SI113, a Specific Inhibitor of the Sgk1 Kinase Activity that Counteracts Cancer Cell Proliferation

Lucia D’Antona a Rosario Amato a Cristina Talarico b Francesco Ortuso a Miranda Menniti a Vincenzo Dattilo a Rodolfo Iuliano b Francesco Gigliotti b Anna Artese b Giosuè Costa a Silvia Schenone c Francesca Musumeci c Claudia Abbrezese e Lorenzo Botta d Francesco Trapasso b Stefano Alcaro a Marco G. Paggi e Nicola Perrotti a

a University “Magna Graecia” of Catanzaro, Dept. of “Scienze della Salute”, Catanzaro, University “Magna Graecia” of Catanzaro, Dept. of “Medicina Sperimentale e Clinica”, Catanzaro, University of Genova, Dipartimento di Farmacia, Genova, University of Siena, Dipartimento Farmaco Chimico Tecnologico, Siena, Experimental Oncology, Regina Elena National Cancer Institute, IRCCS, Rome, Italy

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Abstract
Background/Aims: Published observations on serum and glucocorticoid regulated kinase 1 (Sgk1) knockout murine models and Sgk1-specific RNA silencing in the RKO human colon carcinoma cell line point to this kinase as a central player in colon carcinogenesis and in resistance to taxanes. Methods: By in vitro kinase activity inhibition assays, cell cycle and viability analysis in human cancer model systems, we describe the biologic effects of a recently identified kinase inhibitor, SI113, characterized by a substituted pyrazolo[3,4-d]pyrimidine scaffold, that shows specificity for Sgk1. Results: SI113 was able to inhibit in vitro cell growth in cancer cells derived from tumors with different origins. In RKO cells, this kinase inhibitor blocked insulin-dependent phosphorylation of the Sgk1 substrate Mdm2, the main regulator of p53 protein stability, and induced necrosis and apoptosis when used as a single agent. Finally, SI113 potentiated the effects of paclitaxel on cell viability. Conclusion: Since SI113 appears to be effective in inducing cell death in RKO cells, potentiating paclitaxel sensitivity, we believe that this new molecule could be efficiently employed, alone or in combination with paclitaxel, in colon cancer chemotherapy.

R. Amato, S. Alcaro, M.G. Paggi and N. Perrotti contributed equally and thus share last authorship
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Introduction

The serum- and glucocorticoid-regulated kinase (Sgk) family consists of three members, Sgk1, Sgk2 and Sgk3, all displaying serine/threonine kinase activity and sharing structural and functional similarities with the Akt family of kinases [1]. Sgk1 was originally described as a key enzyme in the hormonal regulation of sodium absorption by the amiloride-sensitive sodium channel (ENaC) [2]. The activity of ENaC, through Sgk1, is regulated by aldosterone, glucocorticoids, insulin and vasopressin at the transcriptional and post-translational level [3, 4].

Sgk1 activity is strictly correlated with its post-translational modifications. It becomes a substrate of the mTOR kinase that phosphorylates its hydrophobic motif (H-motif) at the level of S422. Then, 3-phosphoinositide-dependent kinase-1 (Pdk1) binds to the H-motif of Sgk1, at the level of phospho-S422, and further phosphorylates the protein at T256 [5]. Interestingly, the mTOR inhibitor Torin-1 has been proposed as a powerful drug candidate for the therapy of metastatic colorectal cancer [6].

Sgk1 has been implicated in mediating insulin, IGF1 (Insulin like Growth Factor 1), glucocorticoid and IL2 (Interleukin2)-dependent survival signals in normal and cancer cells [7-11], thus gaining significant attention in the field of molecular oncology. Indeed, the anti-apoptotic function of Sgk1 suggests its possible involvement in human carcinogenesis. Sgk1 increased expression has been found in human tumors, like prostate cancer [12] and non-small cell lung cancer of the squamous subtype, where Sgk1 mRNA level correlates with several clinical prognostic indicators [13]. Sgk1 knockout mice appear to be resistant to the chemical carcinogens used for the induction of colon cancer [14]. Interestingly, the active Sgk1 kinase regulates cell survival, proliferation and differentiation through Mdm2 (Mouse Double Minute 2), that directs p53 to ubiquitylation and proteosomal degradation [15] and, by modulating the expression of RanBP1 (Ran Binding Protein 1), it affects mitotic stability and paclitaxel sensitivity of RKO colon carcinoma cells in culture [16]. Sgk1 interacts with enzymes involved in the metabolism of glycoproteins and lysophospholipids [17, 18], suggesting a role in the development of some of the metabolic alterations that may affect colorectal cancer progression [19-22]. We recently demonstrated that Sgk1 specific RNA silencing in cultured RKO colon carcinoma cells enhances paclitaxel sensitivity, measured as early and late apoptosis [16].

Taken together, these observations suggest an important role for Sgk1 in carcinogenesis and in the mechanisms responsible for resistance to chemotherapy [23]. Sgk1 may thus be considered an interesting molecular target in the therapy of some human tumors [24; 15, 25]. The expression level of Sgk family of proteins may have a central role in tumors that are resistant to Akt inhibition [26]. Notably Sgk1, as compared with its cognate kinase Akt, is highly regulated by steroids. Glucocorticoid receptor antagonism has been recently proposed as a promising therapy in triple negative breast cancer [27], and Sgk1 inhibition is thus expected to be at least as effective as glucocorticoid receptor antagonism.

We recently screened a family of dual Src/Abl inhibitors characterized by a substituted pyrazolo[3,4-d]pyrimidine scaffold [28, 29] for their ability to inhibit Sgk1 and Akt kinase activity, competing with ATP for its binding domain. One of these molecules, compound 3 (henceforth SI113), was particularly effective in inhibiting Sgk1 kinase activity, being much less effective on Akt1 activity, probably because it fits better with the lipophilic area of the ATP binding domain that in Sgk1 is larger than in Akt1 [30]. The structural formula of SI113 is shown in Fig. 1, panel A. We now provide more information on the in vitro specificity of SI113 as a Sgk1 inhibitor, and describe the effects of this compound on the proliferation of some cancer cells and on survival and paclitaxel sensitivity in colon carcinoma cells. The results indicate a conceivable use of this Sgk1 kinase inhibitor in the treatment of colon carcinoma as a single agent or in combination with taxanes.
Materials and Methods

In vitro inhibition of Sgk1 kinase activity by molecules
SI113 was produced and kindly provided by one of us (S.S.). The molecule was first diluted in DMSO as a 50 mM stock solution; then it was further diluted in saline solution at the concentrations indicated in the text. Control cells were treated with vehicle alone.

Active Sgk1, Akt1, Ab1, and Src kinases (all by EMD Millipore, Darmstadt, Germany) were incubated in the appropriate kinase buffers provided by the manufacturer in the absence or in the presence of specific target peptides, as indicated in the Results section, according to previously published methods [30]. The reactions were allowed to occur for 30 min at room temperature, with gentle agitation, prior to adding 10 ml of stopping solution (1 mM ATP, 2% bovine serum albumin, 0.6% w/v HCl). The reaction mix was clarified by centrifugation in an Eppendorf microcentrifuge at room temperature, for 10 min at 14,000 rpm. Supernatants (10 µl) were applied to a 2.1-cm Ø p81 Whatman paper. Filters were dried, washed with 25 mM phosphoric acid, once with acetone, and then counted in a scintillation counter to measure the radioactivity incorporated in the substrate peptide. Background radioactivity was estimated by measuring the amount of radioactivity incorporated in the absence of the active kinase.

Cell culture and transfection

RKO colorectal adenocarcinoma cells, MCF-7 breast cancer cells and A-172 glioblastoma multiforme cells were plated at a concentration of 5 x 10^5 cells/ml in 100 mm Ø plates in DMEM-low glucose medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Aurogene, Rome, Italy). Cells were cultured at 37°C in a humidified atmosphere of CO_2 (5%) and air (95%). After 24 h, when cells were approximately 60% confluent, SI113 or vehicle was added at the desired concentration for the time indicated in Results section. Paclitaxel was used at a concentration 10 nM for 16 h, alone or in combination with SI113. Cell proliferation was evaluated by measuring the total number of cells using a Burker chamber. Cell viability was monitored by trypan blue exclusion. RKO cells were transfected with an expression plasmid (PcDNA4-TO myc Sgk1)(WT) using Lipofectamine 2000 (Life Technologies, Inc.) following the manufacturer’s instructions.

Insulin stimulation and Western blot

Cell cultures were serum-starved for 12 h before stimulation. 1 µM insulin was added 30 min before lysis [Lysis buffer: 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.5% IGEPA, 25 mM NaF, 1 mM DTT, 1 mM Na_3VO_4 plus protease inhibitor cocktail 10 X (Sigma-Aldrich, St. Louis, MO)].

Protein extracts (10-30 µg/well) were separated by 12% SDS- polyacrylamide gel electrophoresis and blotted on a 0.45 µm nitrocellulose membrane (Bio-Rad). Filters were incubated overnight at 4°C in blocking buffer (1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk).

Insulin-stimulated cells were used for western blot detection of phosphorylated Mdm2 (pMdm2) by means of phospho-serine 166 Mdm2 rabbit polyclonal antibody (#35215 Cell Signaling, Danvers, MA). Total Mdm2 was detected by Mdm2 Rabbit immunoglobulins (#C1908 Santa Cruz Biotechnology, Inc. Santa Cruz, CA). Primary antibodies (1:800 dilution) were incubated overnight at 4°C in 1X TBS, 0.1% Tween-20, containing 3% BSA.

After incubation with peroxidase-linked secondary antibodies, bands were detected by chemiluminescence using the ECK kit (GE Healthcare). When membrane stripping was indicated, filters were incubated for 20 min at 37°C with Restore Western Blot Stripping Buffer (Thermo Scientific, # 21059).

Inhibition of Sgk1 kinase activity in an intact cell environment

In these experiments, Myc-Sgk1 was immunoprecipitated using a rabbit anti-Myc antibody (Santa Cruz Biotechnology, Inc.), whereas endogenous Sgk1 was immunoprecipitated by means of a Sgk1-specific rabbit polyclonal antibody (#07-315, EMD Millipore). Immunoprecipitates were employed in the kinase assay reaction as described above, according to previously published methods [3, 15].

Apoptosis and Cell cycle assays

Guava Nexin Assay. Apoptosis was studied in adherent RHO cells. To this end, the culture medium was aspirated and discharged, whereas the remaining adherent cells growing in monolayer were washed.
extensively with calcium- and magnesium-free Dulbecco’s phosphate buffered saline (PBS). Cells were then trypsinized, diluted with complete culture medium (plus serum) to stop the proteolytic activity, centrifuged, washed with PBS and used for the nexin assay following the instructions of the manufacturer. Briefly, 2 x 10^6 RKO cells, untreated or treated with 12.5 µM SI113 for 24 h, were prepared for incubation with 1% BSA.

2 x 10^4 cells were incubated with 100 µl Guava Nexin Reagent (100 tests, Lot No. 14-0032, Merck Millipore), a pre-made cocktail containing Annexin V-PE and 7-AAD in buffer, in a 200 µl final volume. After 20 min incubation at room temperature in the dark, samples were ready to be acquired by the Guava System.

The Guava Nexin Assay utilizes Annexin V-PE to detect phosphatidylserine (PS) on the external membrane of apoptotic cells. The cells impermeant dye, 7-AAD, is also used in the Guava Nexin Assay as an indicator of cell membrane structural integrity. 7-AAD is excluded from live, healthy cells as well as early apoptotic cells.

The assay allows the detection of three populations of cells, i.e.: non-apoptotic cells AnnexinV(-) and 7-AAD(-); early apoptotic cells Annexin V(+) and 7-AAD(-) and late stage apoptotic and dead cells Annexin V(+) and 7-AAD(+).

The results include count and percentage of cells in each of the quadrant-defined populations, as well as the mean fluorescence intensity of Annexin V and 7-AAD for each population.

**Guava Cell Cycle Assay.** RKO cells (5 x 10^4) were plated in 65 mm Ø plates and incubated at 37°C to allow the cells to attach to the plate and to grow up to 60-80% confluence before proceeding. RKO cells were treated with 12.5 µM SI113 for 24 h and paclitaxel 10nM for 16h. Cells were harvested by trypsinization, fixed in ice-cold 70% ethanol and stored at -20°C overnight, prior to staining. Cell pellets were washed twice with cold PBS and incubated with 200 µl of a propidium iodide-based staining reagent (Guava Cell Cycle Reagent. 4700-0160, Merck Millipore) at room temperature for 30 min. All samples were transferred to 1.5 ml microcentrifuge tubes and analyzed on the Guava Instrument.

**Statistical analysis**

All tests were done at least in triplicates, as indicated, and the results were expressed as mean +/- Standard Error (SE). Differences between groups were analyzed by unpaired two tailed t Test (Fig. 1 through 5) and by one-way ANOVA followed by Newman-Keuls post hoc test, using the GraphPad Prism v5.01 software (Fig. 6).

**Results**

**In vitro kinase activity inhibition by SI113**

Sgk1, Akt1, Abl and Src kinase activities were measured as radioactivity incorporated in specific target peptides, i.e.: crosstide (GRPRTSSFAEGKK) for Sgk1 and Akt1, Cdc2 aa 6-20 (KVEKIGEGTYGVVYK) for Src, and ABLtide (EAIYAAPFAKKK) for Abl according to previously published methods [3, 31, 32], in the presence or absence of 12.5 µM SI113 for 30 min. Sgk1 kinase activity was completely abrogated by this compound, whereas variable amounts of residual kinase activity persisted for the other three kinases tested (Table 1). Interestingly, Src kinase activity, at lower SI113 concentration (30 nM), appeared significantly enhanced, possibly representing a consequence of allosteric regulation and/or binding dependent conformational change of this kinase (data not shown).

**Table 1. Inhibition of different kinase activities by SI113.** Residual activity of the respective kinases after exposure to 12.5 µM SI113 for 30 min. Values are the average of three experiments performed in triplicate and are expressed as percent residual kinase activity in the presence of SI113 compared to the respective activity in the presence of the vehicle alone.

| Kinase full name                        | Kinase short name | % residual activity |
|-----------------------------------------|-------------------|---------------------|
| Serum- and glucocorticoid-regulated kinase 1 | Sgk1              | 0 ± 0               |
| RAC-alpha serine/threonine-protein kinase | Akt1              | 87 ± 3.8            |
| Abelson murine leukemia viral oncogene homolog | Abl               | 37 ± 1.6            |
| Sarcoma kinase                          | Src               | 40.3 ± 9.7          |
SI113 effect on cell number and viability in RKO, MCF-7 and A-172 human cancer cell lines

RKO colorectal adenocarcinoma, MCF-7 breast cancer and A-172 glioblastoma multiforme cell lines were plated as indicated in the Methods section. SI113 (12.5 µM) was added for 72 h. Control cells were treated with vehicle alone. Cells were then trypsized, and viable cells were stained with trypan blue and counted. In all three cell lines, SI113 determined a significant reduction in the number of viable cells (Fig. 1 panel B, MCF-7 (top), A-172 (middle) and RKO cells (bottom). The most notable effect in cell number reduction was achieved in the RKO cell line. A dose/response curve describing the inhibitory effect of SI113 on RKO cells proliferation is shown in Fig. 1 panel C.

SI113 was added at concentration of 1, 4, 8, 12.5 and 25 µM. Viable cells were counted after 72 h as above. Half-maximal inhibitory dose corresponded to approximately 8 µM.

Fig. 1. Growth inhibition induced by SI113 in RKO, MCF-7 and A-172 human cancer cell lines. A. Structural formula of the SI113 molecule. B. MCF-7 breast cancer cells (top), A-172 glioblastoma multiforme cells (middle) and RKO colon carcinoma cells (bottom). The bar diagrams represent the number of viable cells, untreated or treated with SI113 (12.5 µM) for 72 h. Values for treated and untreated cells were compared by unpaired two-tailed Student’s t test. Results represent the mean ± S.E. of three independent experiments for each cell line. Statistical significance: RKO cells (P = 4.00452 x 10⁻⁷), MCF-7 cells (P = 0.00069), A-172 cells (P = 0.00024). *** P ≤ 0.001. C. Dose dependent inhibition of RKO cells proliferation. SI113 concentrations (1, 4, 8, 12.5 and 25 µM) are expressed in log scale. Viable cells were counted after 72 h.
**Fig. 2.** SI113-induced apoptosis in RKO cells. RKO cells were treated with SI113 (12.5 µM) for 24 h. The percentage of cells stained with Annexin V (early and late stage apoptotic cells) and 7-AAD (late stage apoptotic and dead cells) was calculated by the Guava Nexin Assay. A. The bar diagrams represent the number of control cells and those treated with SI113 for 24 h in a parallel experiment. Data represent means ± S.E. from triplicates (P = 0.0028). B. Representative FCM analyses are shown. C. The bar diagrams depict the percentage of cells that are Annexin V(+) and 7-AAD(-) (early apoptosis) and the percentage of cells that are Annexin V(+) and 7-AAD(+) (late apoptosis) after exposure to SI113, as indicated. SI113 treatment for 24 h determined a significant increase in the percentage of Annexin(+) cells (early and late apoptosis (P = 0.029) and a significant increase in the percentage of Annexin(+) and 7-AAD(+) cells (late apoptosis, P = 0.035). * P ≤ 0.05; ** P ≤ 0.01.

**SI113 induces apoptosis in RKO cells**

Since several published observations point to a crucial role played by Sgk1 in colon carcinogenesis (see above in the Introduction) we elected to focus on some mechanistic and functional effects of SI113 on the RKO cell line.

Treatment with 12.5 µM SI113 for 24 h induced a significant and important (more than 50%) decrease in total cell number (Fig. 2 panel A). We then examined the effects of SI113 (12.5 µM for 24 h) on the induction of apoptosis in RKO cells. We focused on the early and late phases of apoptosis and analyzed the adherent cells only, as specified in the Methods section. Cells were labelled with Annexin-V-Fluorescein and 7-AAD, and the percentage of Annexin(+) cells including Annexin-V(+)/7-AAD(-) (early apoptotic cells) and Annexin-V(+)/7-AAD(+) (late apoptotic cells) was determined by flow cytometry (Fig. 2, panel B). A significant increase in the percentage of Annexin V(+) cells was recorded (1.53 ± 0.57 % for control cells, 4.8 ± 0.81 % for SI113-treated cells, P = 0.029). Interestingly, the percentage of Annexin V(+)/7-AAD(+) (late apoptotic cells) also increased significantly (0.33 ± 0.11 % for control cells, 1.96 ± 0.32 % for SI113-treated cells, P = 0.035) (Fig. 2 panel C). The increase in the percentage of apoptotic cells is only apparently small. Since solely adherent cells were analyzed, most of the dead cells were discharged with the medium. The increase in apoptotic cells thus represents the percentage of adherent cells that were actually in the process of dying at that specified time point.
Sgk1 inhibition by SI113 in an intact cellular environment

We investigated the effects of SI113 upon the enzymatic activity of a Myc-tagged Sgk1 molecule. After transiently expressing Myc-tagged Sgk1 in RKO colon carcinoma cells, transfected cells were incubated in the presence or absence of 12.5 µM SI113 for 12 h and/or 10^{-6} M insulin for the following 30 min. Cells were then lysed and the chimeric Myc-Sgk1 protein was immunoprecipitated using an anti-Myc antibody. Sgk1 activity was then assayed in the immune complexes. Insulin significantly increased Sgk1 activity, as expected [3]. Incubation of RKO cells in the presence of SI113 led approximately to a 50% inhibition of insulin-stimulated Sgk1 activity, thus suggesting that the molecule was able to permeate the cells and inhibit intracellular Sgk1 activity (Fig. 3, panel A).

Similarly, RKO cells were incubated in the presence or absence of 12.5 µM SI113 for 12 h and 10^{-6} M insulin for the following 30 min. Endogenous Sgk1 activity, immunoprecipitated by a Sgk1 specific antibody, was determined as above. Again, insulin significantly increased the endogenous Sgk1 activity [3]. Incubation of RKO cells in the presence of SI113 led approximately to a 50% inhibition of insulin-stimulated Sgk1 activity, thus suggesting that the molecule was able to permeate the cells and inhibit intracellular Sgk1 activity (Fig. 3, panel B), similarly to what was observed in the experiments with the transfected recombinant kinase.

Effect of SI113 on Mdm2 phosphorylation

Sgk1 regulates p53 expression through direct phosphorylation on serine 166 and subsequent stabilization of its negative regulator Mdm2 [15]. When RKO cells were incubated in the presence of 12.5 µM SI113 for 12 h, basal and insulin-stimulated phosphorylation of Mdm2 on serine 166 was drastically reduced, consistent with the inhibition of Sgk1 activity that is known to phosphorylate and stabilize Mdm2 (Fig. 4, panel A for western blotting and panel B for densitometric evaluation).
Cell cycle distribution in RKO cells after SI113 or paclitaxel treatment

In order to analyze the timing of cell cycle progression in the presence of SI113, cell cycle was analyzed by cytofluorometry in cells growing in the presence of serum after staining with propidium iodide. RKO cells treated with 12.5 µM SI113 for 24 h showed a striking delay in cell cycle progression with a significant accumulation in G0-G1, when compared with control cells (P = 0.0024) (Table 2). Treatment with 10 nM paclitaxel for 16 h gave quite different results, showing, as expected, a significant increase in the percentage of G2-M cells (P = 0.00019), in accordance with the paclitaxel-dependent mitotic arrest [16].

Table 2. Cell cycle analysis. Cell cycle distribution in RKO cells treated with 12.5 µM SI113 for 24 h or 10 nM paclitaxel for 16 h. Values are the average of three experiments performed in triplicate and are expressed as percent distribution ± S. E. ** P< 0.01; ***P< 0.001

|         | % G0/G1   | % G2/M    | % S       |
|---------|-----------|-----------|-----------|
| Control | 61.16 ± 1.28 | 25.986 ± 1.21 | 11.666 ± 0.25 |
| SI113   | 70.036 ± 1.45 | ** 17.686 ± 1.06 | ** 7.153 ± 0.045 |
| Paclitaxel | 32.676 ± 2.35 | ** 53.170 ± 2.52 | *** 13.603 ± 0.41 |

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Time course of SI113- and paclitaxel-induced apoptosis and necrosis in RKO cells

RKO cells treated with 12.5 µM SI113 and/or 50 nM paclitaxel for 2, 4 or 6 h were analyzed by cytofluorometry, as indicated above (Fig. 5, panel A, B and C, respectively).

After treatment with SI113, the percentage of late stage apoptotic and dead cells, i.e. Annexin V(+) and 7-AAD(+), slightly increased at the 4 h time point of treatment (Fig. 5, panel B), but the difference with control cells reached the statistical significance only at the 6 h time point (1.4± 0.4% for control cells, 2.8 ± 0.6% for SI113-treated cells, P= 0.023) (Fig. 5, panel C). Paclitaxel appeared to be more rapidly effective. The percentage of positive

Fig. 4. Inhibition of intracellular Sgk1 kinase activity by SI113. A. RKO colon carcinoma cells were grown in the presence of insulin and/or SI113, as specified. Cells were then lysed and analyzed by Western blot after SDS-PAGE, for the amount of pMdm2, a Sgk1 substrate and a surrogate marker of the Sgk1 kinase activity (upper panel). Loading control was done by determining, after membrane stripping, the amount of the total Mdm2 (phosphorylated and non-phosphorylated) protein (lower panel). MDM2 (approximately 90 kDa) migrates slight above the 80 kDa marker. Phosphorylation decreased the electrophoretic mobility of the protein, as expected. The figures are representative of three different experiments. B. Bar diagram representation of the Western blot experiments by densitometric analysis and semi-quantitative determination of pMdm2 after normalization against loading control and background. Values (A.U. Mean ± Standard Error) were obtained in three different experiments. Insulin significantly increased Sgk1 activity, as expected, in the absence of SI113 (P = 0.027). SI113 significantly reduced basal (P = 0.019) and insulin-stimulated (P = 0.0033) phosphorylation of MDM2. * P≤ 0.05; ** P≤ 0.01.
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**Fig. 5.** Time course of SI113- and paclitaxel-induced apoptosis and necrosis in RKO cells. RKO cells were treated with SI113 (12.5 µM) and/or with paclitaxel (50 nM) for 2, 4 and 6 h. The percentage of cells stained with both Annexin V and 7-AAD (late stage apoptotic cells and dead cells, upper right) was calculated by the Guava Annexin Assay and shown in the representative graphs on the left on each panel. Bar diagrams on the right of each panel depict the percentage of cells undergone apoptosis and necrosis after exposure to the agents, as indicated. To facilitate the interpretation of the data, the full-scale value of the Y axis is the same for all the right panels. A. After 2 h, RKO cells treated with both agents demonstrate an increase in the percentage of Annexin V(+) and 7-AAD(+) cells when compared with control cells (*P = 0.014*), whereas no significant increase was observed with either agents alone. B. After 4 h, RKO cells treated with both agents demonstrate an increase in the percentage of Annexin V(+) and 7-AAD(+) cells when compared to control cells (*P = 0.0158*), whereas no significant increase was observed with either agents alone. C. After 6 h, the percentage of Annexin V(+) and 7-AAD(+) cells was significantly increased in cells treated with either SI113 (*P = 0.029*) or paclitaxel (*P = 0.013*) and with the combination of both agents (*P = 0.00037*). The percentage of Annexin V(+) and 7-AAD(+) cells was significantly higher in cells treated with both agents than the percentage of Annexin V(+) and 7-AAD(+) cells observed after the treatment with either SI113 (*P = 0.033*) or paclitaxel (*P = 0.032*) alone. Data are the average ± S.E. of six individual values. * *P ≤ 0.05; *** *P ≤ 0.001.

cells increased at 2 and 4 h of paclitaxel treatment (Fig. 5, panels A and B, respectively) but, again, the difference with control cells reached significance only at the 6 h time point (1.4 ± 0.4  % for control cells, 2.9 ± 0.52 % for paclitaxel-treated cells, *P = 0.013*) (Fig. 5, panel C). Interestingly, the use of both SI113 and paclitaxel significantly increased the percentage of positive cells already at the 2 h time point (1.7 ± 0.23 % for control cells, and 2.9 ± 0.52 % for paclitaxel-treated cells, *P = 0.013*) (Fig. 5, panel A). The difference was also significant at the 4 h time point (1.4 ± 0.38 % for control cells and 3.66 ± 0.35 % for paclitaxel-treated cells, *P = 0.0158*) (Fig. 5, panel B). After 6 h of treatment with both agents (Fig. 5, panel C), the percentage of positive cells (4.36±0.62) was significantly higher than in control samples (1.4 ± 0.48%, *P= 0.00037*), SI113 alone (2.8 ± 0.61 %, *P = 0.033*) and paclitaxel
alone (2.93±0.52%, *P*= 0.032). Taken together, these results strongly suggest that treatment with SI113 can potentiate the effects of paclitaxel on the survival of RKO cells in culture.

**SI113 and paclitaxel inhibit RKO cell proliferation**

RKO cell number and viability was measured in control cells and in the presence of 12.5 µM SI113 for 24 h, 10 nM paclitaxel for 16 h or both. Either SI113 or paclitaxel significantly reduced the cell number, as assessed by cell counting in the presence of the trypan blue exclusion dye. The addition of both agents further and significantly reduced cell number and viability (Fig. 6), strongly suggesting that SI113 and paclitaxel may be used together to enhance the effects of either agents alone.

**Discussion**

Colorectal carcinoma represents one among the most frequent causes of cancer death. Many epidemiological data support the existence of a link between colon cancer and conditions like obesity, type 2 diabetes, insulin resistance, hyper-insulinemia and insulin therapy [33, 34]. Moreover, enhanced activation and intracellular localization of the insulin receptor appear to play a role in early stages of colon carcinogenesis [35]. The kinase Sgk1, which is activated by insulin, growth factors and steroids, is an interesting candidate to explain the increased cancer risk observed in patients with obesity and insulin resistance, thus representing a potential pharmacological target for these tumors.

The data reported here provide an improved characterization, at a molecular and cellular level, of the SI113 Sgk1 inhibitor previously described by our group [30]. Indeed, this molecule appears effective and selective in inhibiting Sgk1 kinase activity, either evaluating its efficacy using isolated (recombinant or endogenous) proteins in specific kinase assays, or employing Mdm2 as a surrogate marker for Sgk1 kinase activity in an intact cellular environment (RKO cells). Such an inhibitory effect, as expected, influences several major cellular features, enhancing the apoptotic processes, decreasing overall cell viability and, lastly, producing a block in the G1-S transition. All these features appear strictly in accordance with the down-modulation of the signal transduction pathways in which the Sgk1 molecule is actively involved.

Considering the biological role of Sgk1, which can be epitomized as a stress survival factor, associated with the increasing knowledge on the significance of this kinase for cancer cell survival and replication, it is of key importance to make a selective Sgk1 kinase inhibitor available to specifically hit cancer signal transduction pathways and cell survival mechanisms.
mechanisms. The SI113 molecule, indeed, was effective \textit{in vitro} in all the cancer cell lines tested so far. Most of our previous studies were carried out on Sgk1 [14-16]. Sgk2 expression appears to be down-regulated in colon cancer [36], whereas Sgk3 expression appears to have a role in the mechanisms of resistance to Akt inhibitors [26]. At present we cannot rule out that some of the effects described for SI113 may be attributed to the inhibition of other of Sgk1-related kinases (namely Sgk2 and Sgk3).

A previous study by our group describes the Sgk1-dependent regulation of RANBP1, a major effector of the RAN GTPase, and the functional consequences of this regulation on microtubule activity and paclitaxel sensitivity in RKO colon adenocarcinoma cells [16]. According to this notion, we detected an obvious cooperation between paclitaxel and SI113 in inhibiting RKO cancer cell growth \textit{in vitro}.

Given that Sgk1 expression in NSCLC patient samples significantly correlates with cancer malignancy and is thus considered a worse prognostic indicator [13], the \textit{in vivo} use of a small molecule able to inhibit selectively Sgk1 kinase activity can specifically hit cancer cells, contemporarily sparing normal cells, characterized by a modest expression of the kinase Sgk1. On the other hand, the sometimes ambiguous results described for the clinical use of Akt inhibitors in cancer therapy [37, 38], recently attributed to the high expression of Sgk3 [26], can carve out a peculiar role for Sgk inhibitors in blocking the Ras/PI3K/PTEN/Akt(Sgk)/mTOR pathway in specific neoplastic phenotypes [1].

Interestingly, compounds belonging to the same \textit{pyrazolo[3,4-d]pyrimidine} library of SI113, showed no apparent sign of toxicity after oral administration for 15 days in mice [39].

The SI113 molecule has a molecular mass <500 Da [30], and its chemical structure makes it potentially highly available in all body fluids and theoretically able also to cross the blood-brain barrier. Such a feature makes it eligible to perform an exhaustive \textit{in vivo} analysis of its antineoplastic properties in immunocompromised mice carrying human tumors. In particular, this study suggests the use of SI113 as a pharmacological agent in human colorectal cancer, to be employed alone or in combination with paclitaxel and/or other chemotherapeutic agents.

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\textbf{Disclosure Statement}

The authors declare no conflict of interest.

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