EC-70124, a Novel Glycosylated Indolocarbazole Multikinase Inhibitor, Reverts Tumorigenic and Stem Cell Properties in Prostate Cancer by Inhibiting STAT3 and NF-κB

Gianluca Civenni1, Nicole Longoni1, Paula Costales2, Cecilia Dallavalle1, Cristina García Inclán3, Domenico Albino1, Luz Elena Nuñez2, Francisco Morís2, Giuseppina M. Carbone1,4, and Carlo V. Catapano1,4,5

Abstract

Cancer stem cells (CSC) contribute to disease progression and treatment failure in prostate cancer because of their intrinsic resistance to current therapies. The transcription factors NF-κB and STAT3 are frequently activated in advanced prostate cancer and sustain expansion of prostate CSCs. EC-70124 is a novel chimeric indolocarbazole compound generated by metabolic engineering of the biosynthetic pathways of glycosylated indolocarbazoles, such as staurosporine and rebeccamycin. In vitro kinase analyses revealed that EC-70124 acted as a multikinase inhibitor with potent activity against IKKβ and JAK2. In this study, we show that EC-70124 blocked concomitantly NF-κB and STAT3 in prostate cancer cells and particularly prostate CSCs, which exhibited overactivation of these transcription factors. Phosphorylation of IkB and STAT3 (Tyr705), the immediate targets of IKKβ and JAK2, respectively, was rapidly inhibited in vitro by EC-70124 at concentrations that were well below plasma levels in mice. Furthermore, the drug blocked activation of NF-κB and STAT3 reporters and suppressed transcription of their target genes. Treatment with EC-70124 impaired proliferation and colony formation in vitro and delayed development of prostate tumor xenografts. Notably, EC-70124 had profound effects on the prostate CSC subpopulation both in vitro and in vivo. Thus, EC-70124 is a potent inhibitor of the NF-κB and STAT3 signaling pathways and blocked tumor growth and maintenance of prostate CSCs. EC-70124 may provide the basis for developing new therapeutic strategies that combine agents directed to the CSC component and the bulk tumor cell population for treatment of advanced prostate cancer.

Introduction

Prostate cancer is the most common epithelial cancer and the third leading cause of cancer-related death in men in western countries (1, 2). Substantial progress has been made in recent years to define the molecular and genetic mechanisms underlying prostate carcinogenesis (3). Nevertheless, there has been limited improvement in the management of patients with advanced castration-resistant prostate cancer (CRPC) for which many cancer types (8–11). Accordingly, treatment failure in advanced prostate cancer patients may be attributed to the intrinsic resistance of the subpopulation of prostate CSCs to current therapies, such as androgen deprivation and cytotoxic drugs (8, 10).

Development of new treatment strategies for prostate cancer should take into consideration the biologic complexity and cellular heterogeneity intrinsic to the disease. Drugs able to eliminate the prostate CSC subpopulation could have considerable impact on the management of prostate cancer (8). In this context, the signaling pathways controlling the expansion and maintenance of CSCs could provide the ideal targets for development of CSC-directed therapies (11, 12). The CSCs rely both on cell-autonomous signals and the signals coming from the supportive niche formed by stromal cells within the tumor microenvironment (7, 11). Supportive cells include macrophages, fibroblasts, and mesenchymal stem cells that secrete growth factors and cytokines, such as IL6, and create a milieu enabling the cancer cells to acquire and retain stem-like evidence of intratumor heterogeneity in human cancers whereby distinct cell populations sharing common genetic makeup exhibit different tumor-initiating and metastasis-seeding capability (7). A subpopulation of cancer cells, defined as cancer stem cells (CSC) or tumor-initiating cells, which display stem cell-like properties and high tumor-initiating potential, has been implicated in progression and disease recurrence in many cancer types (8–11). Accordingly, treatment failure in advanced prostate cancer patients may be attributed to the intrinsic resistance of the subpopulation of prostate CSCs to current therapies, such as androgen deprivation and cytotoxic drugs (8, 10).

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properties (13–15). Recent reports point to transcriptional regulators, such as NF-kB and STAT3, as key elements in the signaling pathways sustaining CSCs in human tumors (16–18). Constitutive activation of NF-kB is found in many tumors (19, 20). NF-kB and several of its target genes, including the proinflammatory cytokine IL6, promote cancer development and activation of prosurvival pathways (21). IL6 activates the JAK/STAT3 signaling, which promotes the production of prosurvival, proangiogenic, and immunosuppressive factors (22–25). Activation of STAT3 is frequent in human cancers and is often concomitant with the activation of NF-kB (22).

Notably, STAT3 and NF-kB induce highly overlapping sets of protumorigenic genes with important functions in tumorigenesis and CSC biology (22, 26–28). Furthermore, multiple positive and negative feedback loops link the STAT3 and NF-kB pathways leading to reciprocal activation or inhibition, depending on the cell context and nature of the extracellular stimuli (29, 30).

One of the hallmark in prostate cancer is deregulated expression of ETS transcription factors as consequence of genetic and epigenetic events (31–34). Recently, we reported the establishment of a feedback loop that links the ETS factor ESE1/ELF3 with components of the NF-kB complex and leads to constitutive activation of NF-kB in a clinically aggressive subset of prostate cancers (35). Notably, activation of the ESE1/ELF3/NF-kB axis promoted the production of inflammatory and protumorigenic cytokines, including IL6 (35). The activation of NF-kB and cross-talks with the IL6/JAK/STAT3 signaling pathway were essential for the acquisition of the epithelial-to-mesenchymal transition characteristics and the CSC phenotype in aggressive tumors (35). In such a context, combined targeting of NF-kB and STAT3 could provide a very effective strategy to eliminate prostate CSCs and prevent tumor progression.

Combinatorial biosynthesis by genetic rearrangements of key metabolic enzymes in microorganisms is a powerful approach to produce ‘chimeric’ compounds that resemble natural products but possess new and improved pharmacologic properties (36). Genetic manipulation of the biosynthetic pathways of the glycosylated indolocarbazoles, such as staurosponine and rebeccamycin, was recently reported to give raise to novel compounds retaining the indolocarbazole moiety of rebeccamycin and glycosylation patterns similar to staurosponine (37). Many chimeric compounds acted as potent multi-kinase inhibitors with similar or higher activity compared with staurosponine when assayed against a wide spectrum of human kinase inhibitors with similar or higher activity compared with staurosponine (37). Many chimeric compounds acted as potent multi-kinase inhibitors with similar or higher activity compared with staurosponine when assayed against a wide spectrum of human kinase inhibitors with similar or higher activity compared with staurosponine when assayed against a wide spectrum of human kinase inhibitors with similar or higher activity compared with staurosponine when assayed against a wide spectrum of human kinase inhibitors with similar or higher activity compared with staurosponine when assayed against a wide spectrum of human kinase inhibitors with similar or higher activity compared with staurosponine. One of the hallmark in prostate cancer is deregulated expression of ETS transcription factors as consequence of genetic and epigenetic events (31–34).

Constitutive activation of NF-kB and STAT3, as key elements in the signaling pathways sustaining CSCs in human tumors (16–18). Constitutive activation of NF-kB is found in many tumors (19, 20). NF-kB and several of its target genes, including the proinflammatory cytokine IL6, promote cancer development and activation of prosurvival pathways (21). IL6 activates the JAK/STAT3 signaling, which promotes the production of prosurvival, proangiogenic, and immunosuppressive factors (22–25). Activation of STAT3 is frequent in human cancers and is often concomitant with the activation of NF-kB (22).

Materials and Methods

Cell culture, cell transfection, luciferase reporter assays, and selection of stable cell clones

Prostate cancer cell lines were maintained as described previously (34, 40). All cell lines were obtained from the ATCC, which performs cell line characterization based on DNA profiling (short tandem repeat analysis), and were used within 6 months of culturing. ESE1/ELF3 expressing 22Rv1 cells were described previously (35). Prostasphere (PS) cultures were established as described previously (40–42). Luciferase reporter assays from transient transfection were carried out using the pGL4.32(luc2P/NF-kB-RE/Hygro) and SIE pGL4(luc2P/SIE-RE/Hygro) vectors along with pGL3-basic vector (Promega AG) as control and pRL-TK vector to normalize for transfection efficiency as described previously (34). Stable DU145 cell lines were generated with the NF-kB– and STAT3-responsive reporters using pGL4.32(luc2P/NF-kB-RE/Hygro) and precast lentiviral particles with STAT3 reporter construct (Qiagen).

Cell proliferation and cell migration

Cell growth was evaluated using the sulforhodamine B (SRB) assay. Clonogenic, scratch/wound healing, and Boyden chamber assays were performed as described previously (35).

Cell cycle and apoptosis

7-AAD (25 μg/mL, Sigma Aldrich) was used for cell-cycle analysis by flow cytometry on a FACS Fortessa (BD Biosciences). Apoptosis was evaluated measuring the level of activated Caspase-3 (BD Pharmingen Activated Caspase-3 MAb Apoptosis Kit).

Quantitative RT-PCR

Quantitative real-time PCR (qRT-PCR) was performed on total RNA using custom-made primers and analyzed as described previously (35).

Immunoblotting and immunofluorescence microscopy

Cell lysates were prepared and analyzed as described previously (35). Lysates from tumor xenografts were prepared from freshly frozen tissue. Cytoplasmic and nuclear extract were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Scientific). For immunofluorescence microscopy, cells grown on glass coverslips were incubated with primary antibodies followed by incubation with anti-rabbit Alexa 488 or anti-mouse Alexa 594 (Invitrogen) secondary antibodies (35). The following antibodies were used in the study: ESE1/ELF3 (ab1392, AbCam), p50, p65, β-tubulin (Calbiochem), STAT3, pSTAT3 (Tyr705), pSTAT3 (Ser727), JAK2, and phosphorylated JAK2 (pJAK2; Tyr1007/1008; Cell Signaling Technology).
Figure 1. EC-70124 blocks NF-κB and STAT3 activities in prostate cancer cells. A, structure of EC-70124. B, NF-κB and STAT3 transcriptional activities in 22Rv1 (black bars) and DU145 (white bars) determined using NF-κB- or STAT3-responsive luciferase reporters. C, evaluation of luciferase activity in DU145 stably expressing a NF-κB- and STAT3-responsive element luciferase reporters after treatment for 24 hours with EC-70124 (500 nmol/L). D, phosphorylation of JAK2 and STAT3 evaluated by Western blot analysis after treatment of DU145 cells with EC-70124 (250 and 500 nmol/L). E, phosphorylation inhibition of STAT3 by EC-70124 is reversible. DU145 cells were treated with EC-70124 (500 nmol/L) for 2 hours. Cells were lysed at different time points (0, 4, and 24 hours) and phosphorylation of STAT3 was evaluated by Western blot analysis. F, phosphorylation of NF-κB inhibitor IκBα evaluated by Western blot after treatment of DU145 cells with EC-70124 (250 nmol/L, 4 hours). G, level of p65 assessed by Western blot analysis in nuclear and cytoplasmic fractions of DU145 cells after 4-hour treatment with EC-70124 (250 and 500 nmol/L). H, mRNA levels of ESE1/NF-κB target genes evaluated after 4 hours of incubation with EC-70124 (250 nmol/L). I, STAT3 and NF-κB transcriptional activities in 22Rv1 transiently cotransfected with a STAT3- and NF-κB-responsive element luciferase reporter and an ESE1 expression vector (pESE1). J, expression of STAT3/NF-κB target genes (COX2, ST6GAL-5, and MMP10) in 22Rv1-pcDNA and 22RV1-pESE1 cells cultured in presence of EC-70124 (250 nmol/L) was determined by qRT-PCR. P values were determined using t test. *, P < 0.01; **, P < 0.05.
STAT3 and NF-κB Inhibition by EC-70124 Affects Prostate CSCs

Animal studies
Study protocols involving animals were approved by the Swiss Veterinary Authority. Experimental groups consisted of at least 5 animals with equal age (8–10 weeks) and body weight (30–33 g). To monitor tumor growth athymic nude (Balb/c nu/nu) mice with subcutaneous tumor xenografts were treated daily for 5 days for 2 consecutive weeks with EC-70124 [40 mg/kg, orally]. To assess the drug distribution, healthy CD-1 male mice received a single dose of EC-70124 (40 mg/kg, orally) and drug levels in blood and tissues were measured by LC/MS. In vivo imaging was performed on an IVIS Spectrum Imaging System (Caliper LifeSciences; ref. 42). Details are provided in Supplementary Methods.

Statistical analysis
Differences between groups were assessed with an unpaired two-tailed t test and were considered statistically significant for P < 0.01 (*) and < 0.05 (**). To calculate IC_{50} values, means and standard deviations were plotted using GraphPad Prism Version 6. A sigmoidal 4P L model was used to interpolate the data and calculate IC_{50}. Each interpolation with R^2 ≥ 0.8 was considered valid.

Results
EC-70124 inhibits NF-κB and STAT3 in prostate cancer cells
Assays interrogating in vitro a large spectrum of kinases in the human kinome revealed that EC-70124 was highly active against IKK and JAK kinases (37, 38). EC-70124 was also reported to inhibit NF-κB and JAK/STAT signaling, respectively, in glioblastoma cells (43) and in triple-negative breast cancer cells (38). However, the effects of EC-70124 on STAT3 and NF-κB signaling and the consequences of concomitantly targeting these key transcription factors in cancer cells with constitutive activation of both pathways have not been investigated. To address this issue, we tested the effects of EC-70124 in DU145 prostate cancer cells. DU145 cells are a model of androgen receptor (AR) negative and androgen-independent prostate cancer. Furthermore, unlike the AR-dependent LNCaP cells, DU145 cells have constitutive activation of both STAT3 and NF-κB (30, 44, 45). These cells represent also a good model of prostate tumors characterized by concomitant upregulation of ESE1/ELF3 and sustained activation of NF-κB (35).

Activity of transcription factors, such as NF-κB and STAT3, can be accurately assessed in cells using reporters with specific responsive elements for the given factor. Using a reporter system, we confirmed that both NF-κB and STAT3 were highly active in DU145 cells (Fig. 1B). For comparison, NF-κB- and STAT3-responsive reporters had low activity in 22Rv1 prostate cancer cells in which these factors were not constitutively active. To monitor the activity of EC-70124 on STAT3 and NF-κB signaling, we generated derivative DU145 cells with stable expression of the STAT3 (STAT3-RE DU145) and the NF-κB (NFκB-RE DU145) responsive reporters. The activity of the reporters reflected the level of the transcription factor and the status of the signaling pathway responsible for its activation. Treatment with EC-70124 inhibited both the STAT3- and NF-κB–responsive reporters (Fig. 1C).

To determine whether these effects were due to interference with IKKβ and JAK2 kinase, we assessed the phosphorylation state of the immediate targets of these kinases by immuno-blotting. EC-70124 reduced pJAK2 within 30 minutes of incubation (Fig. 1D). Concomitantly, phosphorylation of STAT3 (pSTAT3) at Tyr705 was reduced with similar time and dose dependence. The rapid response of JAK2 and STAT3 (Tyr705) phosphorylation to EC-70124 suggested that the drug acted as a direct inhibitor of this kinase. JAK2 and STAT3 protein levels were not affected. Furthermore, phosphorylation of STAT3 at Ser727 was not reduced, consistent with selective inhibition of the JAK2-mediated Tyr705 phosphorylation. Interestingly, the effects of EC-70124 on JAK2 and STAT3 phosphorylation were rapidly reversed within 4 hours after drug washout (Fig. 1E), suggesting that continuous exposure would be likely required to obtain more persistent inhibition of the target by the drug. Treatment of DU145 cells with EC-70124 reduced also phosphorylated IκBα (Fig. 1F) and the amount of the NF-κB subunit p65 translocated in the cell nuclei (Fig. 1G and Supplementary Fig. S1), a prerequisite for reduced NF-κB transcripational activity. Notably, the effects on STAT3 and NF-κB signaling were seen at nanomolar concentrations (250–500 nmol/L) and relatively short incubation times, emphasizing the potency of EC-70124 in these cellular assays. Moreover, inhibition of STAT3 and NF-κB activation led to significant downregulation of various genes controlled by these transcription factors, such as ST6GAL-5, FN-1, ANGPTL-4, p50, ESE1/ELF3, and il6 (Fig. 1H). These results support the efficacy of EC-70124 as dual inhibitor of STAT3 and NF-κB signaling in these cells.

We showed previously that overexpression of ESE1/ELF3 in 22Rv1 cells resulted in activation of NF-κB and several of its target genes (35). We found that the activity of both NF-κB– and STAT3-responsive reporters was strongly induced by expression of ESE1/ELF3 in 22Rv1 cells (Fig. 1I), indicating concomitant activation of these pathways in ESE1/ELF3 overexpressing 22Rv1 cells. Consistently, the STAT3 and NF-κB reporters were inhibited by EC-70124 in 22Rv1 cells expressing ESE1/ELF3 (Fig. 1I). Furthermore, transcription of NF-κB and STAT3 target genes, which were upregulated in ESE1/ELF3-22Rv1 cells, was inhibited by EC-70124 (Fig. 1I). Interestingly, EC-70124 had no effect on transcription in control 22Rv1 cells indicating that the drug effect depended on the state of NF-κB and STAT3 in the cells.

EC-70124 affects proliferation of prostate cancer cells
Inhibition of NF-κB and STAT3 individually is known to affect proliferation of DU145 cells (30, 44, 46, 47). Given the ability of EC-70124 to block concomitantly both signaling pathways, we examined the compound’s effect on the phenotype of DU145 cells. Proliferation of DU145 cells grown as adherent monolayer was inhibited very effectively by treatment with EC-70124 for 72 hours (IC_{50} = 127 ± 18 nmol/L, Fig. 2A). Inhibition of cell proliferation increased drastically with increasing treatment time from 24 to 72 hours (Supplementary Fig. S2). Consistently, monitoring the number of viable cells over 7 days of continuous treatment showed that EC-70124 inhibited cell proliferation in dose-dependent manner with >50% and >75% inhibition at 75 and 250 nmol/L respectively (Fig. 2B), consistent with the need of prolonged and continuous drug exposure to affect cell proliferation. Other prostate cancer cell lines, like PC3 cells, without concomitant activation of STAT3 and NF-κB, were slightly less sensitive (IC_{50} = 394 ± 36 nmol/L) to EC-70124 (Supplementary Fig. S3).

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Treatment with EC-70124 suppressed also colony formation in DU145 cells. A, cell viability evaluated by SRB assay 72 hours after treatment with EC-70124 in DU145 cells. B, DU145 were treated with EC-70124 (75, 150, 250, and 500 nmol/L) and counted over a 7-day period. C, colony formation in adhesive and in soft-agar conditions (D) after treatment EC-70124 (250 nmol/L) in DU145 cells. E, scratch-wound healing assay with DU145 cells following treatment with 250 nmol/L of NF-κB inhibitors for 4 hours. F, scratch-wound healing assay with control (pcDNA) and ESE1/ELF3-expressing (pESE1/ELF3) 22Rv1 cells after treatment EC-70124 (250 nmol/L) for 4 hours. G, modified Boyden chamber assay with pESE1/ELF3 and pcDNA 22Rv1 cell lines after treatment with EC-70124 (250 nmol/L). H, colony formation in soft-agar conditions after treatment EC-70124 (250 nmol/L) in control (pcDNA) and ESE1/ELF3-expressing (pESE1/ELF3) 22Rv1 cells. P values were determined using t test. *, P < 0.01.

Figure 2.
EC-70124 decreases proliferation, migration, and colonies formation of prostate cancer cells. A, cell viability evaluated by SRB assay 72 hours after treatment with EC-70124 in DU145 cells. B, DU145 were treated with EC-70124 (75, 150, 250, and 500 nmol/L) and counted over a 7-day period. C, colony formation in adhesive and in soft-agar conditions (D) after treatment EC-70124 (250 nmol/L) in DU145 cells. E, scratch-wound healing assay with DU145 cells following treatment with 250 nmol/L of NF-κB inhibitors for 4 hours. F, scratch-wound healing assay with control (pcDNA) and ESE1/ELF3-expressing (pESE1/ELF3) 22Rv1 cells after treatment EC-70124 (250 nmol/L) for 4 hours. G, modified Boyden chamber assay with pESE1/ELF3 and pcDNA 22Rv1 cell lines after treatment with EC-70124 (250 nmol/L). H, colony formation in soft-agar conditions after treatment EC-70124 (250 nmol/L) in control (pcDNA) and ESE1/ELF3-expressing (pESE1/ELF3) 22Rv1 cells. P values were determined using t test. *, P < 0.01.

Treatment with EC-70124 suppressed also colony formation in adherence and in soft agar (Fig. 2C and D) and cell migration in wound-healing assay (Fig. 2E). Interestingly, we detected minimal or no induction of apoptotic cell death at the dose of 250 nmol/L that inhibited substantially cell proliferation (Supplementary Fig. S4). At the dose of 250 nmol/L, there was only a slight decrease of the fraction of cells in the S phase of the cell cycle, consistent with partially reduced proliferative capacity of DU145 cells treated with EC-70124 (Supplementary Fig. S5).

We also examined the effects of EC-70124 in ESE1/ELF3-22Rv1 cells. Overexpression of ESE1/ELF3 in 22Rv1 cells promoted cell proliferation, colony formation, and migration (35). EC-70124 was able to counteract the phenotypic effects induced by ESE1/ELF3 overexpression reducing cell migration (Fig. 2F and G) and colony formation in soft agar (Fig. 2H).
The activity of EC-70124 in parental 22Rv1 cells was minimal in these assays, indicating that ESE1/ELF3 overexpression and consequent activation of STAT3 and NF-κB determined higher sensitivity to the drug.

EC-70124 inhibits STAT3 and NF-κB in prostate CSC cells

Culturing prostate cancer cells, like DU145 cells, at low cell density in nonadherent conditions and serum-free media allows the selection of single-cell-derived floating colonies, called prostate spheres (PS), that are enriched of cancer cells with stem-like and tumor-initiating properties (40–42). PS-forming DU145 cells have high expression of CSC-specific markers and produce tumors in immunodeficient mice with much higher efficiency compared with the bulk cancer cell population grown as adherent monolayer (40–42). Using the stable reporter cell lines, we examined the activity of STAT3 and NF-κB in the CSC-enriched PS and bulk DU145 cells. The activity of both STAT3 and NF-κB reporters was strikingly higher in PS-forming cells compared with adherent DU145 cells (Fig. 3A). The level of target genes, like COX2, IL6, and ESE1, was also increased in the PS-forming cell subpopulation compared with bulk adherent DU145 cells (Fig. 3B). These data suggested that hyperactivity of STAT3 and NF-κB could drive the expansion of CSCs in prostate cancers. Consistently, treatment with EC-70124 drastically reduced STAT3 and NF-κB reporter activity in CSC-enriched DU145 PS cells (Fig. 3C). Furthermore, EC-70124 led to a significant decrease of COX2, IL6, and ESE1 expression in PS-derived cells (Fig. 3D). These results indicated that EC-70124 effectively blocked STAT3 and NF-κB signaling in the CSC subpopulation in this prostate cancer cell line.

Next, we examined whether treatment with EC-70124 affected the number of PS-forming cells in prostate cancer cell cultures. STAT3 and NF-κB are hyperactive in the prostate CSC subpopulation and likely provide survival and growth signals for maintaining the CSC compartment. DU145 cells cultured as adherent monolayer were dissociated, plated in PS-forming conditions and then incubated in the presence or absence of EC-70124 (Fig. 3E). We observed dose-dependent reduction in the number of PS in the presence of EC-70124 compared with control cells (IC_{50} = 139 mmol/L). Thus, treatment with EC-70124 impaired the ability of the CSC subpopulation to expand and generate single-cell–derived PS. To determine whether EC-70124 affected the CSC fraction also within the heterogeneous bulk cancer cell population, we treated DU145 cells in adherent monolayer cultures with the drug for 24 hours. Then, cells were harvested, washed, and plated in PS-forming conditions. Strikingly, the number of PS-forming cells was strongly reduced by the pretreatment with EC-70124 compared with control cells (>90% inhibition; Fig. 3F), suggesting that treatment with EC-70124 and consequent inhibition of NF-κB and STAT3 had a prominent effect on the prostate CSC subpopulation.

Oral EC-70124 has favorable pharmacokinetic and pharmacodynamic profile in mice

To assess the activity of EC-70124 in mouse xenograft models, we examined the compound pharmacokinetic and pharmacodynamic properties to define optimal delivery and schedule of administration. Prior studies indicated that the drug could be given safely by intravenous injection (38). Toxicity studies indicated that EC-70124 was also be well tolerated when given orally. Therefore, we explored the option of giving EC-70124 orally, which could be particularly attractive because of the likely need of continued treatment to maintain durable target inhibition. We assessed the pharmacokinetic profile of EC-70124 given orally at a single dose of 40 mg/kg. Measurement of the drug level in the plasma showed an initial peak after 2 hours and a slow decline of the level of EC-70124 in the following 24 hours (Fig. 4A). Importantly, the concentration of EC-70124 in plasma was 1,107 ng/g (2.4 μmol/L) and 425 ng/g (0.9 μmol/L) at 4 and 8 hours, respectively, well above the drug concentration (250–500 nmol/L) required for target and cell growth inhibition in vitro. Furthermore, the level of EC-70124 in mouse tissues (e.g., prostate) was comparable with the level in the plasma (Fig. 4A), indicating that the drug was rapidly distributed from the bloodstream and efficiently taken up in organs reaching levels compatible with target inhibition.

To verify whether EC-70124 was able to inhibit the expected targets in vivo, we used the reporter cell line and performed in vivo luciferase reporter assays. The NFKB-RE DU145 reporter cells were implanted subcutaneously to generate tumors in mice. Constitutive activity of NF-κB in DU145 cells ensured elevated activity of the luciferase reporter in the subcutaneous tumor xenografts detectable by in vivo bioluminescence imaging. Mice bearing tumors were treated with EC-70124 (40 mg/kg, orally) at day 1. Reporter activity was evaluated in control and drug-treated mice at various times after treatment (2–48 hours, Fig. 4B). Remarkably, a single dose of EC-70124 on day 1 profoundly reduced the activity of the NF-κB reporter within 2 hours (85% of reduction; Fig. 4C). The inhibition of the NF-κB reporter was not sustained and recovered completely at 24 hours. After a second dose of EC-70124 on day 3, the reporter activity was maximally reduced at 6 hours (96% of reduction) and returned slowly to the control level within 24 to 48 hours (Fig. 4B). Thus, the pharmacodynamic behavior was consistent with the pharmacokinetic profile of EC-70124 showing that levels of the drug sufficient to engage the target were achieved within few hours and were maintained for several hours after repeated oral administration.

EC-70124 inhibits tumor growth and depletes CSC in prostate tumor xenografts in mice

Orally administered EC-70124 reached the tumor and inhibited the target as assessed by the in vivo reporter cell assays. The effect, although profound, did not persist suggesting that repeated dosing were required to obtain prolonged inhibition of the target and induce an antitumor response. To evaluate the in vivo antitumor effect of EC-70124, the compound (40 mg/kg) was administered daily for 5 days in 2 consecutive weeks to mice carrying subcutaneous DU145 tumor xenografts. The treatment led to a significant delay of tumor growth (Fig. 5A). EC-70124 was well tolerated and no weight loss or other adverse events were observed. At the last day of treatment, pIkBα and pSTAT3 (Tyr705) were significantly decreased in tumors from EC-70124 treated mice compared with control mice (Fig. 5B and C). In line with the dual inhibition of NF-κB and STAT3 signaling, expression of NF-κB and STAT3 target genes was significantly decreased in EC-70124–treated tumors (Fig. 5D).
We further tested the activity of EC-70124 in tumor xenografts formed by ESE1/ELF3 overexpressing and control 22Rv1 cells. We reported previously that ESE1/ELF3-22Rv1 cells formed larger tumors than control 22Rv1 cells (35). ESE1/ELF3 overexpressing 22Rv1 cells exhibited constitutive activation of NF-κB and upregulation of NF-κB and STAT3 target genes (35). Furthermore, in vitro the effects of EC-70124 depended on the state of activation of the NF-κB and STAT3 pathways, being more pronounced in ESE1/ELF3-22Rv1 than control 22Rv1 cells. Notably, treatment with EC-70124 delayed substantially the growth of 22Rv1-pESE1 xenografts, while 22Rv1-pcDNA xenografts minimally affected (Fig. 6A). Similar to the DU145 tumor xenografts, the level of pIkBα, pSTAT3 (Tyr705; Fig. 6B and C), and expression of ESE1, COX2, MMP10, and IL6 (Fig. 6D) were reduced in 22Rv1-pESE1 tumors from mice treated with EC-70124 compared with tumors from control mice.

In line with the in vitro data, the pattern of inhibition of tumor growth induced by EC-70124 in vivo suggested that the drug had a cytostatic rather than cytotoxic effect, with the drug inducing perhaps a more differentiated phenotype and progressively reducing the fraction of proliferating tumor cells rather than inducing rapid growth arrest or cell death. In line with this hypothesis, the number of tumor cells positive for the proliferation marker Ki67 staining. This pattern could be compatible with the hypothesis that the drug was hitting more effectively the CSC subpopulation than the bulk proliferating tumor cells and progressively depleting the CSC component. This was consistent with the in vitro data showing the high efficacy of EC-70124 on the CSC-enriched PS-forming cells within the bulk tumor cell population (Fig. 3F). This hypothesis was further supported by the significant reduction of Myc and Nanog, two genes that we found consistently associated with the CSC phenotype, in tumor xenografts of DU145 and 22Rv1-ESE1 treated with EC-70124 (Fig. 5E and 6E). To further investigate this aspect, we performed ex vivo PS-forming assays with cells isolated from tumor xenografts at the end of the 2-week treatment with EC-70124. Tumors were excised and dissociated to isolate single cells that were then plated in PS-forming conditions to determine the fraction of cancer cells that retained CSC-like properties in control and drug-treated tumor xenografts. Consistent with our hypothesis, the PS-forming ability of DU145 tumor xenografts from EC-70124-treated mice were significantly reduced compared with control xenografts (Fig. 5F). We observed a similar reduction of ex vivo PS-forming ability in 22Rv1-ESE1 tumor xenografts treated with EC-70124 compared with control xenografts (Fig. 6F). Collectively, these results indicated that treatment with EC-70124 led to inhibition of STAT3 and NF-κB signaling pathways, delayed tumor growth and induced a concomitant depletion of the CSC subpopulation in the tumor xenografts by impairing their self-renewal ability.

**Discussion**

The transcription factors NF-κB and STAT3 are frequently and concomitantly activated in many human cancers (19, 39). In prostate cancer, activation of these transcription factors has been associated with disease progression and poor clinical outcome (28, 46, 48). Both STAT3 and NF-κB have been linked to development of castration resistance and poor responses to targeted therapeutics and cytotoxic drugs. NF-κB and STAT3 contribute to tumor progression also by influencing the intercellular communications between cancer cells and cells in the surrounding stroma and establishing an inflammatory and protumorigenic tumor microenvironment (21, 22, 39). Moreover, both pathways are found to be hyperactive in the CSC subpopulation and have been implicated in the acquisition and maintenance of the CSC phenotype in human tumors (11). Thus, targeting these transcription factors might provide an effective way to contrast the expansion of the highly tumorigenic stem-like tumor cells in advanced prostate cancer. CSCs are intrinsically resistant to androgen deprivation therapy and many cytotoxic drugs causing treatment failures and disease recurrence (8–10, 49). CSC-targeted agents could be most effective in combination with current therapies to increase response rates and prolong survival in advanced prostate cancer patients (11, 12). However, drugs that could selectively and effectively affect prostate CSC behavior are not currently available. CSCs upregulated multiple mechanisms that ensure survival and resistance to treatment (7, 11). Therefore, combined targeting of multiple key oncogenic pathways might be beneficial to effectively block the expansion and survival of CSCs.

In this study, we show that the multikinase inhibitor EC-70124 is able to block concurrently NF-κB and STAT3 signaling in human prostate cancer cells. This approach was particularly effective in the subpopulation of highly tumorigenic and stem cell–like prostate cancer cells that exhibited hyperactivity of the STAT3/NF-κB axis. Drugs targeting prostate CSCs and inducing their elimination or differentiation may represent a major advance for management of CRPC. The availability of such drugs should permit the implementation of combined treatment regimens with agents directed to the CSC components and bulk proliferating cancer cells. We showed recently that blocking c-Myc in prostate CSCs shut down a key oncogenic pathway and led to the progressive loss of self-renewal capacity and induction of cell senescence resulting in effective CSC elimination and inhibition of tumor development in mouse xenografts (42).
Figure 4. Pharmacokinetics and pharmacodynamics of EC-70124 in mice. A, after administrating EC-70124 orally, plasma and prostate tissues were collected at different time points and the levels of the drug were evaluated. B, xenografts generated by DU145 cells constitutively engineered with a NF-κB-responsive element luciferase reporter were treated with EC-70124 (40 mg/kg, orally (PO), red line) and compared with mock-treated mice (black line). Using an IVIS Spectrum Imaging System (Caliper LifeSciences), NF-κB transcriptional activity was evaluated after 2, 6, 24, and 48 hours. At 48 hours, a second dose of EC-70124 (40 mg/kg, orally) was given and, similarly, luciferase activity in vivo was detected after 2, 6, 24, and 48 hours. C, representative pictures of a mouse treated with EC-70124 at time 0 and after 2, 24, and 54 hours of treatment. *P values were determined using t test. † P < 0.01.
STAT3 and NF-κB Inhibition by EC-70124 Affects Prostate CSCs

Using a similar approach, we examined the effects of EC-70124 on the NF-κB/STAT3 axis in the bulk and CSC-enriched cell subpopulation derived from human prostate cancer cell lines. PS formed in vitro by single cells grown in nonadherent and serum-free culture conditions are enriched of prostate CSCs and represent an elective model to study the biology of CSCs and their response to drugs (42, 50). Notably, PS derived from human prostate cancer cell lines, like DU145 cells, exhibited hyperactivity of NF-κB and STAT3 and overexpression of numerous target genes compared with the bulk cancer cells, despite the enhanced activity of these pathways in the bulk tumor cell population. Indeed, hyperactivity of these signaling pathways is a common trait in CSC subpopulations derived from different prostate cancer cell models, although the underlying mechanisms of activation may vary (35, 40). Treatment with EC-70124 reduced the CSC component in the tumor xenografts as demonstrated by ex vivo PS-forming assays. Enhanced activity of NF-κB and STAT3 is common and can be driven by multiple mechanisms in prostate tumors. We recently identified a subgroup of prostate tumors representing 20% to 25% of primary tumors in which upregulation of the ETS factor ESE1/ELF3 was associated with prominent activation of NF-κB

Figure 5. EC-70124 blocks growth of xenografts generated by ESE1/ELF3 high prostate cancer cells. A, DU145 cells were injected subcutaneously into nude mice (n = 5) in the right flank (5 × 10⁶ cells/injection). Treatment with EC-70124 started after 27 days from the subcutaneous injections. EC-70124 (40 mg/kg) was given orally for 5 consecutive days for 2 weeks. At the end of the treatment, mice were sacrificed and tumor tissues were collected. Statistical significance of the differences was evaluated at the end of experiment. B, pIKB and IKB levels were assessed by immunofluorescence in tumor tissues from mice treated as indicated in A and collected at the end of the experiment. pSTAT3 and STAT3 levels were assessed by IHC. C, percentage of cells positive for pIKB and pSTAT3 in control and EC-70124 treated mice are shown in box plots. D, mRNA levels of STAT3/NF-κB targets were evaluated by qRT-PCR in DU145 xenografts treated with EC-70124 (white bars) and control (black bars). E, c-Myc and Nanog expression determined by qRT-PCR in control (black bars) and drug (white bars) treated DU145 tumor xenografts generated in control and EC-70124 (40 mg/kg) treated mice. P values were determined using t test. *P < 0.01.
The establishment of this positive feedback loop led to constitutive activation of NF-κB and increased production of the proinflammatory and protumorigenic cytokine IL6. Interestingly, human prostate cancer cell lines that model this tumor subgroup had concomitant activation of STAT3 and were highly responsive to EC-70124, suggesting that this compound could be very effective in this subgroup of aggressive prostate tumors. Consistently, EC-70124 was remarkably active in vivo in tumor xenografts derived from these cell line models.

EC-70124 is a product of metabolic engineering of the staur osporine and rebeccanycin biosynthetic pathways. In biochemical kinase assays, EC-70124 acted as a highly effective multi-kinase inhibitor (37). We show here that both in cells and tumor xenografts, the efficacy of EC-70124 depended on the cell context and activation state of the STAT3 and NF-κB pathways. The treatment with EC-70124 was more effective in DU145 and 22Rv1-ESE1 cells with hyperactive NF-κB and STAT3 signaling and high dependency on these pathways. However, because of the inherent promiscuity of a multi-kinase inhibitor and the possibility of undesired off-target and toxic effects, appropriate treatment regimens will need to be accurately defined to favor effective and selective inhibition of the proper intracellular targets. In our preclinical trial, we approached this issue by defining the dose and schedule of administration required to obtain a specific biologic response (i.e., inhibition of the NF-κB reporter) in mice with subcutaneous prostate tumor xenografts. Pharmacokinetic and pharmacodynamic studies showed that sufficient drug levels and effective target inhibition were obtained by single oral administration of EC-70124 at a relatively low dose (40 mg/kg), below the MTD of >100 mg/kg. Repeated daily treatment at this dose was well tolerated with no signs of systemic toxicity and resulted in significant target inhibition, antitumor activity, and depletion of the CSC component in tumor xenograft tissues in mice.

Figure 6. EC-70124 highly reduces tumor growth of xenografts generated by ESE1 overexpressing 22Rv1. A. 22Rv1-pcDNA and 22Rv1-pESE1 cells were injected subcutaneously into nude mice (n = 5) in the right flanks (5.0 × 10⁶ cells/injection). Treatment with EC-70124 (red lines, 40 mg/kg) or vehicle (black lines) started after 12 days from the subcutaneous injections. EC-70124 (40 mg/kg) was given orally for 5 consecutive days for 2 weeks. At the end of the treatment, mice were sacrificed and tumor tissues were collected. Statistical significance of the differences was evaluated at the end of experiment. B, pIKB and IKB levels were assessed by immunofluorescence in tumor tissues from mice treated as indicated in A and collected at the end of the experiment. pSTAT3 and STAT3 levels were assessed by IHC. C, percentage of pIKB and pSTAT3 positive cells is shown in box plots. D, mRNA levels of ESE1 targets were evaluated by qRT-PCR in 22Rv1-pESE1 xenografts treated with EC-70124 and control. E, c-Myc and Nanog expression determined by qRT-PCR in control (black bars) and drug (white bars) treated DU145 tumor xenografts. F, sphere-forming capacity was estimated ex vivo in cells derived from 22Rv1-pESE1 tumor xenografts generated in control and EC-70124 (40 mg/kg) treated mice. P values were determined using t test. *, P < 0.01.
Collectively, these findings underscore the ability of EC-70124 to act as a dual inhibitor of STAT3 and NF-kB signaling in vitro and in vivo. This novel kinase inhibitor with its action on two key signaling pathways may represent the prototype of a new class of antinecancer drugs with ability to interfere with CSCs in prostate tumors providing tools for innovative approaches for treatment of advanced prostate cancer. In many other tumor types, CSCs exhibit activation and dependency on NF-kB and STAT3 signaling. Therefore, EC-70124 might be similarly effective in other malignancies.

Disclosure of Potential Conflicts of Interest

F. Morts has ownership interest (including patents) in EntreChem SL. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: G. Civenni, P. Costales, F. Morris, G.M. Carbone, C.V. Catapano
Development of methodology: G. Civenni, P. Costales, C. Dallavalle, C.V. Catapano
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Civenni, N. Longoni, L. Elena Núñez, C.V. Catapano
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Civenni, N. Longoni, P. Costales, C.V. Catapano
Writing, review, and/or revision of the manuscript: G. Civenni, P. Costales, F. Morris, G.M. Carbone, C.V. Catapano
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Civenni, F. Morris, C.V. Catapano
Study supervision: G. Civenni, G.M. Carbone, C.V. Catapano

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Civenni, N. Longoni, P. Costales, C.V. Catapano
Writing, review, and/or revision of the manuscript: G. Civenni, P. Costales, F. Morris, G.M. Carbone, C.V. Catapano
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Civenni, F. Morris, C.V. Catapano
Study supervision: G. Civenni, G.M. Carbone, C.V. Catapano


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