A new synthetic protein, TAT-RH, inhibits tumor growth through the regulation of NFκB activity

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Abstract

Background: Based on its role in angiogenesis and apoptosis, the inhibition of NFκB activity is considered an effective treatment for cancer, hampered by the lack of selective and safe inhibitors. We recently demonstrated that the RH domain of GRK5 (GRK5-RH) inhibits NFκB, thus we evaluated its effects on cancer growth.

Methods: The role of GRK5-RH on tumor growth was assessed in a human cancer cell line (KAT-4). RH overexpression was induced by adenovirus mediated gene transfer; alternatively we administered a synthetic protein reproducing the RH domain of GRK5 (TAT-RH), actively transported into the cells.

Results: In vitro, adenovirus mediated GRK5-RH overexpression (AdGRK5-NT) in human tumor cells (KAT-4) induces IκB accumulation and inhibits NFκB transcriptional activity leading to apoptotic events. In BALB/c nude mice harboring KAT-4 induced neoplasias, intra-tumor delivery of AdGRK5-NT reduces in a dose-dependent fashion tumor growth, with the highest doses completely inhibiting it. This phenomenon is paralleled by a decrease of NFκB activity, an increase of IκB levels and apoptotic events. To move towards a pharmacological setup, we synthesized the TAT-RH protein. In cultured KAT-4 cells, different dosages of TAT-RH reduced cell survival and increased apoptosis. In BALB/c mice, the anti-proliferative effects of TAT-RH appear to be dose-dependent and highest dose completely inhibits tumor growth.

Conclusion: Our data suggest that GRK5-RH inhibition of NFκB is a novel and effective antitumoral strategy and TAT-RH could be an useful tool in the fighting of cancer.

Background

NFκB is a dimeric, ubiquitous transcription factor involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens [1-5]. In basal conditions, NFκB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs, that bind NFκB by means of ankyrin repeat domains masking its nuclear localiza-
tion signals (NLS); such interaction blocks NFκB in an inactive form in the cytoplasm [6]. Activation of NFκB is initiated by the signal-induced phosphorylation of IκB proteins by IκB kinase (IKK), thus inducing IκB ubiquitination and degradation by the proteasome. At this time, NFκB is active and stably localized within the nucleus where it induces the expression of specific genes. The activation of these genes by NFκB then leads to inflammation, immune response, cell survival, or cellular proliferation depending on cell type. The pathogenetic role of NFκB has been clarified in many diseases [7-9], such as type II diabetes and insulin-resistance [10,11]; cardiac hypertrophy [12]; atherosclerosis [13]; chronic heart failure [14]; cancer and angiogenesis [15].

There are many evidences about the role of NFκB in cancer. Indeed, Hanahan and Weinberg identified the six hallmarks that characterized tumor cells (Self-Sufficiency in Growth Signals, Insensitivity to Anti-growth Signals, Evading Apoptosis, Limitless Replicative Potential Sustained Angiogenesis, Tissue Invasion and Metastasis) [16,17] and most of the genes that regulate such effects are under the transcriptional control of NFκB. Moreover, NFκB transcription activity is constitutively increased in many tumors like melanoma [18,19], thyroid [20,21] and colon [22] carcinoma. The mechanisms determining persistent and de-regulated NFκB activity in cancer cells are not well understood but a major role is probably played by the cellular concentration of the inhibitory protein IκB. In particular, the constitutive activation of NFκB in solid tumors has been mainly attributed to decreased IκB levels due to defective IκBα activity, constitutive IKK activity, enhanced proteasome activity, etc.

To date, different approaches have been developed to block NFκB in several conditions. A successful one is using a proteasome inhibitor, PS-341, to treat patients with refractory or resistant multiple myeloma [23]. A protein that disrupts the association of the IKK complex is used to prevent inflammatory bone destruction [24]. The inhibition of IκB kinase by the Bay 11-7082 compound, has been successfully used to prevent tumor growth and leukemic infiltration in a mouse model of adult T cell leukemia [25]. Furthermore, inhibition of NFκB activation by expression of a mutant IκBα, which is resistant to phosphorylation and degradation, increased NFκB dependent apoptosis to stimuli such as TNFa [26,27]. All these approaches open new fields for the management of NFκB-associated diseases like cancer.

G protein coupled receptor (GPCR) kinases (GRKs) regulates GPCRs signaling by inducing receptor desensitization. Recent findings unveil new cellular function for these kinases. Indeed, we have recently demonstrated that GRK5 regulates the activity of the transcription factor NFκB [28]. In particular, in endothelial cells GRK5 is able to bind the inhibitory protein of NFκB, IκBα, by means of the RH domain (GRK5-RH) and stabilize the complex IκBα/NFκB in the nucleus, thus inhibiting NFκB transcriptional activity [28]. Indeed, RH overexpression, which causes GRK5-IκBα interaction, inhibits the transcriptional activity and DNA binding of NFκB both in basal condition and after stimulation with LPS.

Given the notion that the modulation of NFκB transcriptional activity is an effective therapeutic strategy for cancer, we hypothesized that RH, being a potent inhibitor of NFκB, could reveal therapeutic potentialities in oncology. We therefore tested in a human tumor cell line, KAT-4, the effects of GRK5-RH on cell growth either in cultured cells or in tumors in BALB/c nude mice, grown after subcutaneous injection of KAT-4 cells.

**Materials and methods**

**Cell culture**

Human tumor cells (KAT-4) were a kind gift of Dr. Maddalena Illario (Federico II University, Dept. of Cellular and Molecular Pathology). This cell line has been recently authenticated by short tandem repeat (STR) profiling which demonstrated, despite the widespread knowledge, that KAT-4 are not of thyroid cancer origin but share the same STR profile with the HT-29 colon cancer cell line [29]. Cells were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS) at 37°C in 95% air-5%CO₂.

**Plasmid constructs**

In order to synthesize a protein reproducing the RH domain of GRK5, engineered to be actively transported into the cells by means of TAT sequence, we designed the TAT-RH plasmid, using pcDNA3.1-GRK5-RH [28] as template to amplify RH sequence. The primers were designed with the addition of NCO I and KPN I restriction sites sequences:

5'CCCCCATGGCCCGAGATTACTGCGATT3' and 3'ATAAAACTAGCGAAAAAGATCCCATGCGGGA5';

Amplified sequences were purified by gel extraction kit (Invitrogen) and cloned into pTAT-HA vector a kind gift of Dr. Steven Dowdy (Washington University School of Medicine) [30] by means of T4 DNA ligase (Promega). The right frame and orientation were confirmed by restriction analysis and DNA sequencing (Avant 3100, Applied Biosystem). pTAT-HA vector comprises the 11 aminoacid TAT domain, that is the minimum sequence of HIV TAT able to transduce into cells, and histidine and HA as tags.

**Production of adeno viral vectors**

The adeno viral construct GRK5-NT (AdGRK5-NT) used for gene transfer in vivo has been previously described [28]. AdGRK5-NT comprises the N terminal and RH
domains of GRK5 conjugate to Green Fluorescent Protein gene (GFP) under the CMV promoter.

For Lac-z adenoviral construct, we subcloned Lac-Z gene, excised by digestion from pcDNA 3.1/Lac-Z (Invitrogen), into the Ascl site of the pShuttle-linker plasmid, and the resulting plasmid was used to generate the first generation of adenoviruses (AdLac-Z).

First-generation of adenoviral vectors AdGRK5-NT and AdLacZ was amplified in 293N3S cells in monolayer for small-scale amplification and in suspension for large scale amplification. Virus production was performed as follows: 3 liters of 293N3S cells at a concentration of 3-4 × 10⁵ cells/ml were harvested by centrifugation and resuspended in 5% of the volume of conditioned medium and then infected with all the crude lysate obtained from the infection of two 150-mm dishes. Virus adsorption was performed at 37°C on a magnetic stir plate for 2 hours; after this step, medium (25% conditioned and 75% fresh Joklik's MEM supplemented with 5% FBS) was added to a final volume of 2 liters. Infected cells were harvested after 48 hours, lysed and resuspended in TM solution (10 mM Tris-HCl pH 8.0, 2 mM MgCl₂). After three freeze-thaw cycles cells were incubated in 2 M MgCl₂ in presence of DNase (10 mg/ml) for 1 hour at 37°C. After DNAase incubation, cellular debris were eliminated by centrifugation at 3500 rpm for 15 minutes and the remaining lysate was subjected to ultracentrifugation on a continuous CsCl₂ gradient at 35,000 rpm for 2 hours at 4°C. The upper band, which contains empty particles, was eliminated and a second ultracentrifugation on a continuous CsCl₂ gradient (35,000 rpm for 18 hr at 4°C) was performed. The harvested vector was dialyzed twice against 4% sucrose and stored at -80°C until use. Vector concentration (particle number) was determined by UV spectrophotometric analysis at 260 nm.

**Protein synthesis and purification**

TAT and TAT-RH plasmids were transformed into a BL21 (DE3) pLysS (Invitrogen) bacterial strain. 2 L of Luria Broth (LB) culture was grown overnight and then Isopropylthiogalactoside (IPTG, 100 μM, 3 hrs) was added to induce protein expression. For protein purification we used denaturing conditions to recover all the recombinant proteins from bacterial inclusion bodies (Lysis buffer: 8 M Urea, 100 mM NaCl, 20 mM Hepes pH 8). Ni-NTA columns (GE Healthcare) were used for protein purification. Columns were pre-equilibrated with 10 mM imidazole; lysates were clarified by sonication and applied to the columns. After extensive washing with lysis buffer plus 20 mM imidazole, recombinant protein were eluted with increasing amounts of imidazole (100, 200 and 500 mM). Protein refolding was realized by buffer exchanging into Tris 1 M pH 7.5 using Amicon Ultra-4 Centrifugal Filters (Millipore). The recombinant protein TAT-RH was added to the culture medium in KAT-4 cells for the in vitro study and injected intra-tumor for the in vivo study. TAT protein was used as control.

**Nuclear extracts preparation, Immunoprecipitation and western blot and Apoptosis analysis**

The experiments were performed as previously described [28].

**Luciferase assay**

Cells were transfected with plasmid expression vectors containing the luciferase reporter gene linked to five repeats of a NFκB binding site (κB-Luc) or β-galactosidase (β-Gal) and infected with AdGRK5-NT. Transient transfection was performed using the Lipofectamine 2000 (Invitrogen) according to manufacturer’s instruction. Lysates were analysed using the luciferase assay system with reporter lysis buffer from Promega and measured in a β-counter. Relative luciferase activity was normalized against the co-expressed β-galactosidase activity to overcome variations in transfection efficiency between samples.

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed using nuclear extracts. Double stranded NFκB oligonucleotide (5’ AGTTGAGGGGACTT-TCCCAGGC 3’) was end-labelled using [32P]γ ATP (GE Healthcare) and T4 polynucleotide kinase (Roche). Samples were subject to electrophoresis in 8% non denaturing polyacrilamide gels with 0.5% TBE buffer (0.09 M Tris, 0.09 M boric acid, 0.02 M EDTA). Digitalized gels autoradiographies were then quantified (Image Quant).

**In vivo Study Design**

Experiments were carried out, in accordance to NIH guidelines for Animal Investigation, in 6-weeks-old BALB/c immunoincompetent nude mice (Charles River), which had access to food and water ad libitum. For tumor formation, a suspension containing 2 × 10⁶ KAT-4 cells in 200 μl of DMEM were injected subcutaneously in the dorsal side of nude mice. Animals were anesthetized using isoflurane 2%. We used mice that developed tumors of approximately 6 mm in 2 weeks. Mice were divided into 9 groups (5 mice/group) and administered twice a week for 17 days (AdGRK5-NT) or 4 weeks (TAT-RH) with intra-tumor injections of the specific treatment.

In particular, two groups received AdGRK5-NT at either low (10⁵ pfu/ml) or high dosage (10⁷ pfu/ml) while another group received the higher dose of AdLACZ and was used as negative control. Three more groups of mice were treated with TAT-RH protein at the dosage of 8, 12 or 16 mg/Kg. These mice were compared to two control groups treated with either saline solution or the pTAT-HA.
protein lacking the RH sequence. Tumor growth was measured by caliper twice a week and by Ultrasound (VeVo 770, Visualsonics) once a week. At the end of the treatment, mice were sacrificed by cervical dislocation and tumors processed for biochemical or histological analysis. All in vivo experimental protocols were approved by the Federico II University Ethical Committed for Animal Studies.

**Real Time PCR**
Total RNA was isolated using Trizol reagent (Invitrogen) and cDNA was synthesized by means of Thermo-Script RT-PCR System (Invitrogen), following the manufacturer instruction. After reverse transcription reaction, real-time quantitative polymerase chain reaction (PCR) was performed with the SYBR Green real time PCR master mix kit (Applied Biosystems).

The reaction was visualized by SYBR Green Analysis (Applied Biosystem) on StepOne instrument (Applied Biosystem).

Primers for cytokines gene analysis were as follows:

- TNFa: forward, 5’CCAGGAGAAAGTCAGCCTC3’; reverse, 5’CGATAAAGGGGTACAGATTT3’; VEGF: forward, 5’CAGGCTGTCAACGATGAA3’, reverse 5’TTCTTGCGCTTTCGTTTT3’; GAPDH: forward, 5’AGTATGTCGTGGAGTCTACT3’; reverse 5’TGTGG TCATGAGCCCTTCCAC3’.

All values obtained were normalized to the values obtained with the GAPDH primers. The results are expressed as the relative integrated intensity.

**Histology and Immunocytochemistry**
Paraffin embedded sections were processed for the triple layered immunocytological peroxidase anti-peroxidase (PAP) method. PCNA (Cell signalling), Cleaved caspase 3 (Cell signalling) and Lectin (Sigma) antiseraum were used to analyze cell proliferation, death and neo-angiogenesis, respectively. The peroxidase was revealed in presence of 0.03% hydrogen peroxide and of an electron donor, 2,5% diaminobenzidine, which becomes visible as a brown precipitate. For negative controls, the primary antiserum was omitted. Sections were then viewed with an Eclipse E1000 Fluorescence Microscope (Nikon) and acquired using Sigma Scan Pro software (Jandel). For X-GAL staining and GFP visualization, cryostat sections were incubated with β-GAL solution (PBS, 0.02 mM K2Fe(CN)6, 0.02 mM K2Fe(CN)4, 0.02 mM MgCl2, 0.002% NP40, 0.05 mg/ml X-GAL stain) for 90 min at 37°C and counterstained with Eosin. Adobe Photoshop was used for final assembly of the images.

**Statistical Analysis**
All values are presented as mean ± SEM. Two-way ANOVA was performed to compare the different parameters among the different groups. A significance level of P < 0.05 was assumed for all statistical evaluations. Statistics were computed with GraphPad Prism Software (San Diego, California).

**Results**
1. **Adenoviral mediated overexpression of GRK5-RH**
   a) **Cell studies**
   As previously demonstrated in endothelial cells [28], also in KAT-4 cells AdGRK5NT causes transgene expression that can be visualized through GFP fluorescence at green light (Figure 1A). This maneuver leads to IκBα accumulation in whole cell extracts (Figure 1A). In cancer cells NfκB activity inhibits apoptotic events. Thus, we assessed apoptosis in KAT-4 cells with adenoviral mediated overexpression of GRK5-RH. This maneuver increases cleaved caspase 3 levels compared to controls (Figure 1B). Similar results were achieved by Annexin V cell staining (Figure 1C). These responses associate with inhibition of NfκB activity, assessed by luciferase assay (Figure 1D). These data demonstrate that GRK5-RH inhibits NfκB transcriptional activity and biological effects also in cancer cells.

   b) **BALB/c nude mice tumors**
   In nude mice, the injection of 2 × 10^6 KAT-4 cells in the dorsal lateral region results in the development of a ~6 mm diameter tumor in 2 weeks, in about 70% of mice. Tumors were treated with different doses of AdGRK5-NT. One high dose of AdLac-Z was used as control. Low doses of AdGRK5-NT retard tumor growth compared to controls (Figure 2A). High doses are more effective, inducing a complete inhibition of tumor growth (Figures 2A).

   After 17 days of treatment, mice were sacrificed and tumors were taken for biochemical and histological analysis. Expression of AdGRK5-NT transgene is found by GFP fluorescence in the majority of cells within tumor when observed under green light (Figure 2B). In control tumors, LAC-Z expression is confirmed by blue X-GAL staining at direct light (Figure 2B). In order to evaluate the effect of GRK5-RH, we performed a western blot in homogenized tumors to analyze IκBα expression and apoptosis. Figure 2C shows that adenoviral mediated overexpression of GRK5-RH induces an increase of IκBα and cleaved caspase 3 levels in treated tumors compared to controls. NfκB also controls tumor angiogenesis. Therefore, we evaluated VEGF expression and found it significantly decreased in treated tumors compared to controls (Figure 2C). Results suggest that adenoviral mediated GRK5-RH expression...
inhibits NFκB activity in tumor cells. Indeed, EMSA analysis of tumors confirms NFκB inhibition: less NFκB is bound to genomic DNA in treated tumors compared to controls (Figure 2D).

2) Effects of administration of TAT-RH protein

a) Cell studies

To close up to a more pharmacological tool, we designed (Figure 3A) a protein (TAT-RH) reproducing the RH domain of GRK5, engineered to be actively transported into the cells through the retroviral TAT sequence and tested its anti-cancer property. Synthesis of the recombinant TAT-RH was performed in bacteria and column purification was confirmed by SDS-PAGE (Figure 3B).

Effects of GRK5-RH in vitro in KAT-4 cells overexpressed by adenovirus mediated gene transfer. A) IkBα and GRK5-RH levels were analyzed in whole extracts by western blot, in KAT-4 overexpressing GRK5-RH by means of adenovirus mediated gene transfer. GRK5-RH increases IkBα levels. GRK5-RH expression was visualized by WB using anti-GFP antibody. B) To evaluate GRK5-RH effect on apoptosis, we analyzed the cleavage of caspase 3 by WB. The overexpression of GRK5-RH increases cleaved caspase 3 levels, suggesting that GRK5-RH causes an increase of apoptotic responses. C) This result was confirmed by Annexin V staining in fluorescence. GRK5-RH overexpression causes apoptosis as shown by Annexin-V staining compared to live cells (green = Annexin V; blue = nuclei) D) We evaluated GRK5 effects on NFκB activity by luciferase assay, in KAT-4 overexpressing GRK5-RH. Cells were transfected with plasmids coding for a κB-luciferase reporter (κB-Luc) and β-galactosidase (β-Gal) and lysates were analyzed by luciferase assay system (Promega). GRK5-RH inhibits NFκB transcriptional activity (*p < 0.05 vs control).

To verify the ability of TAT-RH protein to translocate into cells autonomously, we added the protein into the medium of cultured KAT-4 cells for 1 hour and verified its internalization by means of precipitation and western blot from cell lysates (Figure 4A). To verify the biological effect of TAT-RH, we evaluated IkBα and cleaved caspase 3 levels. TAT-RH increases both IkBα and cleaved caspase 3 levels (Figure 4B), thus confirming that our protein also in these cells causes apoptosis. Apoptosis was also assessed by Annexin V staining. Using different doses of TAT-RH, we found that the minimum amount of protein needed to exert biological functions is 0.5 μg/ml (Figure 5).
Effects of GRK5-RH in vivo on tumor growth and biochemical analysis: treatment with adenovirus. A) To validate our in vitro results, we studied the effects of adenoviral mediated overexpression of GRKS-RH in a cancer model in vivo. Tumor growth was measured twice a week by a caliper during all the treatment long (17 days of treatment). Low doses (10⁸ pfu/ml in 200 ul) of AdGRK5-NT retard tumor growth compared to controls. High doses (10¹¹ pfu/ml in 200 μl) are more effective, inducing a complete inhibition of tumor growth. Figure also shows a representative image of tumors at the end of the treatment. B) 17 days from starting treatment, mice were sacrificed and tumors were taken for histological analysis. To ascertain GRK5-RH expression, cryostat sections were analyzed by direct observation of the green light at the fluorescence microscope. LAC-Z expression in control tumors was evaluated by X-GAL staining at direct light (blue staining indicates LAC-Z expression; eosin was used for counterstaining in red). C) Tumors were homogenized to analyze IκBα levels, VEGF production and apoptosis. Adenoviral mediated overexpression of GRK5-RH induces an increase of IκBα and cleaved caspase 3 levels and a reduction of VEGF expression in treated tumors compared to controls treated with AdLac-Z. D) Nuclear extracts from tumors were analyzed by EMSA. AdGRK5-NT inhibits NFκB activity compared to controls. Lane 1 = probe alone; Lane 2 = empty; Lane 3-4 = AdLac-Z different treated tumors; Lane 5-6 = AdGRK5-NT treated tumors.
b) BALB/c nude mice tumors
We first tested the safety of in vivo administration of our protein in healthy nude mice. There were no significant changes in body weight among treated and control groups of mice indicating no toxicity of the treatment (Table 1). Internal organs (liver, lung, kidney) were then analyzed by histology. No morphological differences were found in treated mice compared to controls (data not shown). The effect of our protein on tumor growth appears to be dose-dependent. Indeed, high doses (16 mg/kg) lead to regression of tumors, intermediate doses (12 mg/kg) are able to completely inhibit tumor growth, since tumors maintain the same size of starting treatment, and low doses (8 mg/kg) can delay tumor growth (Figure 6A). As a control, we treated a group of 5 mice with the synthetic protein TAT lacking the GRK5-RH domain and a group with saline. Tumor was equal in mice treated with saline solution and those treated with TAT (Figure 6A).

The effects of TAT-RH treatment on IkBα levels, apoptosis and angiogenesis, were assessed in control, 12 mg/kg and 8 mg/kg treated mice since at higher doses (16 mg/kg) of protein the tumor had a too small size for biochemical assessments. Figure 6B shows that TAT-RH treatment leads to a dose-dependent increase of IkBα and cleaved caspase 3 and a reduction of VEGF levels. Such effects is associated with NFκB activity inhibition, as evaluated by EMSA (Figure 6C).

To measure angiogenesis in these tumors, lectin staining was used to visualize microvessels. Figure 7A shows that
TAT-RH reduces the formation of tumor vasculature. Cell death and proliferation were evaluated by analysis of cleaved caspase 3 and PCNA levels. TAT-RH treated tumors show increased cleaved caspase 3 levels (Figure 7B) and reduced cell proliferation (Figure 7C). We also evaluated IκBα subcellular localization by immunohistochemistry. As previously demonstrated in endothelial cells, IκBα mainly localizes in the cytosol in control tumors while TAT-RH treatment causes its nuclear accumulation (Figure 7D).

VEGF and TNFα expression was evaluated by Real Time PCR to assess the ability of TAT-RH to inhibit NFκB-dependent gene expression in cancer cells. Figure 8 shows that at 2 weeks from starting treatment both VEGF and TNFα expression is strongly inhibited and prolonged treatments (4 weeks) increase such effect.

**Discussion**

The major finding of our manuscript is that GRK5-RH causes NFκB inhibition, through a novel mechanism of IκB stabilization, and leads to inhibition of growth and regression of size of tumors both in vitro and in vivo. We provide evidence using two sets of experiment: the first set, gathered through the overexpression of GRK5-RH by AdGRK5-RH, provides the proof of concept that GRK5-RH is able to inhibit cancer growth. It also represents the background for the synthesis and administration of a recombinant protein that resembles the inhibitory features of GRK5-RH, which was used in the second set of data, showing that TAT-RH can be used to obtain pharmacological inhibition of tumor growth.

It is known that a sustained, constitutive activation of NFκB contributes to malignant progression and therapeutic resistance in most of the major forms of human cancer, such as human lymphomas [31], carcinomas of the breast [32], prostate [33], lung [34], colon [22], pancreas [35], thyroid [21], head and neck [36] and cervix [37]. Thus, the modulation of NFκB activity represents an useful therapeutic strategy for cancer, since NFκB inhibition promotes apoptotic events induced by chemotherapy, reduces the high proliferative rate that characterizes tumor cells and inhibits metastasis [13]. We have recently demonstrated that the RH domain of GRK5 is an effective inhibitor of NFκB transcriptional activity in endothelial cells with implications in wound healing and tissue regeneration. In the present study we evaluated the effects of GRK5-RH in cancer. Our study was performed on an aggressive human carcinoma, the KAT-4 cell line. This tumor shows high proliferative rates and is NFκB sensitive [38]. Several strategies have been developed for blocking NFκB in tumors that include the inhibition of NFκB signaling pathway by proteasome inhibitors, IKK inhibitors, antioxidants or recombinant adenovirus-mediated overexpression of the IκBα gene, interfering with NFκB mRNA using specific anti-sense oligonucleotide [39-41]. We synthesized a protein reproducing the RH domain of GRK5, engineered to be actively transported into the cells by means of TAT domain without the support of other vehicles and tested its anti-cancer property. This approach has been already successfully used for protein transport in the treatment of several mouse model of cancer, inflammation and other diseases [42,43]. Our data suggest that TAT-RH protein enters into tumor cells, inhibits NFκB transcription activity and induces apoptosis, reduces tumor angiogenesis,
Effects of TAT-RH in vivo on tumor growth and biochemical analysis. 

A) We evaluated the effects of TAT-RH on tumor growth in BALB/c nude mice. High doses (16 mg/kg in 200 μl) lead to regression of tumors, intermediate doses (12 mg/kg in 200 μl) are able to completely inhibit tumor growth and low doses (8 mg/kg in 200 μl) can only delay tumor growth. Figure also shows a representative image of tumors at the end of the treatment (inset).

B) Tumors were homogenized and the biological effects of TAT-RH treatment were evaluated by WB. TAT-RH leads to a dose-dependent increase of IκBα and cleaved caspase 3 and a reduction of VEGF levels. TAT treated tumors were used as controls.

C) To evaluate the effect of TAT-RH on NFκB activity, we performed an EMSA using nuclear extracts from tumors. TAT-RH treatment reduces NFκB activity. Lane 1 = probe alone; Lane 2 = empty; Lane 3-4 = TAT treated tumors; Lane 5-6 = TAT-RH treated tumors.
blocks cell proliferation and consequently tumor growth in a dose dependent manner.

A clear advantage of our strategy of inhibition of NFκB is the fact that there is no overexpression of a transgene, but rather, the pharmacological inhibition of a mechanism of degradation of IκB which is both efficient and selective. One concern about inhibiting the NFκB pathway is the specificity. In particular, the proteasome which is responsible for IκB degradation has many other important functions. Thus, inhibition of proteasome activity could potentially cause severe side effects [44]. Since our strategy is based on the sterical interaction of TAT-RH and IκB and therefore does not require the inhibition of a general cellular mechanism, such as the proteasome, we hypothesize that the side effects of chronic treatment with TAT-RH, or a small molecule resembling it, will be of minimal intensity. Indeed, TAT-RH protein is not toxic when delivered through a systemic route, as it did not induce organ damage in mice. Thus, our data suggest that TAT-RH protein is a potent inhibitor of tumor growth both in vitro and in vivo, that is safe and well tolerated when administered systematically.

We believe that the anti-tumoral properties of GRK5-RH rely at least on two possible mechanisms. It is well known that apoptosis induced by chemotherapies is attenuated in tumor cells showing enhanced NFκB activity [1,45-47]. Here we demonstrated that GRK5-RH is able to induce apoptotic events in our model of cancer as evidenced by increased levels of the active form of caspase 3 (both by western blot and histological analysis) and Annexin V staining. Another feature that characterizes aggressive tumors is the ability to generate new vessels. Tumor angiogenesis has shown to be dependent on angiogenic factors, like chemokines and growth factors produced by macrophages, neutrophils and other inflammatory cells, all factors that have been shown to be regulated by NFκB [48,49]. GRK5-RH treatment reduces VEGF expression and production compared to controls and inhibits angiogenesis, thus providing another mechanism for retarding the in vivo growth of KAT-4 cells.

**Conclusion**

This study demonstrates the ability of the RH domain of GRK5 to inhibit tumor growth through NFκB antagonism. This effect is achieved both in vitro and in vivo either through gene therapy or injection of TAT-RH protein. Thus, our data propose GRK5-RH as an useful therapeutic tool for cancer.
Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
GI designed research. DS, GS, AC, EL, and LP performed research. GI, DS and BT analyzed data and wrote the paper.

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