other agents (3, 5–7).

MDM2 is an oncogene cloned in a spontaneously transformed cell line (8, 9). MDM2 encodes for a protein containing a p53-binding domain that binds to and inactivates p53 protein (9, 10). This favors its ubiquitination and proteosomal degradation, because it has been recently shown that MDM2 belongs to the RING finger ubiquitin ligase family (10). On the other hand, p53 induces MDM2 transcription and expression (9), indicating that MDM2 and p53 constitute an integral part of a self-regulatory loop. MDM2 overexpression abrogates several p53-dependent functions, including gene transcription and control of cell proliferation and apoptosis (9, 10). Particularly relevant is the fact that MDM2 also plays a p53-independent role. In fact, not only is MDM2 able to bypass p53 by directly binding to p21 protein, favoring its proteasome-mediated degradation (11), but it also interacts

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negatively with tumor suppressor protein $p19^{ARF}$, a product of the frequently mutated (in tumors) *ARF-Ink4a* (9, 12), binds to the retinoblastoma gene product (Rb; Ref. 13) and to E2F (9, 14). Moreover, a relevant direct functional link has been demonstrated between MDM2 and the ras-raf-mitogen-activated protein kinase (MAPK) signaling pathway (15, 16). Therefore, MDM2 has unique features, integrating multiple and independent pathways involved in cell growth and apoptosis. MDM2 is amplified and/or overexpressed in a large number of human tumors, including sarcomas and several hematological and solid tumors (10, 17–19). In a study conducted in a cohort of prostate cancer patients, MDM2 expression has been associated with features of more advanced disease, suggesting that its overexpression inactivates p53 and favors prostate cancer progression (20). Because MDM2 can be stabilized by mutated p53 (21), it may also play a role in tumors harboring a mutant p53, regardless of its amplification status.

For the above reasons, MDM2 is considered a potentially relevant target for cancer therapy, and different approaches have been used to inhibit its expression and function, including antisense oligonucleotides (22–24). An antisense MDM2 (AS-MDM2) of a novel class, defined mixed-backbone oligonucleotides, with hybrid DNA/RNA structure, has shown an ability to inhibit the growth of a large variety of human tumors, including prostate cancer, harboring either wild-type or mutated p53, and to cooperate with several class of cytotoxic drugs (22, 25, 26) or with radiotherapy (27), both *in vitro* and *in vivo*, inducing apoptosis. More recently, this AS-MDM2 has shown activity against human prostate cancer cells *in vitro* and *in vivo* and an ability to cooperate with selected classes of cytotoxic drugs (28).

On the basis of these data, EGFR and MDM2 may play a critical role in the development of hormone-independent prostate cancer and control several key signal transducers. In this study, we investigated the hypothesis that these two pathways may share functional interactions with such signal transducers and that their combined blockade by the selective inhibitors gefitinib and AS-MDM2 may have an impact on prostate cancer growth.

MATERIALS AND METHODS

Cell Cultures. Hormone-refractory PC3 and DU145 and hormone-sensitive LNCaP human prostate cancer cells, purchased from the American Type Culture Collection (Manassas, VA), were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 4 mM glutamine (ICN, Irvine, United Kingdom) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Mixed Backbone Oligonucleotides. The two oligonucleotides used in the study are: a 20-mer mixed-backbone oligonucleotide targeting the human MDM2 (26), **UG**ACACCTGTTCTCAC**UCAC** (AS-MDM2), and the mismatch control **UG**TCACCCTTTTTCAT**UCAC** (Mm-ON). Both oligonucleotides contain 2′-*O*-methylribonucleosides at the 5′ end and the 3′ end (identified by bold face letters), the remaining are deoxynucleosides. Synthesis of oligonucleotides and confirmation of their identity and purity by ^{31}P NMR and capillary gel electrophoresis, were carried out as described previously (29).

Growth in Soft Agar and Analysis of Combination Index. On day 0, cells (10^4 cells/well) were suspended in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson, Lincoln Park, NJ) and were treated on days 0–2 with doses of AS-MDM2, Mm-ON, and gefitinib, alone and in combination, ranging from 0.1 to 5 μM. After 10–14 days, cells were stained with nitroblue tetrazolium (Sigma), and colonies larger than 0.05 mm were counted. Selection of drug doses and combination analysis were performed following the method described by Chou and Talalay (30) and using the Calcusyn software program (Biosoft, Cambridge, United Kingdom).

Western Blot Analysis. Total cell lysates were obtained either from cells cultured *in vitro* or from homogenized tumor specimens. The protein extracts were resolved by 4–15% SDS-PAGE and probed with antihuman monoclonal MDM2 (Oncogene, Cambridge, MA), monoclonal Akt, and monoclonal pAkt (Cell Signaling Technologies, Beverly, MA), monoclonal actin (Sigma-Aldrich, Milan, Italy), monoclonal EGFR (Lab Vision, Fremont, CA), monoclonal Rb, MAPK, pMAPK, and VEGF, and polyclonal basic fibroblast growth factor (bFGF) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Phosphorylated EGFR was detected by immunoprecipitating cell lysates with an anti-EGFR monoclonal antibody (Santa Cruz Biotechnology), then resolving the protein extracts with an anti-pTyr monoclonal antibody (Santa Cruz Biotechnology). Immunoreactive proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL), as described previously (26).

Apoptosis in Cultured Cells. Apoptosis was determined by the Cell Death Detection ELISA Plus kit (Roche Molecular Biochemicals, Mannheim, Germany). Cells (5×10^4 cells/dish in quadruplicate) treated for 4 days with the indicated drugs, alone and in combination, were processed on day 5 and the ratio $A_{405\ nm}$-treated:*A*-untreated cells was defined as apoptotic index, as described previously (26).

Xenografts in Nude Mice. Five-week-old Balb/cAnN-CrlBR athymic (nu+/nu+) mice (Charles River Laboratories, Milan, Italy) were maintained in accordance with institutional guidelines of the University of Naples Animal Care Committee and in accordance with the Declaration of Helsinki. PC3 or DU145 human prostate cancer cells (10^7 cells/mice) were resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA) and were injected s.c. in mice. After 7 days, tumors were detected and groups of 10 mice were randomized to receive the following treatments: oral gefitinib, 150 mg/kg; i.p. or oral AS-MDM2, 10 mg/kg; i.p. or oral Mm-ON, 10 mg/kg; or the combination of gefitinib and either AS-MDM2 or Mm-ON, on days 7–11, 14–18, 21–25, and 28–32. Tumor volume was measured using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as reported previously (26). Two mice were sacrificed at day 32 to perform biochemical analysis.

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RESULTS

Effect of Different Treatments on Cancer Cell Growth. We evaluated a wide range of doses of AS-MDM2, its control oligonucleotide Mm-ON and gefitinib to assess their antiproliferative activity on the soft agar growth of hormone-independent PC3 and DU145 and hormone-dependent LNCaP prostate cancer cells. The effects of drugs, alone and in combination at fixed molar ratios, according to the method of Chou and Talalay, are summarized in the dose-response fit curves generated (Fig. 1). Fig. 1, *A, C,* and *E,* show that AS-MDM2 and gefitinib have dose-dependent antiproliferative effects in all of the three cell lines tested, alone and in combination, the LNCaP cells being the most sensitive to the AS-MDM2. Conversely, Mm-ON caused only a mild growth-inhibitory effect on the three cell lines, even at the higher doses. Moreover, although a positive cooperation was observed with AS-MDM2 in combination with gefitinib, Mm-ON did not significantly modify the inhibitory effect of gefitinib alone (Fig. 1, *B, D,* and *F*). To better evaluate the interaction between gefitinib and either AS-MDM2 or Mm-ON, we performed a combination analysis and generated combination index (CI) and isobologram curves, according to Chou and Talalay (30), using an automated calculation software (Fig. 2). Values of CI <1 indicate synergism. Fig. 2, *A* and *B,* demonstrates a strong synergism of action of AS-MDM2 in combination with gefitinib in hormone-independent PC3 cells (average CI, 0.71) and, particularly, in DU145 cells (average CI, 0.53), whereas the effect was only additive in hormone-dependent LNCaP cells (Fig. 2*E*). Confirming the data observed in the dose-response curves, the addition of Mm-ON to gefitinib had no cooperative effect in any of the cell lines tested (Fig. 2, *B, D,* and *F*).

Effect of Different Treatments on Protein Expression and Apoptosis. We performed a Western blot analysis of cells treated with the different agents, using actin as control for protein loading (Fig. 3). Gefitinib or the control oligonucleotide Mm-ON, even at high doses, had no effect on MDM2 expression, whereas a suboptimal dose AS-MDM2 caused about 30% inhibition of target protein expression. The combination of AS-MDM2 and gefitinib further decreased MDM2 protein levels. We then analyzed the expression of proteins involved in the mitogenic cascade. Gefitinib, but not AS-MDM2, caused an inhibition of tyrosine-phosphorylated EGFR. The combination of these two drugs only moderately enhanced the inhibitory effect caused by gefitinib. Conversely, the expression of total

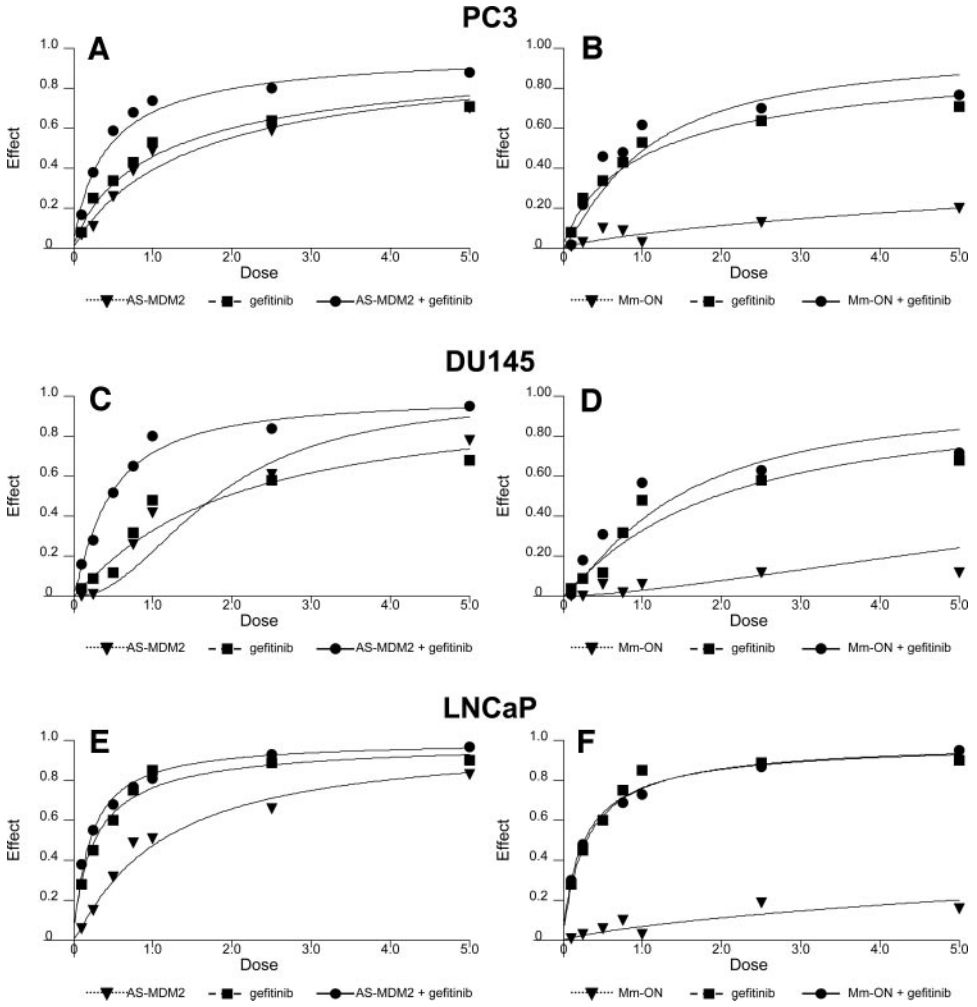


Fig. 1 Effect of human MDM2, **UG**ACACCTGTTCTCAC**UCA-C** [antisense (AS)-MDM2], gefitinib, and mismatch control **UG**TCACCCTTTTTCAT**UCAC** (Mm-ON) on the soft agar growth of PC3, DU145, and LNCaP cells. *A, C,* and *E,* AS-MDM2 and gefitinib, alone and in combination. *B, D,* and *F,* Mm-ON and gefitinib, alone and in combination. Doses of each drug ranged from 0.1 to 5 μM and were used at fixed molar ratio when the drugs were combined, according to the Chou and Talalay method (30). Data are expressed as percentage colony formation and curves are generated using the Calcusyn software. The data represent means and SEs of triplicate determinations of at least two experiments.

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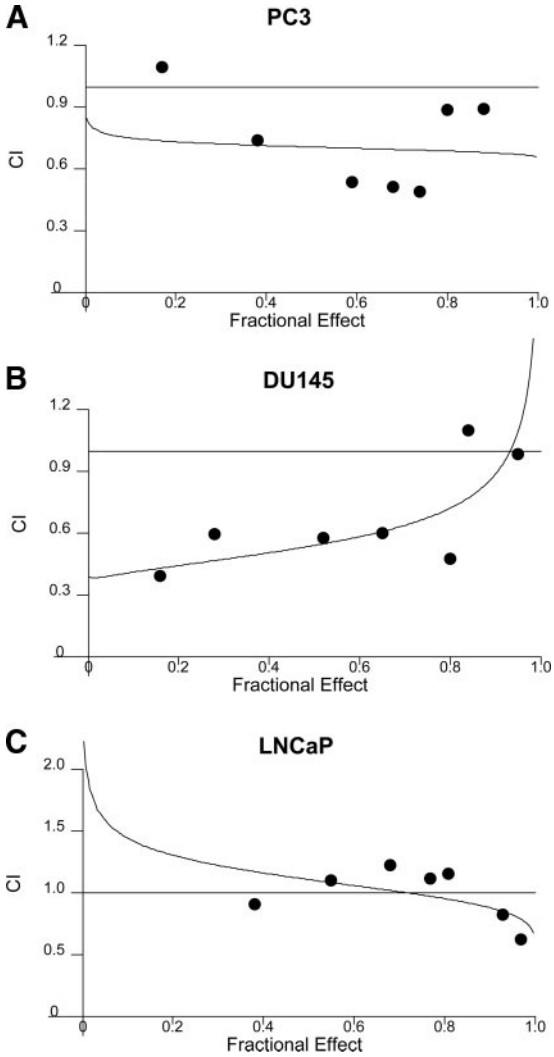


Fig. 2 Western blotting analysis of different proteins expression in PC3 cells. Cells were treated for 4 days with antisense (AS)-MDM2 1 μM, mismatch control (Mm-ON) 1 μM, or gefitinib 0.5 μM, alone and in combination, and cell lysates were processed and incubated with respective antibodies, as described in the "Materials and Methods" section. *CI*, combination index.

unphosphorylated EGFR was unmodified by any of the two agents (data not shown). Unlike total Akt expression, which was only slightly affected by each agent used alone or in combination, the levels of activated pMAPK and pAkt were inhibited by gefitinib and, although to a lesser degree, also by AS-MDM2. The combination of the two agents almost completely suppressed both activated proteins (Fig. 3).

Because MDM2 also interferes with Rb function, we analyzed the expression of Rb protein. AS-MDM2 inhibited the expression of the phosphorylated form of Rb more efficiently than did gefitinib, but the combination of the two agents almost completely inhibited phosphorylated Rb. Finally, we measured the activity of these agents on VEGF expression. VEGF was inhibited by single agent AS-MDM2 or gefitinib and was almost suppressed when the two agents were combined. In none of the conditions, did Mm-ON significantly change the effect of gefitinib alone (Fig. 3).

An analysis of apoptosis showed that the suboptimal doses of gefitinib and AS-MDM2 caused a 1.7-fold and a 1.9-fold increase of apoptosis, respectively, compared with untreated cells. Combination of the two agents caused an approximate 3.5-fold increase of apoptotic cells, thus showing an almost additive effect (data not shown).

Effect of Treatment on Tumor Xenografts in Nude Mice. We investigated the antitumor activity of gefitinib, AS-MDM2, or Mm-ON administered i.p. or orally, alone and in combination, in nude mice bearing hormone-independent PC3 or DU145 prostate cancer xenografts. Groups of 10 mice were treated with the different agents, alone and in combination. Two mice were sacrificed on day 32 to perform biochemical analysis; therefore, tumor growth studies were performed on the remaining eight mice. Within approximately 10 weeks PC3 tumors reached a size not compatible with normal life (Fig. 4 *A*). Treatment with gefitinib or AS-MDM2 alone at the dose of 150 mg/kg and 10 mg/kg, i.p., respectively, caused about 30–40% inhibition of tumor growth. Gefitinib and AS-MDM2, given in combination, caused a tumor growth inhibition of ~80–90%. Tumor growth was absent or moderate for almost 5 weeks after treatment withdrawal, up to 10 weeks after tumor cell injection (Fig. 4*A*). At this time point, pathological evaluation showed that 5 of 8 mice were still tumor free. Combination of gefitinib

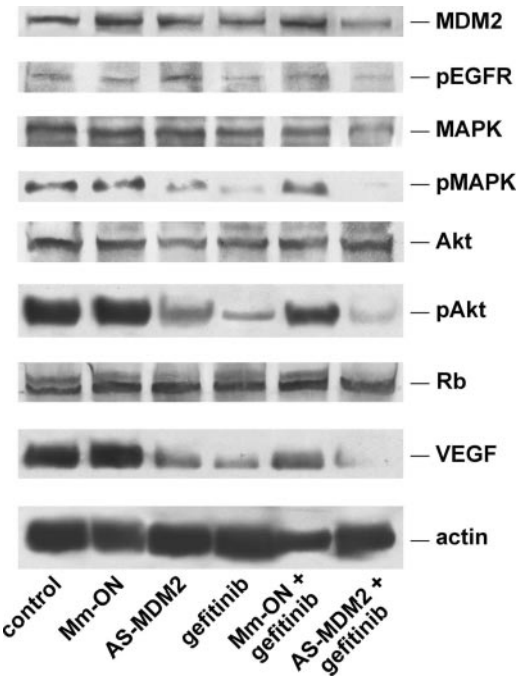


Fig. 3 Effect of the treatment with antisense (AS)-MDM2, gefitinib, or mismatch control (Mm-ON) on the growth of PC3 and DU145 tumor xenografts. The schedule and doses of each single agent, alone or in combination were: AS-MDM2, 10 mg/kg; Mm-ON, 10 mg/kg; gefitinib, 150 mg/kg; daily, on days 7–11, 14–18, 21–25, and 28–32. *pEGFR*, phosphorylated epidermal growth factor receptor; *MAPK*, mitogen-activated protein kinase; *pMAPK*, phosphorylated MAPK; *Rb*, retinoblastoma; *VEGF*, vascular endothelial growth factor.

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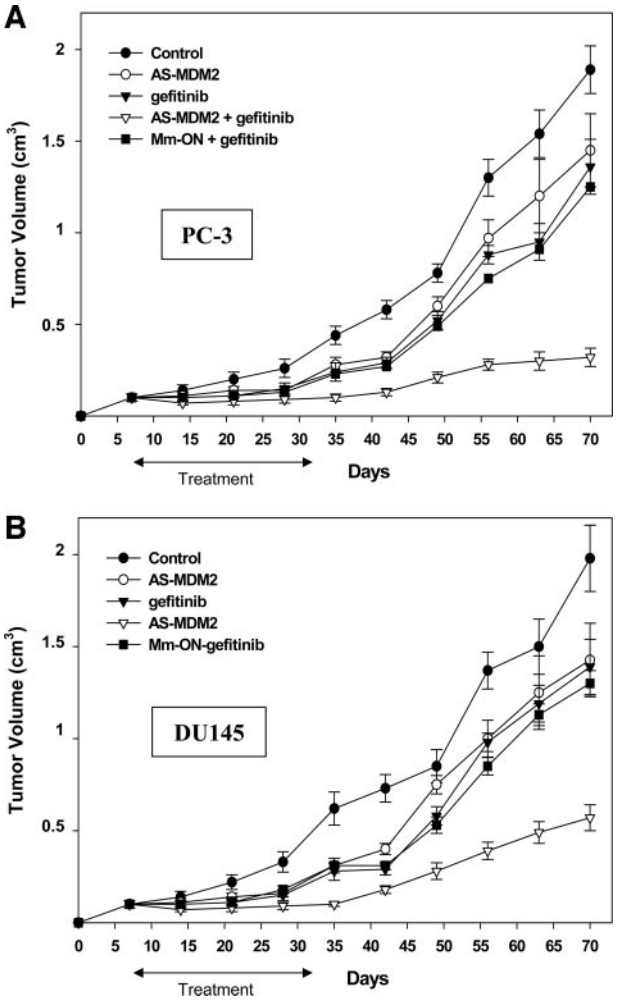


Fig. 4 Western blotting analysis of protein extracts from PC3 tumor specimen. Analysis was performed on total lysates from tumor specimens of two mice sacrificed at day 32 and treated as described in the "Materials and Methods" section.

with Mm-ON resulted in a modest increase of the effect observed with gefitinib. The combined treatment was well tolerated; no weight loss or other signs of acute or delayed toxicity were observed. Similar results were obtained when the AS-MDM2 was administered orally (data not shown).

The antitumor activity of gefitinib and AS-MDM2, alone and in combination, was also studied in mice bearing DU145 xenografts. An inhibition of tumor growth of 27% and 30% was observed with AS-MDM2 and gefitinib, respectively, at 10 weeks after tumor injection. At the same time point, the combination of the two agents together caused about 70% inhibition of tumor growth, whereas the addition of control oligonucleotide Mm-ON to gefitinib resulted only in a slight increase of the effect produced by gefitinib alone (Fig. 4*B*). Preliminary short-term experiments in nude mice bearing LNCaP tumors show an increased inhibitory effect when AS-MDM2 and gefitinib are used in combination, although to a lesser degree when compared with hormone-independent PC3 and DU145 (data not shown).

As represented in Fig. 5, Western blot analysis of PC3 tumors removed at the end of treatment, on day 32, demonstrated an inhibition of MDM2 protein by the specific antisense and a marked inhibition by AS-MDM2 and gefitinib used together. As observed also *in vitro*, both pMAPK and pAkt were inhibited by each single agent and cooperatively inhibited by the two agents in combination. Analysis of VEGF and bFGF expression showed an inhibitory activity by gefitinib and only a moderate reduction with AS-MDM2, whereas the combination of the two agents resulted in a marked reduction of VEGF and a suppression of bFGF expression. Tumor specimens from animals treated with gefitinib and Mm-ON revealed minor changes as compared with animals treated with gefitinib alone (Fig. 5).

DISCUSSION

The majority of patients with relapsed or metastatic prostate cancer are initially responsive to hormonal manipulation; however, failure of first-line hormonal therapy leads to a fatal and rapid progression of androgen-independent disease. For these reasons, the past few years have witnessed an extensive search for biological factors responsible for the shift to the hormone refractory status. EGFR, whose overexpression is associated with growth and induction of angiogenesis in different types of cancer (1), has been recently associated with poor prognosis and with progression of prostate cancer to hormone-independence (2, 4); for these reasons, there is growing interest

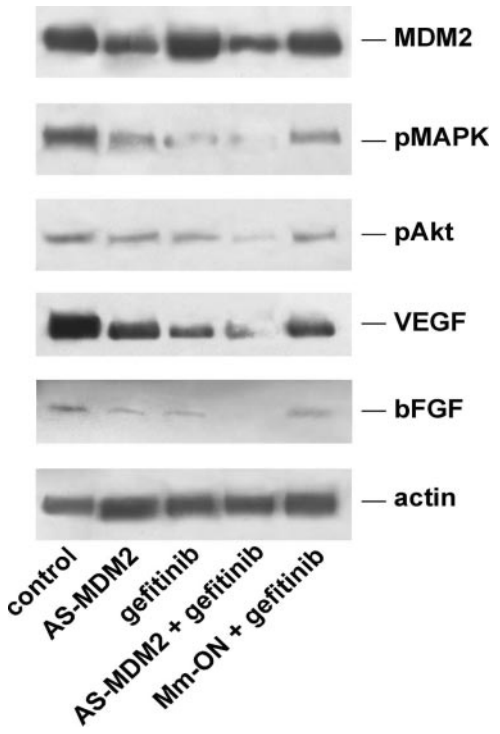


Fig. 5 Protein expression of PC3 tumor specimens. *pEGFR*, phosphorylated epidermal growth factor receptor; *pMAPK*, phosphorylated mitogen-activated protein kinase; *pAkt* phosphorylated Akt; *VEGF*, vascular endothelial growth factor; *bFGF*, basic fibroblast growth factor; *AS-MDM2*, antisense-MDM2; *Mm-ON*, mismatch control.

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in using an anti-EGFR approach in the treatment of hormone-refractory prostate cancer patients. Gefitinib has shown potent activity against prostate cancer cell lines and an ability to cooperate with anti-androgen drugs in hormone-refractory cancer cells (3, 5).

A potentially relevant role in prostate cancer progression and prognosis has also been attributed to two other proteins involved in multiple functions, such as bcl-2 (31) and MDM2 (20). In the past few years MDM2 has gathered increasing attention after the demonstration that, in addition to its function as a master regulator of p53, it is also structurally or functionally linked to other critical molecules, such as Rb, E2F, $p19^{arf}$ and *ras*, thus connecting the main pathways controlling both proliferation and apoptosis (10). MDM2 is deregulated and/or overexpressed in a large number of human tumors and has been associated with prostate cancer progression (20). For the above reasons, MDM2 has been recognized as a potentially relevant target for cancer treatment. Different selective inhibitors have been developed to interfere with the MDM2-p53 module (such as synthetic peptides, natural agents, and, more recently, a small molecule) or to inhibit MDM2 expression [such as antisense oligonucleotides (22–24, 32)]. In this regard, a second generation oligonucleotide antisense MDM2 has shown a potent antitumor activity against a large variety of human cancer types *in vitro* and in mice, regardless of the p53 status, cooperating with several classes of cytotoxic drugs (22, 26). The antiproliferative effect is associated with modulation of p53 and/or $p21^{waf-1}$, and with induction of apoptosis (22). Recently, it has been reported that this compound has a potent antiproliferative effect in prostate cancer cells and the ability to cooperate with selected chemotherapeutic agents and with radiotherapy, *in vitro* and *in vivo* (27, 28).

On the basis of the above studies, we have hypothesized that there is an interplay between the multifunctional EGFR and MDM2 pathways and that their combined blockade could greatly affect tumor growth, particularly in hormone-independent prostate cancer cells. Therefore, in the present study we have investigated this therapeutic opportunity by combining gefitinib and AS-MDM2 in different human prostate cancer cells.

We have demonstrated a synergistic antiproliferative effect of the two agents in combination on soft agar growth of androgen-independent PC3 and DU145 cells, although the effect was mostly additive on hormone-dependent LNCaP. Analysis of protein expression has shown an inhibition of the target proteins MDM2 and phosphorylated EGFR. Increasing interest has been drawn by signaling molecules acting downstream from the activated EGFR, such as MAPK and Akt, for their potential role in cancer progression. Treatment of prostate cancer cells with gefitinib or AS-MDM2 caused inhibition of the activated proteins pMAPK, pAkt, and phosphorylated Rb, an effect which was highly enhanced by the use of the two agents together, suggesting a relevant participation of EGFR and MDM2 pathways in the control of the proliferative machinery. Moreover, expression of VEGF was completely suppressed by the two agents in combination.

Analysis of apoptosis demonstrated the ability of AS-MDM2 or gefitinib to induce apoptosis and an additive effect when the two agents were used together.

We translated this strategy *in vivo*, in nude mice bearing hormone-independent PC3 and DU145 tumors. Treatment with AS-MDM2 or gefitinib caused a similar significant inhibition of tumor growth, delaying the death of mice by about 2 weeks. The use of the two drugs in combination, despite the short treatment, determined a marked inhibitory effect and a delay of several weeks for tumor growth recovery. Moreover, the majority of mice were tumor-free at pathological evaluation. These effects were not reproduced when gefitinib was combined with the control Mm-ON.

We analyzed the tumor specimens by Western blot to evaluate the effect of treatment on protein expression. We observed that the AS-MDM2 alone, as previously reported (28), was able to inhibit MDM2 target protein expression and that the inhibition was further enhanced by the addition of gefitinib to AS-MDM2. The two agents also produced a cooperative inhibition of pMAPK expression. We have previously shown that gefitinib is able to inhibit the expression and secretion of a variety of growth and angiogenic factors, including VEGF and bFGF (33). On the other hand, it has been demonstrated that Akt and the angiogenic growth factors may represent escape pathways for tumor progression and may be implicated in the acquisition of resistance to treatments, particularly with certain biological agents (34, 35). We have demonstrated a complete suppression of pAkt, VEGF and bFGF in specimens from mice treated with gefitinib and AS-MDM2 together.

The present study suggests that EGFR and MDM2 represent critical signaling pathways controlling a broad range of key molecules involved in prostate cancer. Their combined blockade by noncytotoxic selective agents, such as the EGFR tyrosine kinase inhibitor gefitinib and the second generation hybrid oligonucleotide AS-MDM2, causes the down-regulation of these critical proteins involved in cell growth and angiogenesis, resulting in a potent antitumor activity. Moreover, because both agents are active by oral administration, this strategy may be worthy of investigation in a clinical setting either to treat androgen-independent prostate cancer or to prevent the fatal tumor progression from hormone-dependent to hormone-independent status.

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