

Blockade of Rac1 Activity Induces G₁ Cell Cycle Arrest or Apoptosis in Breast Cancer Cells through Downregulation of Cyclin D1, Survivin, and X-Linked Inhibitor of Apoptosis Protein

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Abstract

Rac1 GTPase regulates a variety of signaling pathways that are implicated in malignant phenotypes. Here, we show that selective inhibition of Rac1 activity by the pharmacologic inhibitor NSC23766 suppressed cell growth in a panel of human breast cancer cell lines, whereas it had little toxicity to normal mammary epithelial cells. NSC23766 elicits its cytotoxicity via two distinct mechanisms in a cell line-dependent manner: induction of G₁ cell cycle arrest in cell lines (MDA-MB-231, MCF7, and T47D) that express retinoblastoma (Rb) protein or apoptosis in Rb-deficient MDA-MB-468 cells. In MDA-MB-231 cells, Rac1 inhibition induced G₁ cell cycle arrest through downregulation of cyclin D1 and subsequent dephosphorylation/inactivation of Rb. By contrast, MDA-MB-468 cells underwent substantial apoptosis that was associated with loss of antiapoptotic proteins survivin and X-linked inhibitor of apoptosis protein (XIAP). Rac1 knockdown by RNAi interference confirmed the specificity of NSC23766 and requirement for Rac1 in the regulation of cyclin D1, survivin, and XIAP in breast cancer cells. Further, NF- κ B, but not c-Jun NH₂-terminal kinase or p38 pathways, mediates the survival signal from Rac1. Overall, our results indicate that Rac1 plays a central role in breast cancer cell survival through regulation of NF- κ B-dependent gene products. *Mol Cancer Ther*; 9(6); 1657–68. ©2010 AACR.

Introduction

Rac1 is a member of the Rho family of small GTPases that act as molecular switches in regulation of cellular functions. Activation of Rac1 regulates cell morphology (1, 2), cell cycle and gene expression (3–5), and survival and apoptosis (6–8), which is achieved through its ability to control a multitude of signaling pathways, including the extracellular signal-regulated kinase (ERK; ref. 9), c-Jun NH₂-terminal kinase (JNK), p38 kinase (10–12), and NF- κ B pathways (13–15). These pathways stimulate expression of a variety of genes that are related to cell cycle and survival. For instance, NF- κ B activation is known to induce transcription of its target genes such as *cyclin D1*, *Bcl-2*, and *Bcl-XL*. In human cancers, Rac1 is found to be frequently hyperactivated due to elevated protein

expression in itself (16, 17) or alteration in its regulatory proteins (18–20). The dysregulated Rac1 activity has been implicated in several aspects of malignant phenotypes such as tumorigenic transformation and outgrowth (21–25). Rac1 is also integral in the regulation of tumor angiogenesis through its control over epithelial cell motility (26–28) and endothelial cell permeability (29, 30). We (6–8) and others (31–34) have shown that Rac1 activity contributes to the resistance of cancer cells to apoptosis by different chemotherapies. Rac1 and its signaling components have thus been proposed as a therapeutic target for treating human cancers (reviewed in ref. 35).

As a molecular switch, Rac1 alternates between an inactive GDP-bound and an active GTP-bound state. An essential step for Rac1 activation is its interaction with guanine nucleotide exchange factors (GEF) that catalyze GDP exchange for GTP. Thus, one strategy for inhibiting Rac1 function is to block its interaction with GEFs. NSC23766 is such an inhibitor that targets a subset of GEFs specific to Rac1 (e.g., Tiam1 and Trio; ref. 36). Repression of Rac1 activity by NSC23766 diminished Rac-dependent cell proliferation of PC3 prostate cancer line (36) and leukemia cell lines both *in vitro* and *in vivo* (37–40); it also inhibited cell migration of several breast cancer cell lines (41) and induced apoptosis in leukemia (37–40) and glioma (42) cell lines. Increasing evidence shows that Tiam1 and Trio are overexpressed in different types of human tumors (18–20), including human breast (42) carcinomas,

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which is believed to contribute to Rac1 hyperactivity. This promoted us to investigate whether targeting Rac1 could be beneficial in eliminating cancer cells.

In this study, we examine the effect of Rac1 inhibition in a panel of human breast cancer cell lines. Suppression of Rac1 by NSC23766 inhibited cell growth of all four cancer lines tested but not the MCF12A normal mammary epithelial cells. The growth inhibition was mediated by induction of G₁ cell cycle arrest or rapid apoptosis in a cell line-dependent manner. These effects correlated with a decrease of NF- κ B activity and subsequent down-regulation of its target genes *survivin*, X-linked inhibitor of apoptosis protein (*XIAP*), and *cyclin D1*. Rac1 seems to regulate a NF- κ B-dependent survival pathway in breast cancer cells, and suppression of its activity is beneficial in eliminating cancer cells.

Materials and Methods

Cell lines and reagents

The human breast cancer cell lines MDA-MB-231, MDA-MB-468, T47D, and MCF7 as well as the MCF12A immortalized normal mammary epithelial cell line were obtained from the American Type Culture Collection and cultured as recommended. Rac1 inhibitor NSC23766 was purchased from Calbiochem. Monoclonal antibodies specific to human Rac1, Cdc42, Bcl-XL, p21WAF1, cyclin D1, retinoblastoma (Rb), and its underphosphorylated form were from BD Biosciences. Antibodies against human caspase-3 and caspase-8 and the cell-permeable I κ B kinase 2 (IKK2) inhibitor V [N-(3,5-bis-trifluoromethylphenyl)-5-chloro-2-hydroxybenzamide] were from Calbiochem. Anti-actin, anti-survivin, horseradish peroxidase-conjugated goat anti-rabbit IgG, and anti-mouse IgG1 were from Santa Cruz Biotechnology. Monoclonal antibodies to cIAP1, cIAP2, and XIAP as well as polyclonal antibodies to JNK, phospho-JNK (Thr¹⁸³/Tyr¹⁸⁵), p38, phospho-p38 (Thr¹⁸⁰/Tyr¹⁸²), ERK, and phospho-ERK (Thr²⁰²/Tyr²⁰⁴) were from Cell Signaling. The cell-permeable inhibitor for JNK (SP600125) was from Alexis Biochemicals. The validated Stealth RNAi duplex for Rac1 (5'-UGGA-GAAUUAUCCCUACUGUCUUU-3' and 5'-AGGGU-CUAGCCAUGGCUAAGGAGAU-3') was purchased from Invitrogen. pCMV6-XL plasmid for expression of human survivin, pCMV6-XL-BIRC5, was obtained from OriGene Technologies. Transfections of RNA interference duplexes were carried out using Lipofectamine RNAiMAX or Neon electroporation transfection system (Invitrogen). Expression plasmids for human Rb (pCMV6-Rb) and survivin (pCMV6-XL-BIRC5) were from OriGene Technologies. Transfections of plasmid were done using FuGENE 6 reagent (Roche Applied Science).

Cell viability and apoptosis assays

Cell viability was analyzed by the MTS colorimetric assay using tetrazolium reagent (Promega; ref. 43). Briefly, cells (1.5×10^4 /mL) were seeded in each well of 96-well

tissue culture plates with 200 μ L of medium. This results in 40% to 50% confluence of the cells after 24 hours of plating. The medium was then replaced with 200 μ L of fresh medium containing NSC23766 at the indicated concentrations. At the end of the treatment period (48 h), 20 μ L of MTS solution were added to each well and incubated at 37°C for 2 hours. Absorbance at 490 nm, which is directly proportional to the number of living cells, was read on a 96-well plate reader. Data are expressed as a percentage of the untreated cells cultivated under the same conditions. To determine apoptosis, cells were grown on six-well plates to 70% to 80% confluence. After treatment, cells were harvested, incubated with FITC-conjugated Annexin V and propidium iodide (PI), and analyzed by flow cytometry on BD FACSCalibur Flow Cytometer (BD Biosciences; ref. 7).

Rac GTPase activity assay

The levels of active GTP-Rac1 and GTP-Cdc42 were determined by a pull-down assay using the Cdc42/Rac1-interactive binding domain of human p21-activated kinase 1 (GST-PAK1) as a probe (6, 7). Briefly, cells were grown to 80% confluence in a 10-cm dish and treated with NSC23766 at the indicated concentrations for 24 hours. Afterwards, cells were harvested and lysed in a buffer containing 50 mmol/L HEPES, 150 mmol/L NaCl (pH 7.5), 1 mmol/L EGTA, 1% Triton X-100, 10% glycerol, 10 mmol/L MgCl₂, and protease inhibitor cocktail (Calbiochem). Equal amounts of cell lysates were incubated with agarose-immobilized GST-PAK1 at 4°C for 30 minutes. The coprecipitates were subjected to immunoassays using antibodies specific to Rac1 or Cdc42.

Cell cycle analysis

Cells (1×10^5) were harvested, washed with PBS, and fixed with 70% ethanol on ice for 2 hours. Subsequently, cells were centrifuged and resuspended in a solution containing 25 μ g/mL PI, 0.1% Triton X-100, and 100 μ g/mL RNase A for 15 minutes at 37°C. The DNA content and percentage of cells in each phase of cell cycle were analyzed by flow cytometry.

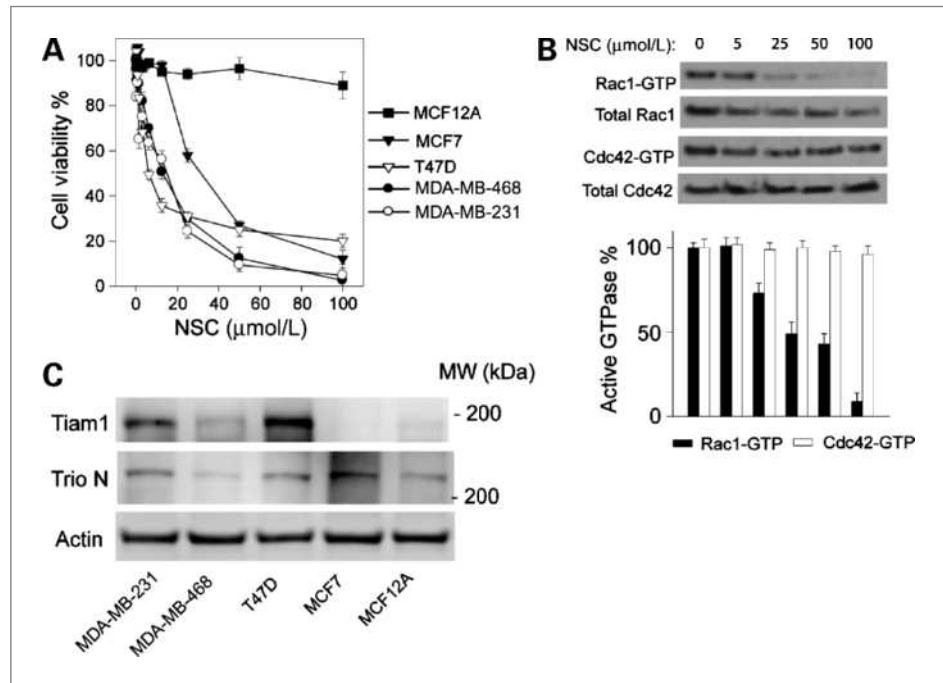
NF- κ B activity

NF- κ B activation was accessed by an ELISA assay using TransAM NF- κ B kit (Active Motif) per the manufacturer's instruction. Briefly, equal amounts of nuclear extracts were incubated in 96-well plates that were coated with NF- κ B consensus oligonucleotide sequence 5'-GGGACTTTCC-3'. The bound, active form of NF- κ B was detected by incubation with antibodies specific to p65, p50, p52, c-Rel, or RelB followed by horseradish peroxidase-conjugated secondary antibody. Absorbance was measured at 450 nm.

Western blotting

Cells (1×10^6) were lysed in SDS lysis buffer containing 50 mmol/L Tris-HCl (pH 7.0), 2% SDS, and 10%

Figure 1. Cytotoxicity of NSC23766 in human breast cancer cell lines. **A**, cells were cultured in the presence of increasing doses of NSC23766 (NSC) for 48 h. Cell viability was determined by MTS assay and reported as a percent of untreated cells. **B**, levels of active Rac1-GTP and Cdc42-GTP were analyzed by a PAK1-binding domain pull-down assay in MDA-MB-231 cells 24 h after exposure to NSC23766. Total levels of Rac1 and Cdc42 protein were used as control. Bottom, levels of GTP-bound GTPases were estimated by densitometry analysis of the blots in the top and normalized to the total amount of each protein in the lysates. **C**, Tiam1 and Trio N protein expression determined by Western blotting. Results shown are representative of three independent experiments.



glycerol and incubated for 20 minutes at 95°C. Protein concentrations were estimated using the bicinchoninic acid protein assay (Pierce). Equal amounts of cell lysates (20 μg per lane) were resolved by electrophoresis using a 4% to 12% NuPAGE Bis-Tris gel (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore) for immunoblot analysis with an appropriate dilution of antibodies (1:1,000 to 1:2,000). When necessary, the membranes were stripped by Restore Western Blot Stripping Buffer (Pierce) and re-probed with appropriate antibodies. Immunocomplexes were visualized by chemiluminescence using ECL (Santa Cruz Biotechnology).

Results

Blockade of Rac1 activity by NSC23766 inhibits cell growth of human breast cancer cell lines but not normal mammary epithelial cells

The aberrant Rac1 activity has been correlated with several aspects of malignancy in human breast cancers (17, 27, 44–48). We sought to determine whether blockade of Rac1 activity could be beneficial in suppressing breast cancer cell growth and survival. To this end, Rac1 inhibitor NSC23766 was applied to a panel of human breast cancer cell lines. After 48 hours, cell viability was analyzed using a MTS assay. As shown in Fig. 1A, treatment with NSC23766 decreased cell viability in a dose-dependent manner in all cancer lines tested. The most profound effect was seen in MDA-MB-468 and MDA-MB-231 cells; both showed an IC_{50} of ~10 μmol/L.

Importantly, the growth inhibition was not correlated with the status of estrogen receptor (ER), progesterone receptor (PR), Her2, and p53 mutation (Table 1). In contrast, NSC23766 had little effect on the survival of the MCF12A normal mammary epithelial cells. Consistent with previous reports (36, 41), NSC23766 selectively inhibited Rac1 activation without interfering with the activity of the closely related small GTPase Cdc42 in MDA-MB-231 (Fig. 1B) and other cell lines (data not shown). Repression of Rac1 activity seems to selectively induce growth inhibition in breast cancer lines over normal mammary epithelial cells.

NSC23766 was shown to inhibit Rac1 activation by blocking its interactions with a subset of GEFs, including Tiam1 and Trio N (36). When tested for their protein expression, Tiam1 was expressed in MDA-MB-231, MDA-MB-468, and T47D cells, but it was almost

Table 1. Status of ER, PR, Her2 expression, p53 mutant or wild-type, and Rb protein expression in the indicated cell lines

	ER	PR	Her2	p53	Rb
MDA-MB-231	-	-	-	m	+
MDA-MB-468	-	-	-	m	-
T47D	+	+	+	m	+
MCF7	+	+	+	wt	+

Abbreviations: m, p53 mutant; wt, wild-type.

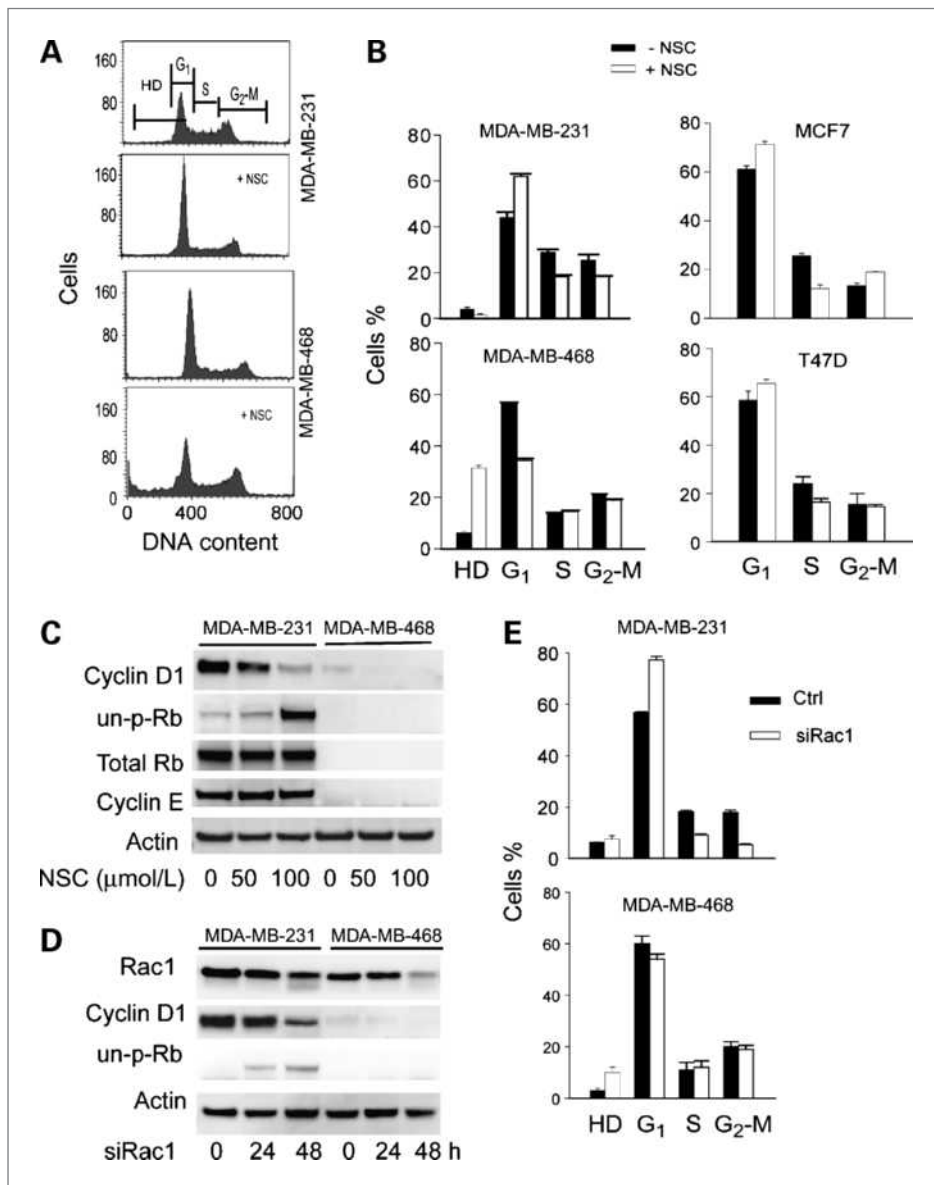


Figure 2. Effects of Rac1 inhibition on cell cycle and cell cycle regulatory proteins. A and B, cells were treated with NSC23766 (100 $\mu\text{mol/L}$) for 48 h and stained with PI and analyzed by FACS to estimate the percentage of cells in each phase of the cell cycle. The experiment was repeated thrice ($n = 3$). Columns, mean for each phase; bars, SD. HD, hypodiploid population. C, equal amounts of total lysates from cells treated with different concentrations of NSC23766 (0, 50, or 100 $\mu\text{mol/L}$) for 48 h were analyzed by immunoblotting using antibodies specific to cyclin D1, the underphosphorylated form of Rb protein (un-p-Rb), total Rb, and cyclin E. Actin was detected as a loading control. D and E, knockdown of Rac1 elicited similar effects as NSC23766 treatment. Cells were transfected with siRNA specific to Rac1 transcript (siRac1) using Lipofectamine RNAiMAX. At the indicated times (hours), cells were harvested and analyzed for protein expression (D) and cell cycle (E).

undetectable in MCF7 and MCF12A cells (Fig. 1C). Trio N was expressed in all cell lines tested. There seemed to be an association between cellular sensitivity to NSC23766 and the expression of Tiam1 and Trio N.

Inhibition of Rac1 induces G₁ cell cycle arrest in MDA-MB-231 cells

The decrease in cell viability could result from reduced cell proliferation and/or apoptosis in the target cells. To distinguish these effects, we first analyzed cell cycle distribution following NSC23766 treatment. After 24 hours, MDA-MB-231 cells showed an increase from 41% to 65% in G₁ phase and a concomitant decrease in S and G₂-M phases (Fig. 2A and B). Similar effect was observed in MCF7 and T47D cell lines (Fig. 2B, right).

These results agree with an established role for Rac1 in promoting cell cycle progression through G₁ (4, 5). By contrast, MDA-MB-468 cells displayed a decrease in G₁ and an accumulation of sub-G₁ population, whereas S and G₂-M populations remained unchanged. We then examined the effect of Rac1 inhibition on several cell cycle regulatory proteins, including cyclin D1, cyclin E, and Rb protein (Fig. 2C). The most remarkable change was the decrease in cyclin D1 protein after NSC23766 treatment. Cyclin D1 is known to regulate cell cycle by stimulating phosphorylation of Rb protein, which subsequently triggers transcription of various genes required for G₁ progression. In line with cyclin D1 reduction, levels of underphosphorylated Rb were significantly increased in MDA-MB-231 cells following

NSC23766 treatment, whereas total Rb level was not changed.

To determine the specificity of NSC23766, MDA-MB-231 and MDA-MB-468 cells were transfected with small interfering RNA (siRNA) specific to Rac1 transcript. Rac1 protein was decreased in a time-dependent manner after siRNA transfection, which was accompanied by a decrease in cyclin D1 and an increase of underphosphorylated Rb (Fig. 2D). As observed for NSC23766, knockdown of Rac1 induced G₁ cell cycle arrest in MDA-MB-231 cells (Fig. 2E, top) but not in MDA-MB-

468 cells (Fig. 2E, bottom). These results support the specificity of NSC23766 in blocking Rac1-dependent cell cycle events.

Loss of Rac1 activity induces apoptosis in MDA-MB-468 cell line via downregulation of survivin and XIAP

Next, we used flow cytometry to assess apoptosis in response to Rac1 inhibition. MDA-MB-468 cells underwent massive apoptosis 24 hours after NSC23766 exposure, as indicated by the positive staining of Annexin

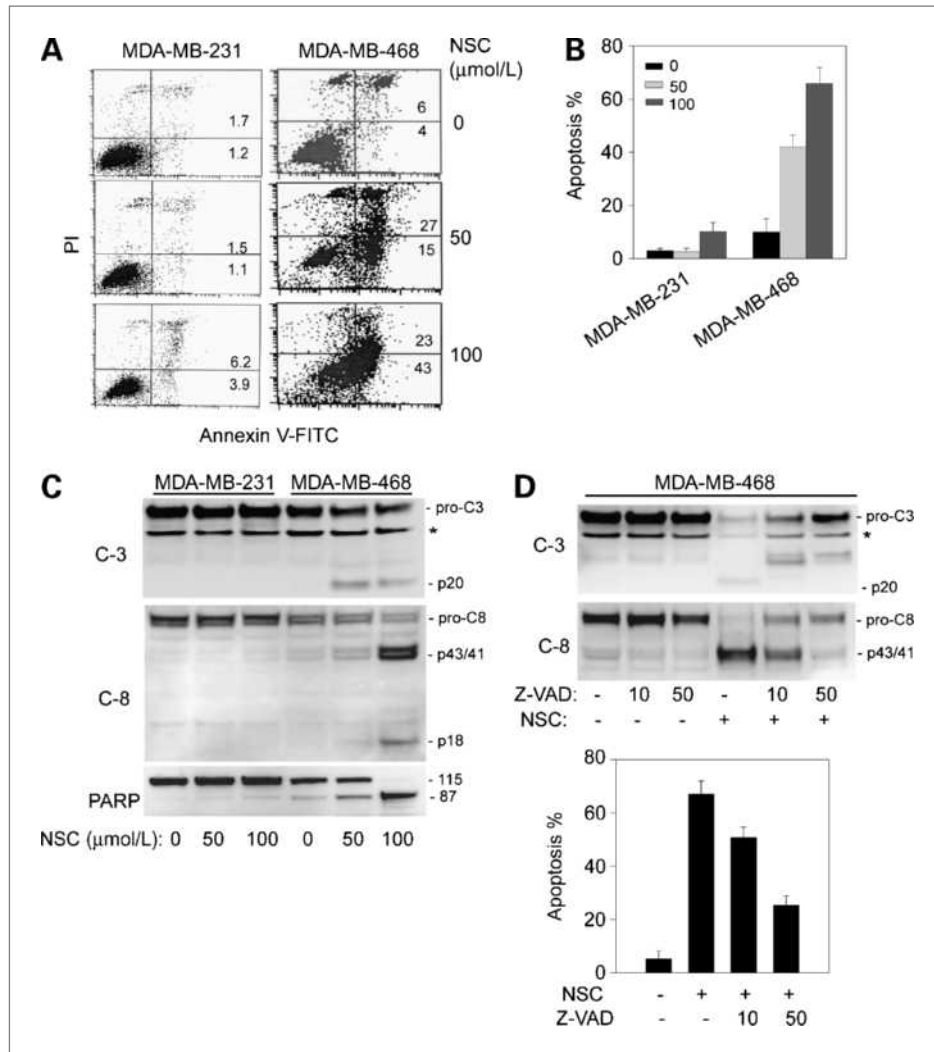


Figure 3. Treatment with NSC23766 induces apoptosis in MDA-MB-468 cells but not in MDA-MB-231 cells. **A**, cells were treated with NSC23766 at the indicated concentrations for 24 h and analyzed by flow cytometry after staining with FITC–Annexin V and PI. Shown are representative dot plots. The insert numbers indicate percentage of cells per quadrant. Bottom right quadrant, early apoptotic cells with exposed phosphatidylserine (Annexin V positive) but intact membrane (PI negative); top right quadrant, necrotic or late apoptotic cells with positive staining of both Annexin V and PI. **B**, quantification of apoptosis as shown in **A**. The results represent the total percentage of the cells in the right-hand quadrants. Columns, mean ($n = 3$); bars, SD. **C**, activation of caspase-3 (C-3) and caspase-8 (C-8) was analyzed by Western blotting in the indicated cell lines 24 h after exposure to Rac1 inhibitor. Caspase activity is indicated by the decrease of procaspases (pro-C3 and pro-C8) and appearance of their fragments p20 and p43/p41, and p18, respectively. *, nonspecific band. Cleavage of poly(ADP-ribose) polymerase (PARP), a caspase-3 substrate, is also shown. **D**, NSC23766-induced apoptosis is dependent on caspase activation. Cells were pretreated with or without a general caspase inhibitor (Z-VAD-fmk) at 10 or 50 μmol/L for 1 h and then incubated with NSC23766 (100 μmol/L) for an additional 24 h. Top, Western blots of caspase-3 and caspase-8; bottom, apoptosis measured as in **A**. Columns, mean ($n = 3$); bars, SD.

V-FITC and PI (Fig. 3A and B) as well as a dose-dependent activation of caspase-3 and caspase-8 and cleavage of the caspase substrate poly(ADP-ribose) polymerase (Fig. 3C, right). In contrast, only little apoptosis (<10%) was detected in MDA-MB-231 cells under the same treatment conditions, which was confirmed by the absence of caspase activation (Fig. 3C, left). Similar effect was observed in T47D and MCF7 cells, showing a G₁ arrest without significant apoptosis (data not shown). Taken together, inhibition of Rac1 activity seems to induce apoptosis in MDA-MB-468 cells, whereas it causes G₁ arrest in other three cancer cell lines (Fig. 2B). A general caspase inhibitor Z-VAD effectively blocked NSC23766-induced apoptosis in MDA-MB-468 cells (Fig. 3D), showing that NSC23766 induces apoptosis by activation of a caspase cascade.

We attempted to identify determinants of cellular responses (apoptosis versus G₁ arrest) to Rac1 inhibition. Rb protein is a key regulator of cyclin D1-dependent cell cycle, and it also plays a role in regulation of apoptosis (49). As previously shown (50) and in Fig. 2C, Rb is deficient in MDA-MB-468 cells but it was found to be expressed in all other cell lines studied (Fig. 4A). The status of Rb seemed to be associated with the observed phenotype changes in the target cells: apoptosis in Rb-negative MDA-MB-468 cells and G₁ arrest in Rb-positive cell lines (MDA-MB-231, T47D, and MCF7). Interestingly, ectopic expression of Rb almost completely blocked NSC23766-induced apoptosis in MDA-MB-468 cells as indicated by lack of caspase-3 activation (Fig. 4B). These results suggest that Rb plays an important role in Rac1-regulated cellular activities. However, knockdown of Rb in MDA-MB-231 cells was unable to enhance caspase activation in response to NSC23766 treatment (Fig. 4C), suggesting that other factors such as levels of antiapoptotic proteins (XIAP and survivin; Fig. 5A) are also involved in switching between Rac1-dependent cell cycle arrest and apoptotic cell death.

To elucidate the mechanism by which NSC23766 induces apoptosis, we assessed expression of several regulatory proteins of caspases. Survivin, a distinct member of the IAP family, was found to be downregulated in a dose-dependent manner of NSC23766, with a stronger effect in MDA-MB-468 cells (Fig. 5A and B). Similar observation was made for XIAP, whereas cIAP1 and cIAP2 were essentially unchanged. In addition, NSC23766 did not affect the expression of Bcl-2 and Bcl-XL in the two cell lines; however, both were previously shown to be downstream of Rac1 signaling in other cell types (39, 51). Knockdown of Rac1 mimicked the effect of NSC23766 on expression of the above-mentioned proteins (Fig. 5C). On the other hand, ectopic expression of a dominant-active Rac1 mutant (Rac1V12) in MDA-MB-231 cells inhibited NSC23766-mediated cellular effects, such as dephosphorylation of Rb and G₁ cell cycle arrest (Supplementary Data I). Taken together, these results show not only the specificity of NSC23766 toward Rac1 but also a critical role for Rac1 in breast cancer cell survival.

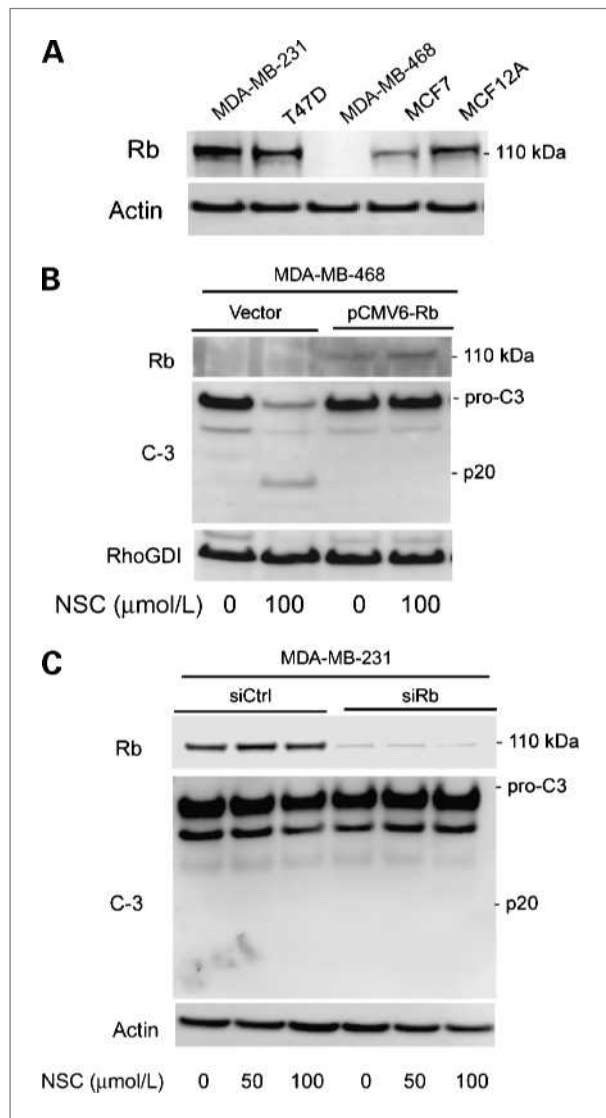


Figure 4. Effects of Rb expression on NSC23766-induced cytotoxicity. **A**, Western blots for Rb protein expression in the indicated cell lines. **B**, ectopic expression of Rb in MDA-MB-468 cells blocks NSC23766-induced caspase activation. Cells were transfected with pCMV6-Rb or empty vector for 24 h, treated with NSC23766 (100 μmol/L) for additional 24 h, and analyzed by immunoblotting for Rb expression (top) and activation of caspase-3. Rho GDP dissociation inhibition (RhoGDI), a cellular protein that is resistant to caspases (8), was used as a loading control. **C**, knockdown of Rb in MDA-MB-231 cells had no effect on caspase-3 activity. Cells were transfected by electroporation with a control siRNA (siCtrl) or siRNA specific to Rb transcript (siRb). After 24 h, cells were treated with NSC23766 at the indicated concentrations for an additional 24 h and immunoblotted for Rb and caspase-3. Representative of three independent experiments.

JNK/p38 and ERK kinase pathways are not required for Rac1-mediated expression of survivin and cyclin D1

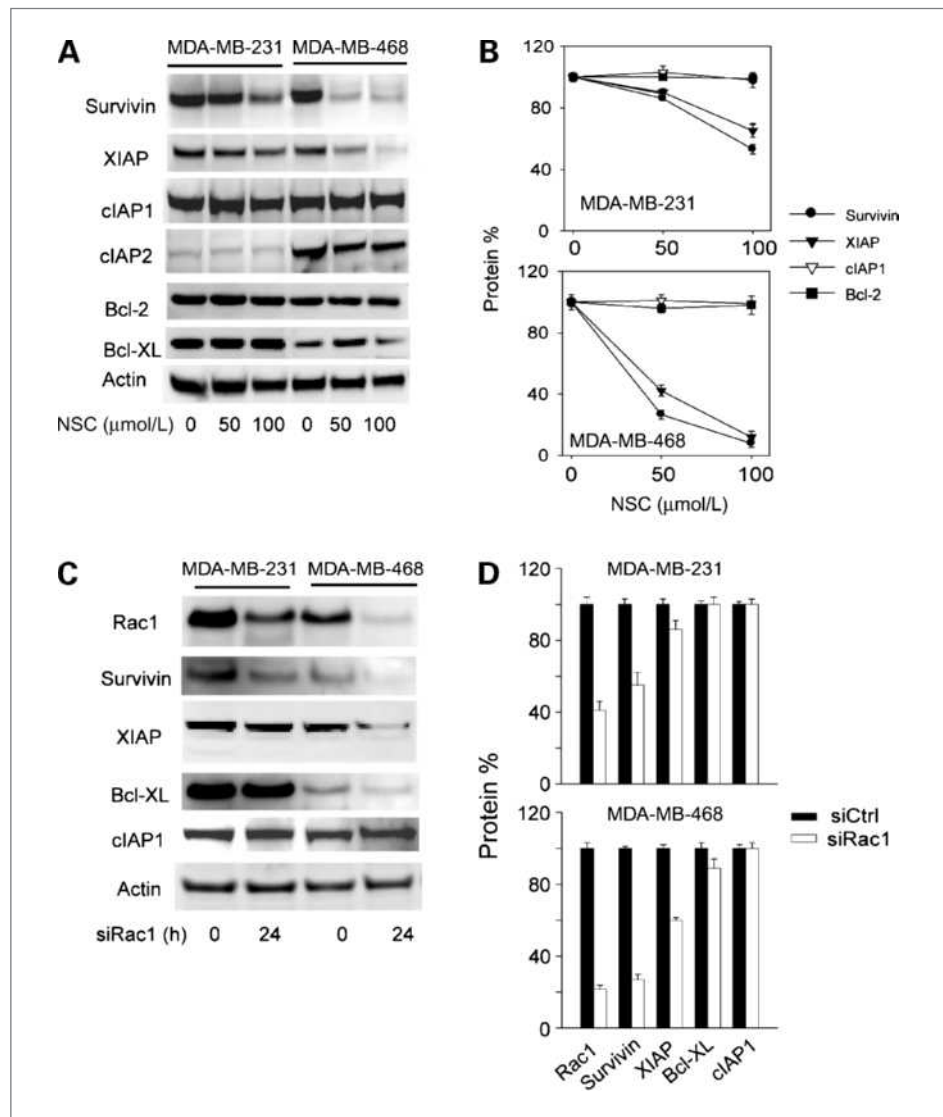
Because Rac1 is an upstream activator of JNK/p38 kinase that is involved in apoptosis in different cell

types (10–12), we tested whether these pathways were affected by Rac1 inhibition. This was done by immunoblotting using antibodies specific to the phosphorylated species of each enzyme. Surprisingly, Rac1 inhibition by NSC23766 resulted in a dose-dependent increase in phosphorylation of JNK, but not p38 or ERK, in both cell lines with a stronger effect in MDA-MB-468 cells (Fig. 6A and B). However, blockade of JNK by the specific inhibitor SP600125 (52), as indicated by loss of c-Jun phosphorylation, failed to inhibit NSC23766-induced reduction of survivin and cyclin D1 as well as apoptosis progression (Fig. 6C and D). These data suggest that JNK/p38 and ERK pathways play a minor role, if any, in NSC23766-mediated cellular responses. XIAP is a negative regulator of JNK activity (53, 54). Thus, JNK activation is likely a bystander effect of NSC23766 treatment and may be related to NSC23766-induced downregulation of XIAP.

NF- κ B acts downstream of Rac1 in promoting survival of breast cancer cells

Rac1 has been shown to stimulate NF- κ B activation (13, 15), and NF- κ B is involved in transcriptional regulation of many target genes, including *XIAP* (54, 55) and *cyclin D1* (14). We therefore tested NF- κ B activity in cells treated with NSC23766. As shown in Fig. 7A, NSC23766 induced a dose-dependent decrease in phosphorylation of p65 subunit in both cell lines, with a stronger effect in MDA-MB-468 cells. This was confirmed by results from a DNA-binding assay (Fig. 7B), revealing a decrease in p65 and p50 activity but not in p52, c-Rel, or RelB. These data show that Rac1 inhibition selectively suppressed p65 and p50 subunit activities in the target cells. In line with these data, pharmacologic inhibition of NF- κ B resulted in a decreased expression of survivin, XIAP, and cyclin D1 (Fig. 7C). In MDA-MB-231 cells, there was also a concomitant increase in the levels of

Figure 5. Blockade of Rac1 selectively downregulates the expression of antiapoptotic proteins XIAP and survivin. **A**, cells were treated with different doses of NSC23766 for 48 h and analyzed by immunoblotting for the indicated proteins. Survivin and XIAP, but not cIAP1, cIAP2, Bcl-2, or Bcl-XL, were reduced in response to NSC23766 treatment. **B**, estimation of changes in protein levels of XIAP, cIAP1, and Bcl-2 by densitometry analysis of the bands in **A**. **C**, siRac1 mimics NSC23766 in suppressing XIAP and survivin expression. Cells were transfected by electroporation with siRNA against human Rac1 transcript (siRac1) and analyzed by immunoblotting after 24 h after transfection. **D**, relative changes in expression levels of the indicated proteins determined by densitometry of the blots in **C**. Representative of three independent experiments. Columns, mean ($n = 3$); bars, SD.



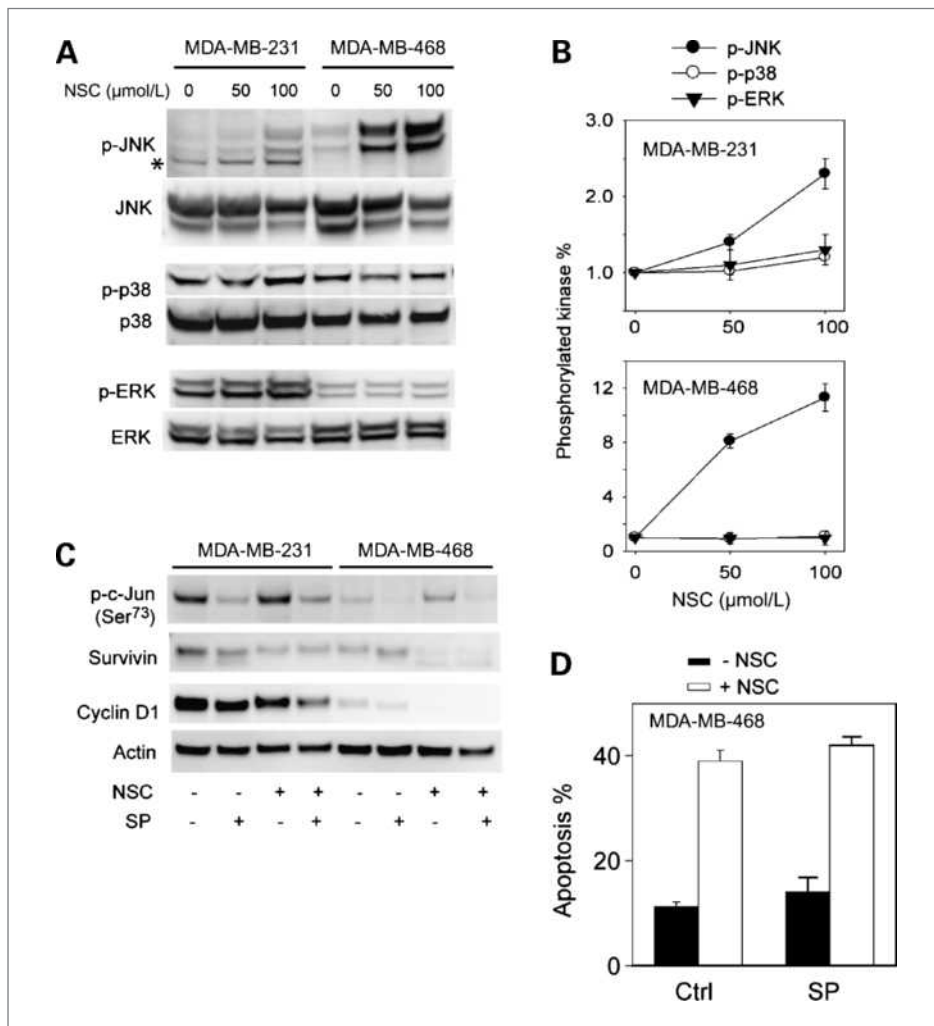


Figure 6. JNK/p38 and ERK kinase pathways are not involved in Rac1-induced expression of survivin and cyclin D1. **A**, Western blots for the phosphorylation status of JNK, p38, and ERK in MDA-MB-231 and MDA-MB-468 cells after NSC23766 treatment for 24 h at the indicated concentrations. *, nonspecific band. **B**, band intensities were quantified by densitometry and are represented as the ratio of phospho-JNK (p-JNK) to total JNK, with the ratio found in the untreated cells being set as 1.0. Activated p38 and ERK were analyzed in a similar manner. **C**, cells were treated with the JNK-specific inhibitor SP600125 (SP; 20 μmol/L) with or without NSC23766 (100 μmol/L) for 24 h and immunoblotted for the indicated proteins. Inhibition of JNK activity is indicated by a decrease in the phosphorylated c-Jun (p-c-Jun). **D**, FACS analysis of apoptosis in MDA-MB-468 cells after treatment for 24 h with NSC23766 or SP600125, alone or in combination. Shown are representatives of three independent experiments.

dephosphorylated Rb (Fig. 7C). These data suggest that NF- κ B, but not mitogen-activated protein kinase pathway, is a primary mediator of Rac1 signaling in stimulating survival and growth of breast cancer cells.

Survivin and XIAP have been shown to directly bind and inhibit the activity of caspase-3 and caspase-7. Consistent with this role, loss of survivin and XIAP correlated with an enhanced activation of caspases (Fig. 3C). Furthermore, ectopic expression of survivin (Fig. 7D) or XIAP (data not shown) inhibited NSC23766-induced caspase activation and apoptosis in MDA-MB-468 cells. Collectively, Rac1 is essential for breast cancer survival and growth. Inhibition of Rac1 activity seems to be beneficial in eliminating breast cancer cells by inducing apoptosis or cell cycle arrest via downregulation of NF- κ B activity and its target gene expressions (Fig. 7E).

Discussion

Rac1 is essential for normal cell function and, when improperly activated, contributes to tumor cell growth,

invasion, and angiogenesis (35). In breast cancer cells, the aberrant Rac1 activity has been related to the elevated expression of its GEF proteins, such as Tiam1 and Trio. Here, we show that repression of Rac1 activity by the pharmacologic inhibitor NSC23766, which blocks interaction between Rac1 and Tiam1 and/or Trio, elicits cytotoxicity to a panel of breast cancer cell lines over normal counterparts. The cytotoxicity of NSC23766 seems to be independent of the status of p53 mutations or expression levels of ER, PR, and Her2 in the target cells. These results suggest that Rac1 could be a therapeutic target in the treatment of breast cancers that are refractory to the conventional chemotherapies. Consistent with this notion, Ushio-Fukai and Nakamura (31) have proposed the Rac1-regulated NADPH oxidase, which is implicated in tumor angiogenesis, as target for cancer therapy.

Blockade of Rac1 activity elicited cytotoxicity to breast cancer cells through distinct mechanisms in a cell line-dependent manner. Consistent with a role for Rac1 in cell cycle progression through G₁ phase (4, 5), loss of Rac1 activity induced G₁ arrest in Rb-positive

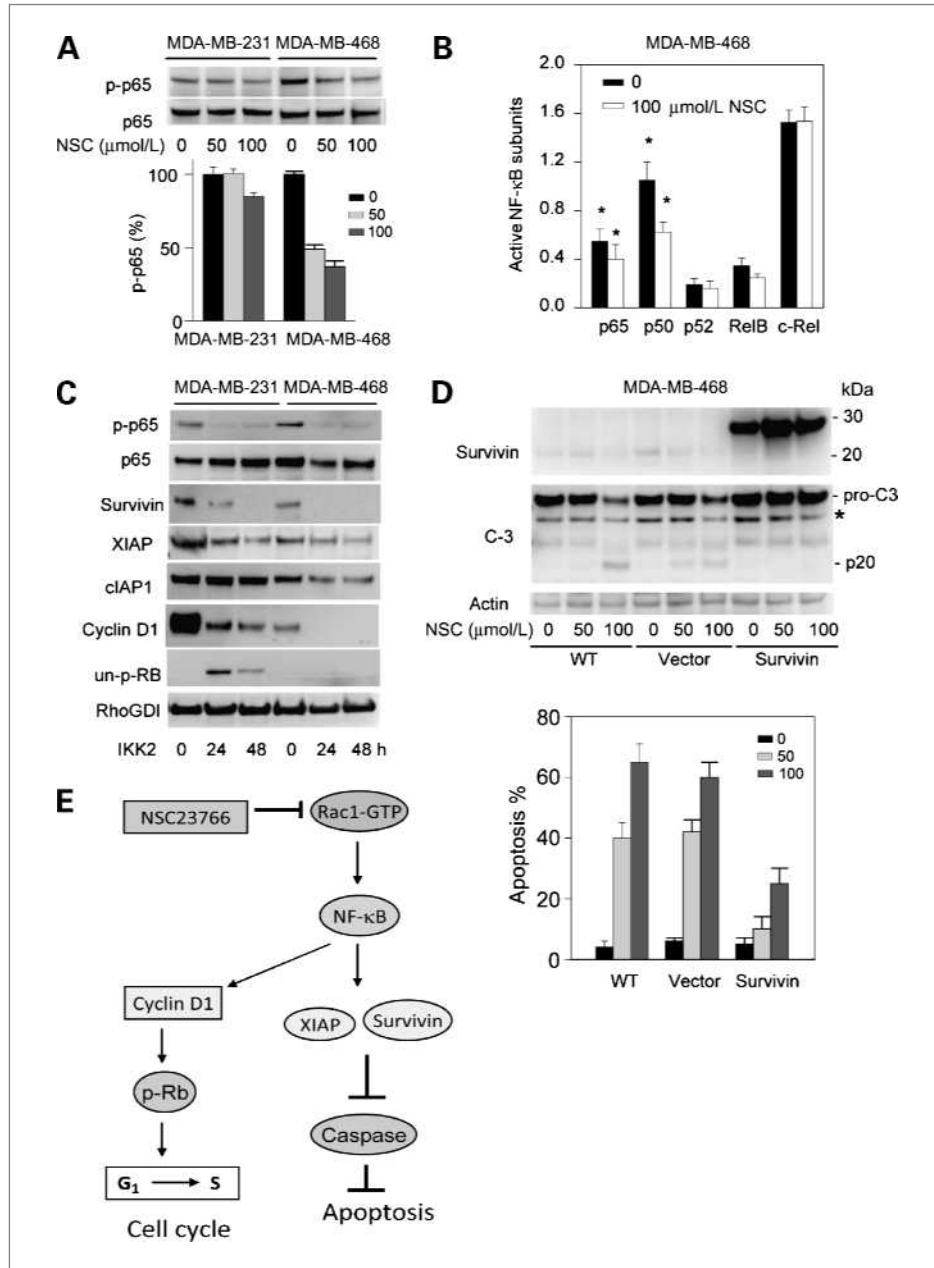


Figure 7. NF-κB activity is required for Rac1-dependent cell survival of breast cancer cells. **A**, status of p65 phosphorylation after Rac1 inhibition. The indicated cell lines were left untreated or treated with NSC23766 at 50 or 100 μmol/L for 48 h and analyzed for phosphorylation of p65 subunit in the two cell lines by Western blotting using antibodies specific to the phosphorylated species. **B**, relative levels of active NF-κB subunits in MDA-MB-468 cells after treatment with 100 μmol/L NSC23766 for 48 h, determined by DNA-binding assays. Columns, mean (*n* = 3); bars, SD. *, *P* < 0.01 (for p65) and *P* < 0.001 (for p50), determined by Student's *t* test versus the corresponding value for untreated cells. **C**, pharmacologic inhibition of NF-κB activity suppresses protein expression of survivin, XIAP, and cyclin D1 but not cIAP. Cells were treated with the cell-permeable IKK2 inhibitor V (1.0 μg/mL) that selectively blocks IκB phosphorylation and prevents the induction of NF-κB p65 nuclear translocation (66). After 24 h, cells were analyzed for expression of the indicated proteins. RhoGDI was used as a loading control. Shown is representation of three experiments. **D**, ectopic expression of survivin inhibits NSC23766-induced apoptosis in MDA-MB-468 cells. Cells were stably transfected with an empty pCMV6-XL vector or pCMV6-XL-BIRC5 expressing human survivin, treated with 50 or 100 μmol/L NSC23766 for 24 h, and analyzed by Western blotting (top) and flow cytometry (bottom). **E**, a model showing the role of Rac1 in regulating NF-κB in regulating cell growth and survival. Rac1 is an upstream activator of NF-κB, which regulates the transcriptional expression of cyclin D1, survivin, and XIAP. Repression of Rac1 activity by NSC23766 or RNAi interference suppresses NF-κB and its target gene expressions. In MDA-MB-231 cells, loss of cyclin D1 results in dephosphorylation and activation of Rb, a negative regulator of G₁ progression, leading to G₁ cell cycle arrest. By contrast, MDA-MB-468 cells undergo apoptosis as a result of downregulation of survivin and XIAP proteins. In the latter, the cell cycle profile is not affected by Rac1 inhibition, likely due to deficiency in Rb and cyclin E proteins (Fig. 2).

cell lines, including MDA-MB-231, MCF7, and T47D. G₁ arrest was associated with a downregulation in cyclin D1 expression and concomitant increase in unphosphorylated Rb protein (Fig. 2). Cyclin D1-dependent phosphorylation of Rb is known to promote transcription of various genes required for G₁ to S transition (49). By contrast, Rac1 inhibition had no effect on cell cycle but it rather induced massive apoptosis in Rb-deficient MDA-MB-468 cells (Fig. 3). These results suggest that Rb deficiency, which is found in many tumor cells, may play a role in rendering apoptosis following Rac1 inhibition. However, targeted knock-down of Rb in MDA-MB-231 cells failed to enhance apoptosis in response to NSC23766 treatment (Fig. 4C). Additional studies are required to identify factors that cooperate with Rb in switching between cell cycle arrest and apoptotic cell death.

The cytotoxicity of most chemotherapy is derived from their ability in inducing activation of a caspase cascade. As with previous studies (37–40, 56), treatment of MDA-MB-468 cells with NSC23766 also induced cleavage and activation of caspase-3 and caspase-8 (Fig. 3C). NSC23766-induced apoptosis was associated with a decrease in Bcl-2 proteins in leukemia cell lines (39, 51). This seemed not to be the case in MDA-MB-468 breast cancer cells, where the expression levels of Bcl-2 and Bcl-XL were not changed in the progression of apoptosis (Fig. 5). In addition to the Bcl-2 family proteins, the caspase activity is also regulated by members of the IAP family, including XIAP, cIAP1, cIAP2, and survivin (57). These proteins form stable complexes with individual caspase, thereby inhibiting their cleavage and activation. On Rac1 inhibition, XIAP and survivin were markedly reduced in both MDA-MB-231 and MDA-MB-468 cell lines (Fig. 5). Ectopic expression of survivin (Fig. 7D) or XIAP (data not shown) protein protected MDA-MB-468 cells from NSC23766-induced apoptosis. In contrast, protein levels of cIAP1 and cIAP2 were not altered by Rac1 inhibition, reflecting the complexity of the regulation of the IAP family proteins. These data suggest that Rac1 is a positive regulator of XIAP and survivin expressions in breast cancer cells. To our knowledge, this is the first evidence that Rac1 is involved in the regulation of IAP family proteins. This result is of importance because XIAP and survivin are frequently overexpressed in human tumors. For instance, survivin was found to be expressed in 71% of breast cancers, whereas the surrounding tissues were negative (58, 59). Survivin has also been proposed as an independent predictor of breast cancer patients with poor prognosis (60). In addition, XIAP was markedly upregulated in a subset

of the breast tumor samples compared with normal tissue (58). Consequently, inhibitors specific to XIAP or survivin are currently in clinical trials for treating various malignancies (61, 62). The ability of Rac1 in regulating XIAP and survivin expressions suggests that targeting Rac1 could be more potent in killing cancer cells than individual inhibitor against XIAP or survivin.

Activation of Rac1 has been implicated in triggering the mitogen-activated protein kinase and NF- κ B pathways in the context of cell survival and apoptosis. In the latter, Rac1 was shown to induce phosphorylation of NF- κ B via PAK, leading to nuclear translocation of NF- κ B and thereby initiating gene transcription (13). In agreement, activity of NF- κ B, particularly the p65 and p50 subunits, was decreased in breast cancer cells treated with NSC23766 (Fig. 7A and B). NF- κ B is involved in the regulation of survivin and XIAP at transcriptional level (55, 63). Consistently, NSC23766-mediated downregulation of NF- κ B activity was correlated with a diminished expression of XIAP and survivin. These results show that NF- κ B mediates Rac1 signaling in regulation of XIAP and survivin expressions in breast cancer cells. This notion is supported by a direct inhibition of NF- κ B activity by the pharmacologic inhibitor IKK2 (Fig. 7C). Unexpectedly, NSC23766 treatment resulted in a significant increase of JNK activity (Fig. 6). The underlying mechanism is not clear, but it may be related to loss of XIAP that was reported as a negative regulator of JNK (53, 54). Our observation is distinct from those showing that Rac1 inhibition led to prolonged JNK activation, which was linked to mitochondria damage and induction of apoptosis of other cell types (65, 66). Nevertheless, inhibitor specific to JNK had no significant effect on NSC23766-induced apoptosis in MDA-MB-468 cells (Fig. 6C and D). Thus, JNK activation is likely a bystander effect of Rac1 inhibition by NSC23766.

Together, these results reveal a novel function of Rac1 in regulating cell cycle and survival in breast cancer cells, providing a strong rationale for development of next-generation Rac1 inhibitors with improved potency and safety profiles for the treatment of breast cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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