

# hERG1/Kv11.1 activation stimulates transcription of p21<sup>waf/cip</sup> in breast cancer cells *via* a calcineurin-dependent mechanism

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## ABSTRACT

The function of Kv11.1 is emerging in breast cancer biology, as a growing body of evidence indicates that the hERG1/Kv11.1 potassium channel is aberrantly expressed in several cancer types including breast cancers.

The biological effects of Kv11.1 channel blockers and their associated side effects are very well known but the potential use of Kv11.1 activators as an anticancer strategy are still unexplored. In our previous work, we have established that stimulation of the Kv11.1 potassium channel activates a senescent-like program that is characterized by a significant increase in tumor suppressor protein levels, such as p21<sup>waf/cip</sup> and p16<sup>INK4A</sup>. In this study we investigated the mechanism linking Kv11.1 stimulation to augmentation of p21<sup>waf/cip</sup> protein level. We have demonstrated that the Kv11.1 channel activator NS1643 activates a calcineurin-dependent transcription of p21<sup>waf/cip</sup> and that this event is fundamental for the inhibitory effect of NS1643 on cell proliferation. Our results reveal a novel mechanism by which stimulation of Kv11.1 channel leads to transcription of a potent tumor suppressor and suggest a potential therapeutic use for Kv11.1 channel activators.

## INTRODUCTION

The human ether a-go-go related gene 1 (hERG1) encodes for the voltage gated Kv11.1 potassium channel which is traditionally known to play a fundamental role in controlling membrane potential in neurons, myocytes [1] or in gland cells [2-4]. Interestingly, Kv11.1 has been found to be expressed in several cancer cell types including carcinomas of the breast in which Kv11.1 current activity appears to be essential for cell proliferation [5-8]. Although these data suggest that Kv11.1 has oncogenic properties, very little is known about the contribution of Kv11.1 activity to the mechanisms controlling cell cycle.

The *CDKN1A* gene encodes for the cyclin-dependent kinase inhibitor p21<sup>waf/cip</sup> which plays a fundamental role in controlling cell-cycle progression [9]. It is generally accepted that p21<sup>waf/cip</sup> transcription is activated by oncogenic stimuli to prevent dysregulation of the cell cycle process. By inhibiting the activity of cyclins, p21<sup>waf/cip</sup> determines the arrest of the cell cycle progression at G1 and S-phase [10]. Changes of p21<sup>waf/cip</sup> protein level can be controlled mostly at the transcriptional level and depend

on cellular context. In quiescent cells, increased p21<sup>waf/cip</sup> protein level is temporary as these cells are waiting for the proliferative stimuli that induces inhibition of p21<sup>waf/cip</sup> synthesis [11]. In contrast, increased p21<sup>waf/cip</sup> protein level that is associated with augmentation of p16<sup>INK4A</sup> and beta-galactosidase, lead to a permanent arrest of the cell cycle as they become a part of the activation of a senescence program [12]. However, recent investigations have also revealed that activation of p21<sup>waf/cip</sup> can be associated with tumorigenesis [13] or apoptosis [14].

Generally, p21<sup>waf/cip</sup> transcription can occur via activation of tumor protein 53 (p53-dependent) and p53-independent mechanisms [15]. For example, hyperactive Ras or Raf determine p53-dependent transcription of p21<sup>waf/cip</sup> via activation of the E2F1 transcription factor [16, 17]. Mutations in the p53 gene leading to the production of non-functional protein greatly increases the risk of developing breast cancer due to lack of p21<sup>waf/cip</sup> function. In contrast, nuclear receptors such as androgen receptors can activate p21<sup>waf/cip</sup> transcription independently of p53 by directly binding *CDKN1A* gene [18-20]. Interestingly, Ca<sup>2+</sup> appears to cooperate with specific transcription

factors such as SP1, AP2 or NFAT in transcribing p21<sup>waf/cip</sup> independently of p53 [21].

Calcineurin (CN) is a ubiquitously expressed serine/threonine phosphatase that plays a fundamental role in many physiological or pathological states [22-24]. CN is activated by calmodulin upon increased intracellular Ca<sup>2+</sup> and acts on numerous substrates including transcription factors such as the nuclear factor of activated T cell (NFAT) proteins. Therefore, CN is considered as a major player in the signaling pathways that couple changes in Ca<sup>2+</sup> homeostasis and gene transcription. However, the role of CN in cancer biology is still debated as the effects of its activation in cells can favor tumor progression or suppression [25, 26].

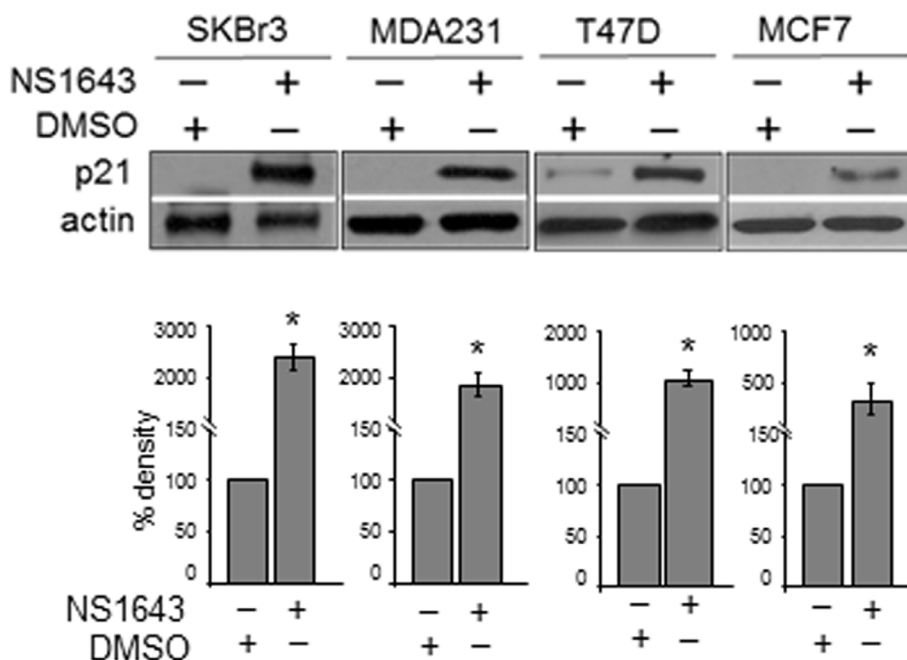
In our previous works, we have demonstrated that application of hERG1/Kv11.1 potassium channel activator NS1643 to breast cancer cells caused an increase in Ca<sup>2+</sup> entry [6] and increased p21<sup>waf/cip</sup> protein level that resulted in a senescence-like phenotype while no effect was observed in non-transformed cells [6, 7]. However, we did not investigate the mechanism by which NS1643 augmented p21<sup>waf/cip</sup> protein level. In this work we demonstrate that NS1643 stimulates p21<sup>waf/cip</sup> transcription via activation of CN.

## RESULTS

### Stimulation of hERG1/Kv11.1 channel activity increases p21<sup>waf/cip</sup> protein level in molecularly diverse breast cancer cells

In our previous work we have shown that chronic stimulation of hERG1/Kv11.1 potassium channel determined a strong increase of p21<sup>waf/cip</sup> protein level in breast cancer cells that lack expression of estrogen receptor (ER<sup>neg</sup>). In the current study we have expanded our investigation on the effect of chronic stimulation of Kv11.1 on the tumor suppressor p21<sup>waf/cip</sup> by testing the effect of NS1643 on molecularly different breast cancer cells representative of the following phenotype: Luminal A and p53-positive (MCF7), Luminal A and p53-negative (T47D), claudin-low breast cancer cells (MDA-MB-231) and HER2-overexpressing (SKBr3) breast cancer cells [27].

We found that application of NS1643 determined a significant increase of p21<sup>waf/cip</sup> protein level that was similar in all breast cancer cells that we have examined (Figure 1). This suggests that the stimulation of Kv11.1 channel increases p21<sup>waf/cip</sup> expression level independently from molecular heterogeneity of breast cancers.



**Figure 1: NS1643 increases p21<sup>waf/cip</sup> protein level in breast cancer cells.** **A.** Representative western blot analyses of extracts (n=3; 32µg loading) from SKBr3, MDA-MB-231 (MDA231), T47D or MCF7 cells treated with DMSO (control) or NS1643 (50 µM) for 48 hr using p21 antibody. Anti-actin immunoblot was used as a loading control. Antibody were purchased from Cell Signaling. **B.** Bar graphs summarizing the observed effects of NS1643 exposure on p21 protein expression (n = 3; \*p < 0.01). SKBr3 (HER2<sup>+</sup>, ER<sup>-</sup>, PR<sup>-</sup>, p53-null), MDA-MB-231 (HER2<sup>+</sup>, ER<sup>-</sup>, PR<sup>-</sup>, p53-null), MCF7 (HER2<sup>+</sup>, ER<sup>+</sup>, PR<sup>+</sup>, p53-WT), T47D (HER2<sup>+</sup>, ER<sup>+</sup>, PR<sup>+</sup>, p53-null) breast cancer cells were exposed to NS1643 (50µM) for 24 hr.

## Suppression of p21<sup>waf/cip</sup> function counteracts the inhibitory effects of NS1643 on cell proliferation

To understand the contribution of p21<sup>waf/cip</sup> in mediating the inhibitory effect of chronic stimulation of Kv11.1 channel on cancer cell growth we monitored the proliferation rate of cells in which p21<sup>waf/cip</sup> function have been suppressed (Figure 2A) and treated with NS1643. Interestingly, the inhibitory effect of NS1643 on cell proliferation was completely repressed in cells expressing siRNA targeting p21<sup>waf/cip</sup> compared to naïve cells (Figure 2B). These data suggest that p21<sup>waf/cip</sup> plays a fundamental role in controlling the inhibitory effect of NS1643 on cancer cell growth.

### NS1643 induces p21 gene transcription

We first assessed whether the NS1643-dependent increased p21<sup>waf/cip</sup> was a consequence of augmented protein synthesis. Therefore, we monitored mRNA level for p21<sup>waf/cip</sup> in cells that were treated with or without Kv11.1 agonist (Figure 3). We found that application of NS1643 for 24hr determined a strong increase of mRNA level suggesting that activation of Kv11.1 channel stimulates p21<sup>waf/cip</sup> gene induction.

To further validate this hypothesis we transfected cells with a luciferase-based reporter construct that contains p21<sup>waf/cip</sup>-promoter region [28] and then we treated these cells with NS1643 (Figure 4A). We found that stimulation of Kv11.1 channel strongly induced the p21<sup>waf/cip</sup> promoter as showed by increased luciferase activity.

### NS1643-dependent increase of p21 protein level is blocked by calcineurin inhibition

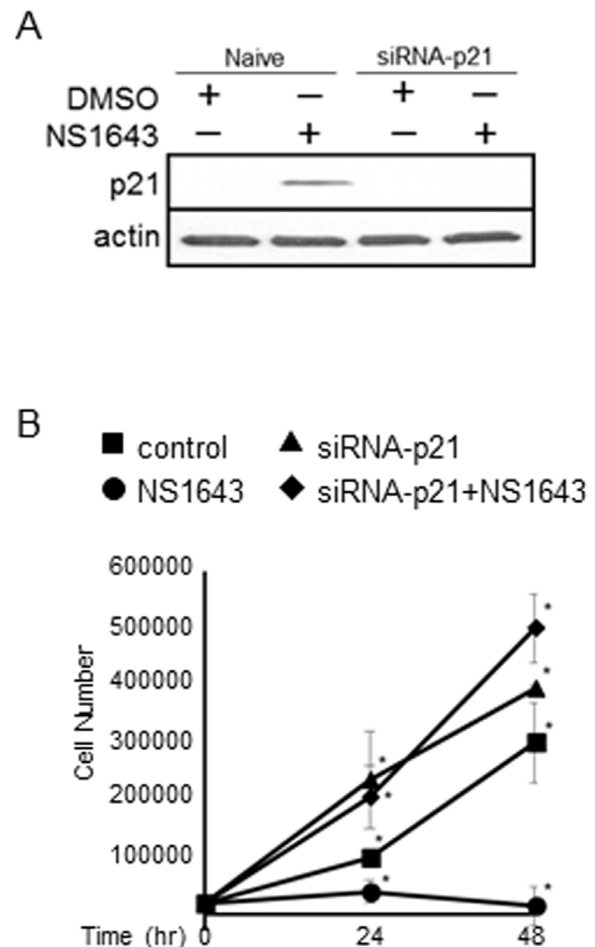
It is well understood that changes in cytosolic Ca<sup>2+</sup> can activate the serine/threonine protein phosphatase calcineurin which in turn can activate biochemical pathways including nuclear transcription. Therefore, based on our previous discovery that NS1643 can increase cytosolic calcium [7], we monitored the effect of the calcineurin inhibitor cyclosporine A (CsA) on NS1643-dependent p21<sup>waf/cip</sup> expression.

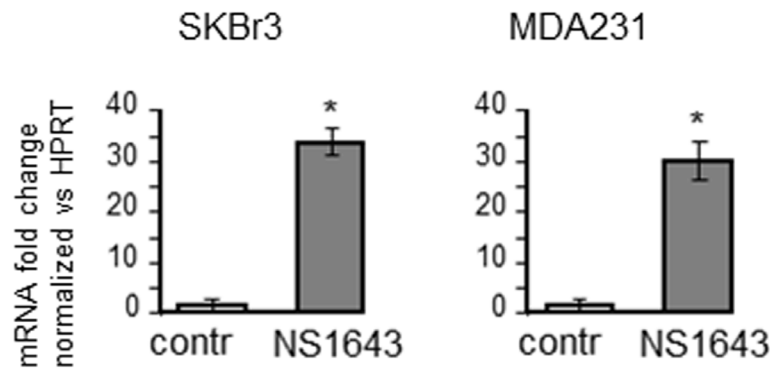
**Figure 2: Suppression of p21<sup>waf/cip</sup> counteracts the inhibitory effect of NS1643 on cell proliferation.** A. Immunoblot analysis showing efficacy of siRNA-mediated p21<sup>waf/cip</sup> knockdown in SKBr3 cells. The blot shows the effect of NS1643 on p21<sup>waf/cip</sup> in naïve cells (control) and in transfected cells. Actin immunoblots are utilized as loading controls (lower panels). B. Graph comparing the cell number over time of SKBr3 cells expressing siRNA targeting p21<sup>waf/cip</sup> alone (siRNA-p21<sup>waf/cip</sup>; square), siRNA-p21<sup>waf/cip</sup> and NS1643 (circle, 50 µM), NS1643 alone (diamond) or DMSO (control; triangle). (*n* = 6 each treatment; \**p* < 0.01).

Strikingly, our luciferase assay revealed that CsA significantly blocked NS1643-dependent induction of p21<sup>waf/cip</sup> gene (Figure 4A). Similarly, western blot analysis showed that application of CsA strongly impairs NS1643 to increase p21<sup>waf/cip</sup> protein level Figure 4B). Altogether, these data suggest that stimulation of Kv11.1 potassium channel determine increased p21<sup>waf/cip</sup> protein synthesis that is mediated by CN.

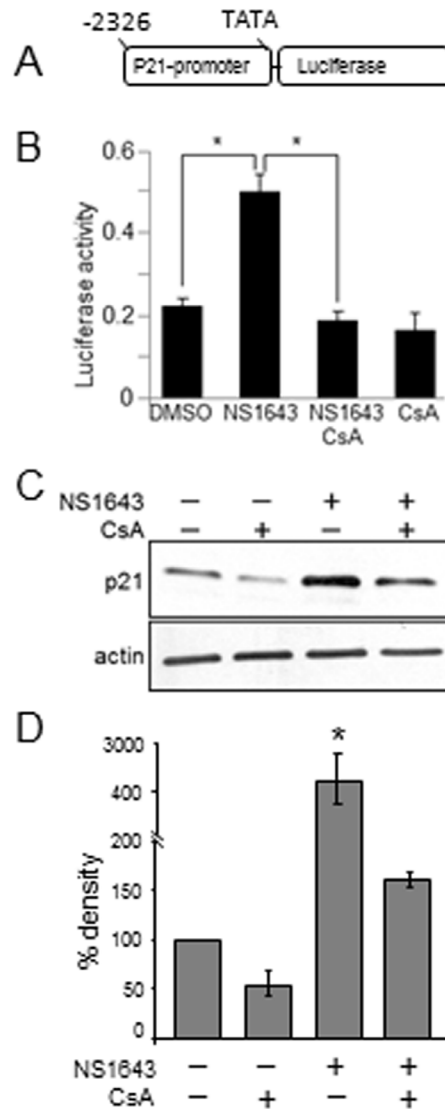
### NS1643 determines CN-dependent NFAT activation

To further test the hypothesis that the effect of NS1643 on p21<sup>waf/cip</sup> protein level is mediated by CN-dependent gene transcription we monitored the activity of one of the best characterized CN-controlled gene regulators, NFAT [29, 30]. Consequently, to assess whether NFAT is activated by NS1643, we transfected cells with a luciferase-based reporter construct that contains NFAT response elements (NFAT-RE) [31] and we discovered that treating transfected cells with NS1643 strongly stimulates NFAT activation. Application of cobalt (Co<sup>2+</sup>), a generic surface membrane Ca<sup>2+</sup> channel blocker, or the calcineurin





**Figure 3: NS1643 increases p21 mRNA level.** Graph showing fold changes of p21 mRNA level in SKBr3 or MDA-MB-231 cells treated with or without NS1643 (50 $\mu$ M) for 24hr.  $p < 0.001$  compared with control.



**Figure 4: Cyclosporine-A inhibits NS1643-dependent stimulation of p21 induction.** **A.** Schematic illustration of the luciferase-based reporter construct that contains p21<sup>wal/cip</sup>-promoter region (Addgene plasmid #21723). **B.** Fold changes of p21-luciferase activity in SKBr3 expressing p21-luciferase reporter and treated with DMSO (control), NS1643, NS1643+Cyclosporine-A (CsA, 10  $\mu$ M) or CsA alone. **C.** Western blot analyses of extracts from SKBr3 cells treated with CsA alone, NS1643 alone or CsA+NS1643 for 24hr. NS1643 for the indicated times, using p21 antibody. Antibody were purchased from Cell Signaling.

blocker cyclosporine A (CsA) strongly inhibited the effect of NS1643 on NFAT activity suggesting that stimulation of the Kv11.1 channel activity leads to activation of NFAT-dependent transcription.

Furthermore, we assessed the ability of NS1643 in activating NFAT by monitoring nuclear translocation of endogenous NFAT. By using immunofluorescence/confocal microscopy we discovered that application of NS1643 determined an increased localization of the endogenous NFAT in the nucleus. To confirm this hypothesis, we performed western blot analysis of nuclear or cytoplasmic cell fractions from cells treated with NS1643. We found that application of NS1643 strongly increased protein level in the nuclear fraction compared to untreated cells.

These data indicate that stimulation of Kv11.1 channel promotes CN-dependent NFAT nuclear translocation and suggest that NFAT might contribute to the NS1643-dependent p21<sup>waf/cip</sup> gene induction.

## DISCUSSION

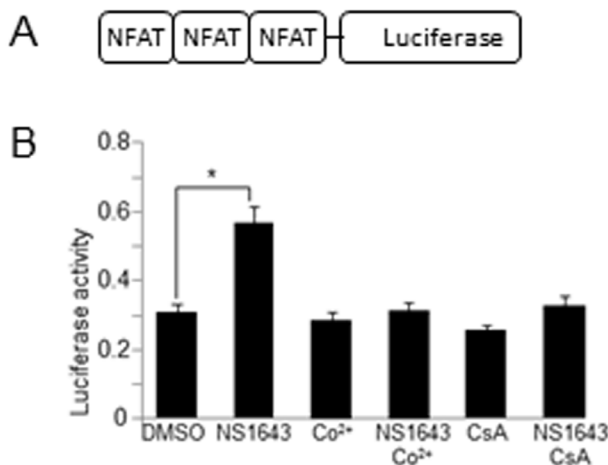
Recent anticancer pharmacological approaches that have been developed into successful strategies have strongly reduced mortality related to breast cancers. For example, the discovery that anti-estrogens such as Tamoxifen can effectively inhibit the proliferative effects of estrogen (E2) on breast cancer cells dramatically improved breast cancer therapy. However, breast cancer is still the second leading cause of cancer death in women. Several reasons can account for the inefficacy of anti-breast cancer therapy. For example, a substantial fraction of breast cancers do not express estrogen receptors (ER,

ER-negative) or can develop resistance to the therapeutic drug. This indicates that, molecular heterogeneity among breast cancers poses a severe limitation to therapy and underlines the urgent need for better pharmacologic approaches.

In our previous works, we discovered that use of a Kv11.1 channel activator (NS1643) determined a cell cycle arrest by activation of a senescence program, which coincided with a strong increase of the tumor suppressor p21<sup>waf/cip</sup> and p16<sup>INK4A</sup> protein level. In contrast, no significant consequences were observed in non-transformed cells following NS1643 application suggesting that, the effect of NS1643 was not due to the activity of the drug on unrelated/unreported targets. However, we did not explore the mechanism linking stimulation of the Kv11.1 potassium channel with expression of tumor suppressors.

In this study we have assessed that, the stimulating effects of NS1643 on p21<sup>waf/cip</sup> protein level occurs in several types of breast cancer cell lines that are categorized by different molecular characteristics. For example, NS1643 application increased p21 protein level independently of the expression of ER or HER2. This suggests that, use of NS1643 as a potential anticancer molecule could be beneficial for treating breast cancers that are molecularly distinct.

The mechanism by which NS1643 increases p21 protein level appears to be related to a p53-independent activation of p21 gene transcription. In our experiments we tested the effect of NS1643 on p21 in several breast cancer cells lines including those in which mutations in the p53 gene rendered p53 non-functional (T47D; SKBr3; MDA-MB-231) or in cells in which was wild p53 was expressed (MCF7). We found that the effect of NS1643



**Figure 5: NS1643 stimulates NFAT-dependent gene induction.** A. Schematic illustration of the luciferase-based reporter construct that contains NFAT response elements (Addgene plasmid #10959) B. Fold changes of NFAT luciferase activity in SKBr3 expressing NFAT luciferase reporter and treated with DMSO (control), NS1643, Cobalt (Co<sup>+</sup>, 10 μM), NS1643+Co<sup>2+</sup>, Cyclosporine-A (CsA) or NS1643+CsA.

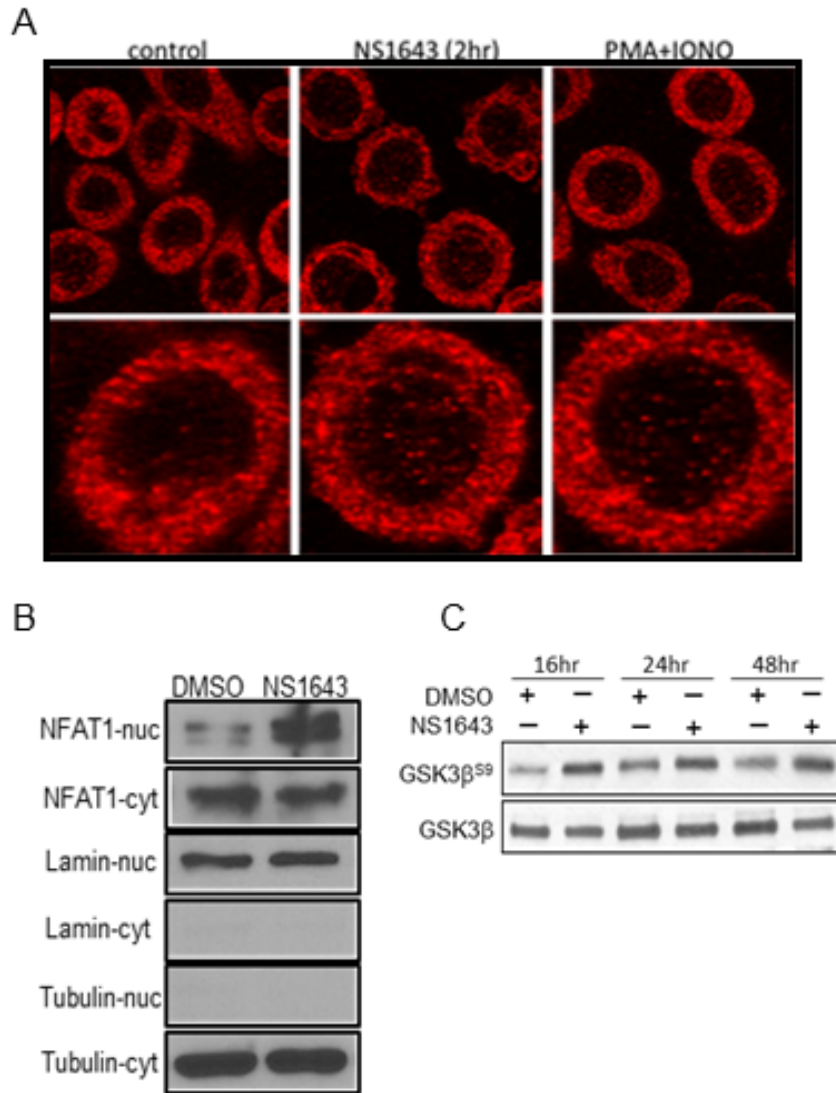


was similar in all the cell lines suggesting that, the possible contribution of p53 in mediating the effect of NS1643 on p21 is minimal or irrelevant.

Our RT-PCR and luciferase assay investigations revealed that NS1643 application strongly increases p21<sup>waf/cip</sup> transcription. Interestingly, this event appears to be controlled by Ca<sup>2+</sup> entry as application of Co<sup>2+</sup> (a generic surface Ca<sup>2+</sup> channel blocker) completely inhibited the effect of NS1643 on p21<sup>waf/cip</sup> transcription.

How can stimulation of a potassium channel drive Ca<sup>2+</sup>-dependent gene transcription?

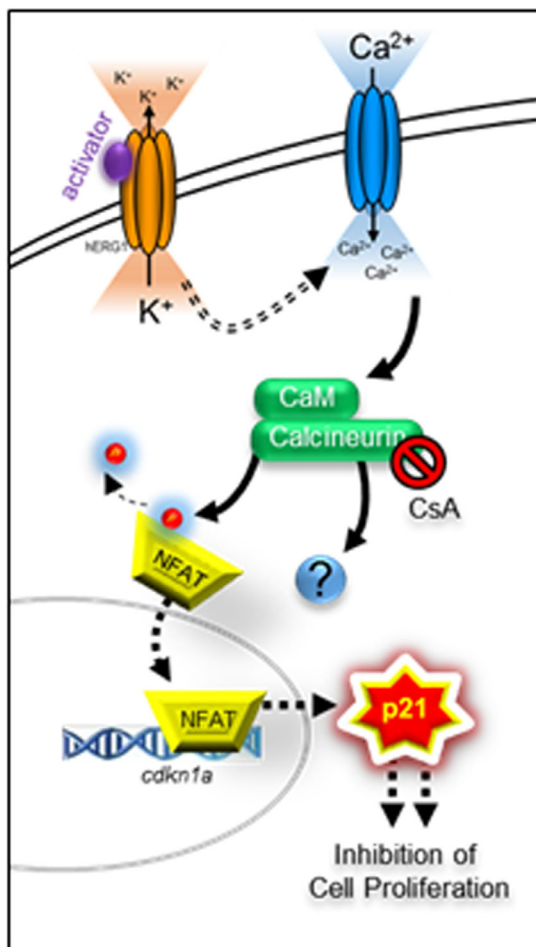
We have previously shown that stimulation of Kv11.1 potassium channel results in build-up of an intracellular negative charge (hyperpolarization) resulting from the outflow of positive charged ions due to activation of the Kv11.1 potassium channel. Correspondingly, Hernandez et al. [32] found that inhibition of the Kv11.1 channel activity in osteoblastic osteosarcoma cells with the selective blocker E4031 determined membrane depolarization. Interestingly, although the effects of the activator and the blocker on the membrane potential were diametrically opposed, they both lead to increased



**Figure 6: NS1643 promotes NFAT nuclear translocation.** **A.** NFAT (red) nuclear translocation in SKBR-3 cells with or without treatment with NS 1643 as observed by confocal microscopy. PMA (100 nM)/ionomycin (2 μM) treated cells served as positive control. **B.** Quantification of NFAT nuclear translocation by image J in control cells, cells treated with NS 1643 and cells treated with PMA/Ionomycin. Data expressed as Mean ± SEM (N = 37 cells). \*Significant at *p* < 0.05 **C.** Nuclear and cytosol localization of NFAT in SKBR-3 cells treated with or without NS 1643 as observed by nucleus and cytosol fractionation and Western blot. The nucleus and cytosol extracts did not reveal any contamination as observed by the lack of tubulin in the nucleus and laminin in cytosol extracts. **D.** Western blot analyses of extracts from SKBr3 cells treated with DMSO (control) or NS1643 for the indicated times, using Phospho-GSK3β (Ser9) antibody (upper panel) or GSK3β (lower panel). Antibody were purchased from Cell Signaling.

cytosolic calcium and inhibition of cell proliferation. In the case of breast cancer cells NS1643-dependent hyperpolarization allows passive moving of  $\text{Ca}^{2+}$  ions from the extracellular solution into the cytoplasm. Interestingly, osteoblastic osteosarcoma cells express the L-type voltage-gated calcium channel (VGCC) [33]. Opening of this channel is dictated by depolarization. Therefore, E4031 can stimulate calcium entry via activation of the L-type calcium channel. Then, in osteoblastic osteosarcoma cells and breast cancer cells,  $\text{Ca}^{2+}$  activates a plethora of intracellular signaling, including membrane and/or nuclear biochemical cascades (e.g. NFAT) that can lead to a common outcome such as inhibition of cell proliferation.

Interestingly a study from Hegle et al. showed that gating of Kv10.1 potassium channel, which belongs to the same superfamily of the Kv11.1 channel, can stimulate proliferation in cancer cells by a mechanism that is independent from  $\text{K}^+$  flux through the channel and/or changes in intracellular calcium [34]. This suggests that



**Figure 7: Schematic representation of hERG1/Kv11.1 activator-dependent stimulation of  $\text{p21}^{\text{waf/cip}}$ .** Stimulation of hERG1/Kv11.1 potassium channel with NS1643 increases passive  $\text{Ca}^{2+}$  entry due to hyperpolarization of the cell. Augmented intracellular  $\text{Ca}^{2+}$  stimulates CN which induces *CDKN1* gene transcription via activation of transcription factor(s) i.e. members of the NFAT family.

members of the EAG superfamily of potassium channels can influence cell proliferation independently from their ability to control membrane potential. However, previous investigations conducted by the Mitcheson's Lab have clearly indicated that, in contrast to EAG channel, the oncogenicity exhibited by the hERG potassium channel appears to be strictly dependent by its ion conduction. In addition, in our experiments, changes of intracellular calcium concentration are crucial for the effects of NS1643 on cell proliferation. We think that altogether, our data suggests that conduction properties of Kv11.1 are fundamental for determining the inhibitory effects upon Kv11.1 chronic activation.

The protein phosphatase CN plays a multifunctional role in cell biology by linking the interaction between  $\text{Ca}^{2+}$ /calmodulin to activation of transcription factors. These events have been originally studied in neurons or immune cells but the discovery that, the immunosuppressant cyclosporine A (CsA) targets CN, provided pharmacological reagents that allowed to demonstrate that, CN plays a major role in "sensing" calcium homeostasis in all eukaryotic cells.

Interestingly, results from our experiments revealed that, the effect of NS1643 on  $\text{p21}^{\text{waf/cip}}$  gene induction is completely inhibited by application of CsA suggesting that, CN can play an important role in mediating the effects of Kv11.1 activation via  $\text{p21}^{\text{waf/cip}}$  gene induction. Nevertheless, we cannot rule out at this time that CN could also modulate other mechanisms that for example control mRNA stability and more defined experiments are needed.

P53-independent regulation of  $\text{p21}^{\text{waf/cip}}$  expression involves several  $\text{Ca}^{2+}$ -activated transcription factors including four of the five members of the NFAT family (NFAT1-4). NFAT1-4 exist inactive in the cytoplasm as phosphorylated proteins.  $\text{Ca}^{2+}$  entry stimulates calcineurin that dephosphorylates NFATs and allow its translocation into the nucleus. Then, NFATs can induce transcription of several genes either by acting as homo/hetero dimers, alone or in collaboration with other transcription factors such as AP1, SP3. Termination of NFAT signaling can occur by decreasing concentration of intracellular  $\text{Ca}^{2+}$  or/and phosphorylation by nuclear kinases such as GSK3 $\beta$  resulting in subsequent NFAT translocation to the cytoplasm and/or degradation. Interestingly, our experiments revealed that NS1643 can strongly activate NFAT as revealed by luciferase assay, detection of nuclear translocation of the endogenous NFAT protein and inhibition of the NFAT negative regulator, GSK3 $\beta$ . These data suggest that, NFAT might contribute to the effect of NS1643 on  $\text{p21}^{\text{waf/cip}}$  in breast cancer cells. However, at this time we don't know which members of the NFAT family can regulate p21 transcription or if they cooperate with other  $\text{Ca}^{2+}$  activated transcription factors.

Several scientific reports have revealed that in human, cellular senescence is mostly driven by expression of 16<sup>INK4A</sup> [35-37]. However, we do not know whether the

role of p16<sup>INK4A</sup> is also predominant in NS1643-activated cellular senescence.

Our experiments have revealed that, proliferative cells (untreated cells) express very low levels of p21<sup>waf/cip</sup> or p16<sup>INK4A</sup> (undetectable by western blot analysis). Nevertheless, cells in which p21<sup>waf/cip</sup> function has been genetically suppressed, present an increased ability to proliferate compared to naïve cells. In our view this suggests that, even if expressed at a low level, p21<sup>waf/cip</sup> can strongly influence proliferation.

In addition, our experiments have also revealed that, the inhibitory effect of NS1643 on cell proliferation was completely removed by suppressing p21<sup>waf/cip</sup> function, suggesting that even though NS1643 treatment leads to upregulation of both p21<sup>waf/cip</sup> and p16<sup>INK4A</sup>, the role of p21<sup>waf/cip</sup> appears to predominate. However, we cannot rule out that p16<sup>INK4A</sup> can still play a major role in NS1643-driven senescence by acting as a downstream effector of p21<sup>waf/cip</sup> and better dedicated experiments need to be designed.

In conclusion, we think that, we have discovered a novel mechanism through which stimulation of hERG1 potassium channel leads to inhibition of breast cancer cell proliferation via activation of NFAT1. In addition, our results offers a compelling opportunity to study Ca<sup>2+</sup>-dependent mechanism in cancer biology which can provide basis for anticancer therapeutic strategies [38].

## MATERIALS AND METHODS

### Cell culture

SKBr3 were cultured in RPMI-1640 (Thermo Scientific). MDA-MB-231, T47D and MCF7 were cultured in DMEM (Corning). All media contained 10% FBS (Serum Source International) and 100 µl/ml penicillin/streptomycin (Thermo Scientific) with the exception of T47D that also contained 1x NEAA (Thermo Scientific). All cells were grown at 37°C in 5% CO<sub>2</sub>.

### Western blot

SKBr3 cells were harvested for western blot analysis by trypsinization with 0.25 % trypsin-EDTA (Thermo Scientific). Cells were washed with 1x PBS (Thermo Scientific) and resuspended in cold radioimmunoprecipitation assay buffer (RIPA) 50 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% Tergitol (Sigma-Aldrich, St. Louis, MO, USA ), 0.5% Na-deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 × Halt Protease Inhibitor Cocktail (Thermo Scientific). Protein concentration was determined by BCA assay (Thermo Scientific). 4x Laemmli buffer containing 10% 2-mercaptoethanol was added to lysates

and boiled at 95 °C for 5 minutes. Lysates were loaded on 8% SDS-PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad).

### Nuclear/cytosol fractionation

Nuclear and cytosol extracts from SKBR3 cells following various treatments were prepared as described below. Cells were washed with 1X PBS and cell pellets were lysed with a hypotonic buffer (10 mmol/L HEPES buffer pH 7.9, 1.5 mmol/L MgCl<sub>2</sub>, 10 mmol/L KCl, 10% glycerol, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 mmol/L dithiothreitol (DTT)). The lysates were kept on ice for 15 min, 5 µl of 10% NP 40 was added to each lysate, vortexed briefly followed by centrifugation at 13200 rpm for 10min to pellet the nuclear fractions. The supernatant was collected as the cytosol extract. The nuclear pellet were lysed with buffer solution containing 20 mmol/L HEPES buffer pH 7.9, 1.5 mmol/L MgCl<sub>2</sub>, 400 mmol/L NaCl, 0.2 mmol/L EDTA, 20% glycerol, 0.5 mmol/L PMSF, and 1 mmol/L DTT. The nuclear lysates were clarified by centrifugation at 13200 rpm for 10 min at 4°C.

### Immunofluorescence microscopy

SKBR3 cells were grown on poly D-Lysine coated coverslips and treated with vehicle alone, 50 µM NS1643 or 80nM PMA and 2µM Ionomycin in RPMI 1640 media. Following treatment, the cells were washed with 1X PBS, fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.3% Triton X-100 for 15 min at room temperature. Nonspecific binding was blocked with 5% bovine serum albumin (BSA) for 1h at room temperature and then incubated with 1:100 primary rabbit monoclonal anti-NFAT-1 antibody (Cell signaling Technology, Danvers, MA) diluted in 1% BSA at 4°C, overnight, while cells incubated with 1% BSA alone, served as negative controls. Following incubation with the primary antibody, the cells were washed with 1X PBS and incubated with Alexa fluor 594 conjugated anti-rabbit secondary antibody (Life technologies, Carlsbad, CA). Following washes, cells were incubated with 50ng/mL DAPI to stain the nuclei. The coverslips were mounted on VECTASHIELD reagent (Vector Laboratories, Burlingame, CA) and fluorescent images were taken using confocal microscopy (Carl Zeiss Meditec, Inc., Thornwood, NY). Nuclear translocation of NFAT-1 across groups was analyzed and quantified using the analyze particle tool from Image J.

### RNA isolation, RT-PCR and quantitative PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was



synthesized using iScript (Biorad, Life Science Research, Hercules, CA, USA). For p21 amplification, primers p21-F (5'-TGA GCC GCG ACT GTG ATG-3') and p21-R (5'-GTC TCG GTG ACA AAG TCG AAG TT-3') were used.

For HSRP amplification, primers HSRP-F (5'-AGC CTC AAG ATC ATC AGC AAT G-3') and GAPDH-R (5'-ATG GAC TGT GGT CAT GAG TCC TT-3') were used.

To determine p21 mRNA levels and stability, quantitative RT-PCR was performed on a Mastercycler<sup>®</sup> ep Realplex (Eppendorf, Hauppauge, NY, USA) using a total volume of 20 µl containing 200 nM primers and 1X Absolute<sup>™</sup> Blue QPCR SYBR<sup>®</sup> Green Mix (ABgene, Thermo Fisher Scientific, Rockford, IL, USA).

### Luciferase assay

Dual-luciferase reporter assay was done according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, cells ( $4 \times 10^4$ ) were plated on a 24-well plate, for 24 hr. Then cells in triplicate wells were co-transfected with 5 µg plasmid DNA encoding firefly luciferase driven by the NFAT or p21 promoter and 1 µg of DNA encoding a constitutively expressed Renilla luciferase gene.

The p21-promoter luciferase reporter luciferase plasmid was generated by Toren Finkel group. This construct was a gift from Toren Finkel (Addgene plasmid # 10959)

The luciferase reporter gene for the studies of NFAT activity contains three tandem repeats of a 30-bp fragment (GGAGG AAAAA CTGTT TCATA CAGAA GGCGT) of the IL-2. This construct was a gift from Wafik El-Deiry (Addgene plasmid # 21723).

The firefly luciferase plasmid contains a luciferase reporter gene driven by a tandem repeat of NFAT binding elements and served as reporter of NFAT activation. Renilla luciferase serves as the control reporter to normalize the activity of the experimental reporter based on transfection efficiency and cell viability. Two days after transfection, the cells were exposed to drugs as indicated. Cells were harvested, and lysates were tested for luciferase activity using a dual-luciferase assay system. Y axis indicates fold change of relative luminescence which denotes the ratio of firefly luciferase activity (NFAT-dependent) to Renilla luciferase (control) activity to standardize for transfection efficiency.

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### CONFLICTS OF INTEREST

There is no conflict of interest to declare.

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