Synuclein γ Compromises Spindle Assembly Checkpoint and Renders Resistance to Antimicrotubule Drugs

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Abstract

Defects in the spindle assembly checkpoint (SAC) have been proposed to contribute to the chromosomal instability in human cancers. One of the major mechanisms underlying antimicrotubule drug (AMD) resistance involves acquired inactivation of SAC. Synuclein γ (SNCG), previously identified as a breast cancer–specific gene, is highly expressed in malignant cancer cells but not in normal epithelium. Here, we show that SNCG is sufficient to induce resistance to AMD-caused apoptosis in breast cancer cells and cancer xenografts. SNCG binds to spindle checkpoint kinase BubR1 and inhibits its kinase activity. Specifically, the C-terminal (Gln106-Asp127) of SNCG binds to the N-terminal TPR (tetratricopeptidic fold) motif of BubR1. SNCG–BubR1 interaction induces a structure change of BubR1, attenuates its interaction with other key checkpoint proteins of Cdc20, and thus compromises SAC function. SNCG expression in breast cancers from patients with a neoadjuvant clinical trial showed that SNCG-positive tumors are resistant to chemotherapy-induced apoptosis. These data show that SNCG renders AMD resistance by inhibiting BubR1 activity and attenuating SAC function. Mol Cancer Ther; 13(3); 699–713. ©2014 AACR.

Introduction

The microtubule cytoskeleton is an effective and validated target for cancer chemotherapeutic drugs. Currently, antimicrotubule drug (AMD) taxane, e.g., docetaxel, is the first-line chemotherapeutic agent to treat patients with locally advanced or metastatic breast cancer and is also the effective chemotherapeutic agent in clinical use in six different cancers (breast, ovarian, non–small cell lung, prostate, gastric, and head and neck; ref. 1). Cytotoxicity stems from its ability to promote tubulin polymerization and formation of stable microtubules. The stabilized microtubules are resistant to disassembly by physiologic stimuli, so cells accumulate disorganized arrays of microtubules. This results in arresting the cell in the G2–M phase, which ultimately results in apoptosis. Nearly half of the treated patients with breast and ovarian cancer, however, do not respond to this microtubule-disrupting chemotherapy (2). Identification of cellular factors that are associated with the sensitivity to AMD treatment would have great clinical implications. Although general mechanisms of drug resistance may apply to AMD resistance (3, 4), more specifically, the current research paradigm on AMD resistance focus on acquired β-tubulin mutations and altered expression of β-tubulin isotypes (5, 6). Because tubulin is the structural protein of microtubule and is supposed to be the main target of docetaxel action, alternations of tubulin expression are thought to induce docetaxel resistance. However, it is not clear what effect the expression of a particular β-tubulin isotype, or acquisition of point mutations, has on the stability of microtubule and their relationship to docetaxel resistance. Furthermore, several recent studies searching for biomarkers predictive of clinical response to taxane are largely contradictory and, in any case, very limited on the predictive role that tubulin characteristics could have biologic effects of these drugs (7, 8). One of the mechanisms underlying AMD resistance involves acquired inactivation of mitotic checkpoint function. AMD works by perturbing spindle assembly, which activates spindle assembly checkpoint (SAC), resulting in cells arrested in the mitotic phase without entering anaphase (9). Prolonged treatments with these agents lead to cell death by undergoing apoptosis. Because AMD is thought to induce mitotic catastrophe, which activates SAC, if SAC is defective or inhibited in cancer cells, the cells will arrest transiently and proceed through mitosis without undergoing apoptosis. In fact, cancer cells can resist such killing by premature exit, before cells initiate apoptosis, due to a weak or inactivated SAC (10).
Although genetic mutations in the key SAC components, e.g., BubR1, are associated with the cancer susceptible disorder mosaic variegated aneuploidy (11), mutations in the known SAC genes are not very often seen in human carcinomas, suggesting that SAC inactivation in human tumors may also be driven by an epigenetic mechanism.

We previously identified a breast cancer–specific gene BCSG1, also known as synuclein γ (SNCG; ref. 12). Synucleins are a family of small proteins consisting of three known members, synuclein α (SNCA), synuclein β (SNCB), and SNCG (13). Although synucleins are highly expressed in neuronal cells and have been specifically implicated in neurodegenerative diseases (14, 15), SNCG is not clearly involved in neurodegenerative diseases but is primarily involved in neoplastic diseases. So far, the abnormal expression of SNCG protein has been demonstrated in many different malignant diseases, including breast (12, 16, 17), liver (18, 19), esophagus (18), colon (18, 20, 21), gastric (18), lung (18), prostate (18), pancreas (22), bladder (23), cervical cancers (18), ovarian cancer (24), and giall tumors (25). In these studies, SNCG protein is abnormally expressed in a high percentage of tumor tissues but rarely expressed in tumor-matched nonneoplastic adjacent tissues. The clinical relevance of SNCG expression on breast cancer prognosis was confirmed in clinical follow-up studies (16, 17). Patients with an SNCG-positive tumor had a significantly shorter disease-free survival and overall survival compared with the patients with no SNCG expression. SNCG is a new unfavorable prognostic marker for breast cancer progression and a potential target for breast cancer treatment. At the cellular level, SNCG increases metastasis (26) and hormone-dependent tumor growth (27–29), and promotes genetic instability (30, 31). Investigations aimed to elucidate the molecular mechanisms underlying the oncogenic functions of this protein revealed that SNCG functions like a tumor-specific chaperone and regulates many pathways in cancer progression, which include cell motility (26) and estrogen receptor (ER) signaling (27–29, 32).

Previous studies conducted through yeast two-hybrid screening revealed an interaction of SNCG with the mitotic checkpoint kinase BubR1 (30). BubR1 was originally characterized as a kinase that controls the activation of the anaphase-promoting complex (APC) by binding and inhibiting Cdc20 (33), the major APC regulatory protein. BubR1 is recognized as an essential component of the mammalian checkpoint machinery that monitors the proper assembly of the mitotic spindle. Because the working mechanism of AMD heavily relies on the normal function of the SAC in which BubR1 is a critical component, we reason that the SNCG–BubR1 interaction may represent a novel mechanism for inactivation of the mitotic checkpoint and thus renders AMD resistance. In this study, we investigated the role of SNCG on BubR1 function, its interaction with other key members in SAC, and on inhibition of docetaxel-induced mitotic checkpoint function. The in silico interaction between SNCG with the crystal structure of human BubR1 was analyzed. We also investigate whether SNCG is a new biomarker for predicting resistance of patients with breast cancer to docetaxel in neoadjuvant treatment.

**Materials and Methods**

**Materials and cells**

Docetaxel was acquired from North Carolina Chemlabs. Nocodazole was acquired from Sigma. A stock solution of docetaxel [dissolved in 5% Tween 80, 5% DMSO (dimethyl sulfoxide) in PBS] was stored at –80°C before use. For *in vitro* use, docetaxel was diluted in serum-free medium at the required concentration. For *in vivo* use, docetaxel was diluted in normal saline to a final dose of 25 mg/kg in 150 μL per injection. All of the cell lines used in this study were originally obtained from the American Type Culture Collection as we described before (27, 28). No authentication was done. SNCG stably transfected MCF-7 (MCF-S6, MCF-S2; refs. 27, 28), and MDA-MB-435 (SNCG-435-3; ref. 26) cells were established in 2003 and 1999. SNCG knock-down MDA-MB-231 and SKBR3 cells were previously established in 2010 (32). Docetaxel-resistant MCF-7 and MDA-MB-231 cells were generated by prolonged and repeated exposure to increasing doses of docetaxel. Cells were initially exposed to 50 nmol/L for 1 week, increasing to 200 nmol/L for another 1 week. After this point, the cells were exposed to 0.6 μmol/L docetaxel for 3 months.

**Determination of apoptotic cells and mitotic index**

Apoptotic cells were determined by propidium iodide staining and flow cytometry as we previously described (32). For mitotic index, cells grown on coverslips were treated with docetaxel (80 nmol/L, 20 hours), fixed with 70% ethanol, and incubated with 5 mg/mL of anti-PM-2 antibody [in PBS/0.5% BSA (bovine serum albumin) for 1 hour] that recognizes mitosis-specific phosphorylated proteins (Upstate). Cells were then washed and incubated with Alexa Fluor 488–conjugated antibody (Invitrogen) diluted in PBS containing 50 mg/mL RNase A and 50 mg/mL propidium iodide and analyzed on a LSR II (Becton Dickinson).

**In vitro BubR1 kinase activity**

Cellular extracts from docetaxel-arrested (80 nmol/L, 30 hours) cells were immunoprecipitated with specific anti-BubR1 antibody (Santa Cruz Biotechnology; sc-54). The immunoprecipitated complex was washed twice with immunoprecipitation buffer (1% Triton X-100, 10 mmol/L Tris pH 7.4, 0.5% NP-40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA) containing protease inhibitor cocktail. BubR1 immunoprecipitates were incubated at room temperature for 30 minutes with 25 mmol/L Hepes (pH 7.5), 10 mmol/L MgCl2, 200 μmol/L ATP, and 1 μCi[γ-32P]ATP, and 100 μg histone H1 as exogenous substrates. The kinase reaction was terminated by addition of SDS sample buffer and boiling. The phosphorylated H1
was separated by SDS-PAGE and detected by exposing to a screen of PhosphorImager.

**Molecular docking study**

The *in silico* interaction study between SNCG and the crystal structure for N-terminal region of human BubR1 was carried out using the easy interface option of high ambiguity driven biomolecular docking (HADDOCK) online server. The GIG motif of BuBR1 (146–148) and Glu110, Glu117, and Asp127 of SNCG were defined as the initial active site residues and residues surrounding them within the radius of 6.5 Å were defined as the passive residues. To confirm the interactions between the C-terminal region residues of SNCG and N-BubR1, the docking study between the SNCG C-terminal region and BubR1 was also carried out using HADDOCK using the same parameters as provided above. The PyMOL molecular visualization tool version 0.99 was used to analyze and to prepare the images and DIMPLOT was used to plot the interactions between SNCG and BubR1.

**Molecular dynamics study**

Molecular dynamics simulations of SNCG-BubR1 and C-terminal region of SNCG-BubR1 structure complexes and the stability of the interactions were analyzed using the molecular dynamic simulation studies carried out using the GROMACS 4.0 software package. Initially, the protein structures were typed with the optimized potentials for liquid simulations (OPLS) all atom force field and later they were placed in a cubic box and solvated with the water molecules using the spc216 water models. Later, the energy of the structures were minimized using the steepest descendent method and simulation was carried out for 3 nanoseconds at the temperature (keeping the pressure of the system). The whole procedures were carried out in a HP workstation (HP xw4600) provided with the Intel core 2 dual core processors. The programs carried out in the molecular dynamic simulation studies carried out were required to have adequate metabolic functions before every cycle chemotherapy and fertile women had to use effective contraception. All patients accepted the modified radical mastectomy 2 weeks after the last cycle of chemotherapy.

**Immunohistochemical analysis**

As previously described (17), deparaffinized human breast sections (5-μm thick) were treated with H2O2, trypsin, and blocked with normal goat serum. Sections were incubated with specific mouse monoclonal antibodies against with ER, PR, p53, HER-2, and Ki67 (American Diagnostica Inc.) followed by the incubation with the biotin-conjugated secondary anti-mouse antibodies (Dako). The colorimetric detection was performed by using a standard indirect streptavidin–biotin immunoreaction method by Dako’s Universal LSAB Kit. SNCG was detected by using affinity-purified sheep anti-SNCG polyclonal antibody (Chemicon International Inc.). Approximately 100 tumor cells per field were counted under a Nikon microscope at ×200 amplification and eight fields were randomly selected in each slide. The negative cases were confirmed with two independent experiments. All the slides were scored by a breast cancer pathologist without knowledge of the clinical outcome.

TUNEL staining (ApopTag Peroxidase In Situ Apoptosis Detection Kit from Chemicon International, Inc.) for apoptotic index was scored by point counting of at least 500 cancer cells, and results were presented as percent-positive tumor cells.

**Statistical analyses**

For *in vitro* and tumor xenograft studies, results were reported as the mean ± SD for typical experiments done in three replicate samples and compared by the Student t test. All experiments were done at least twice to ensure reproducibility of the results. Clinical data were analyzed by using SPSS 17.0. Results were reported as the mean ± SD. Differences in tumor response between the
Results

**SNCG confers cellular resistance to docetaxel-induced apoptosis**

We determined whether alternation of SNCG expression in breast cancer cells affects cell response to docetaxel-mediated cytotoxicity. We first used SNCG-negative MCF-7 and its stably SNCG-transfected clones (27, 28). Stable expression of SNCG compromised the sensitivity to docetaxel-induced killing in MCF-7 cells in a concentration-dependent manner (0.05–0.8 μmol/L; data not shown). As demonstrated in Fig. 1A, although docetaxel treatment induced a 45% and 52% of apoptosis in parental MCF-7 cells and neo-transfected control MCF-neo1 clone, respectively, expression of SNCG rendered cellular resistance to docetaxel, which resulted in only 22% and 18% of apoptotic cells in SNCG stably transfected MCF-S2 and MCF-S6 clones, respectively. Because SNCG activates ERα transcriptional activity (27, 28) and taxol downregulated ERα expression in MCF-7 cells, it is possible that the observed SNCG-mediated anti-docetaxel effect in MCF-7 cells is mediated indirectly by stimulation of ERα activity. To exclude this possibility, we used previously established ER-negative and SNCG stably transfected MDA-MB-435 cells (26). The similar antiapoptotic effect of SNCG was also observed in docetaxel-treated SNCG-transfected MDA-MB-435 cells. Compared with parental MDA-MB-435 and its neo control neo-435-1 cell, the stable SNCG-transfected clone SNCG-435-3 cells were 3.2-fold less sensitive than that of the docetaxel-treated parental or vector-transfected control cells (Fig. 1A).

To address whether antiapoptosis was involved in SNCG-mediated resistance to docetaxel, we examined the presence of cleaved PARP as an apoptotic cell death marker. Western blot analysis showed a significant increase in cleaved PARP in parental MCF-7 cells treated with docetaxel. As shown in Fig. 1B, we found that the full-size PARP protein (116 kDa) was cleaved to yield an 85-kDa fragment after treatment of cells with docetaxel. In SNCG-expressing MCF-7 (MCF-S6) cells, however, PARP cleavage was observed at significant decreased levels upon treatment with docetaxel.

The effect of SNCG expression on docetaxel resistance was further demonstrated by inhibiting endogenous SNCG expression in SKBR3 and MDA-MB-231 cells. SNCG siRNA significantly reduced endogenous SNCG expression in SKBR3 (insert, Fig. 1C) and MDA-MB-231 (insert, Fig. 1D) cells. In nontreated cells, there was no significant difference in apoptosis between siSNCG-infected and control siCtrl-infected cells. Treatment of siCtrl-infected SKBR-3 cells with docetaxel led to a 28% apoptotic cells. This docetaxel-mediated apoptosis was significantly increased in the SNCG-knockdown cells, resulting in a 63% of apoptotic cells (Fig. 1C). For MDA-MB-231 cells, although docetaxel treatment resulted in 32% apoptotic cells, treatment of SNCG-knockdown MDA-MB-231 cells with docetaxel resulted in 56% apoptotic cells (Fig. 1D).

We determined IC50 in response to docetaxel in several breast cancer cell lines, which have been genetically modified for SNCG expression. As shown in Table 1, overexpression of SNCG into MCF-7 and MDA-MB-435 cells increased IC50 values 3.3- and 2.4-fold, respectively. We previously established stable T47D-derived AS-3 clone by knockdown endogenous SNCG expression (27). Knockdown endogenous SNCG in T47D cells reduced IC50 values from 68 to 29 nmol/L. These data suggest that SNCG renders resistance to docetaxel-induced apoptosis.

We also tested the effect of SNCG on AMD resistance using a different microtubule disruptor nocodazole. Unlike docetaxel, which is a microtubule-stabilizing agent, nocodazole is a microtubule inhibitor. The similar antiapoptotic effect of SNCG was also observed in nocodazole-induced apoptosis in SNCG-transfected MDA-MB-435 cells (Fig. 1E). These data suggest that SNCG has a broad effect on AMD resistance.

**SNCG renders tumor resistance to docetaxel**

To determine whether SNCG-mediated docetaxel resistance could be administered in the *in vivo* tumor xenograft model, we studied the tumor growth of MDA-MB-435 and MCF-7 and their SNCG stably-transfected cells in response to docetaxel. Treatment of MDA-MB-435 and 435-neo-1 tumors resulted in >65% tumor growth inhibition. Consistent with apoptotic data in cell culture, SNCG-435-3 tumors were resistant to the treatment with only 34% tumor growth inhibition (Fig. 1F). To test whether SNCG-mediated tumor resistance to docetaxel *in vivo* also occurs through antiapoptotic effect, we used a TUNEL assay to compare apoptotic index in docetaxel-treated samples from SNCG-435-3 tumors versus MDA-MB-435 and 435-neo-1 tumors (Fig. 1G). Few TUNEL-positive cells were detected in MDA-MB-435 and 435-neo-1 tumors without treatment. However, treatment with docetaxel remarkably increased TUNEL-positive cells (MDA-MB-435: from 7% to 36% positive cells; MDA-435-neo-1: from 5% to 42% positive cells). Although docetaxel also enhanced TUNEL-positive cells in the samples obtained from SNCG-435-3 tumors, the increase was significantly reduced (from 5% without docetaxel to 17% with docetaxel).

For MCF-7 xenografts, the growth of MCF-S6 tumor was stimulated much more by E2 than parental MCF-7 tumor, which is consistent with the chaperon function of SNCG on ERα transcriptional activation (27, 28). As expected, the growth MCF-7 xenograft was significantly inhibited by docetaxel. At 25 days following treatment, docetaxel inhibited E2-stimulated tumor growth by 58%. Although the tumor growth of MCF-S6 cells was also inhibited by docetaxel, the magnitude of growth inhibition reduced with a slight 28% growth inhibition (Fig. 1H).
This SNCG-mediated tumor resistance to docetaxel is consistent with its antiapoptotic effect in tumor as judged by a significant reduction of TUNEL-positive cells from 34% in MCF-7 xenografts to 14% in MCF-S6 xenografts (Fig. 1). These results indicate that expression of SNCG significantly inhibited antitumor and antiapoptotic effect of docetaxel in the tumor xenograft model.

**Molecular modeling of interaction between SNCG and BubR1**

BubR1 is a well-defined guardian of the mitotic spindle, initiating mitotic arrest in response to the lack of tension and/or chromosome alignment across the mitotic plate. BubR1 is a 120-kDa multi-domain protein, having a conserved N-terminal region, a central nonconserved region and a C-terminal serine/threonine kinase domain. The C-terminal kinase domain is involved in the phosphorylation of critical components of a mitotic checkpoint, whereas N-terminal is involved in interaction with SAC component Cdc20. Previous studies conducted through yeast two-hybrid screening revealed an interaction of SNCG with BubR1 (30). To gain more insight into their interacting mechanism and to determine whether such interaction will cause the structural changes of BubR1, a docking analysis was carried out to understand the interaction between SNCG and the crystallographic structure of human BubR1. The result of HADDOCK study had given many clusters of which the top ranked one was selected for final analysis. As shown in Fig. 2, the confirmation of SNCG with the highest binding score among docking results is the C-terminal of SNCG (AA 106–127) and the N-terminal TPR (tetratricopeptidelike folds) motifs 2 and 3 of BubR1. The C-terminal tail region of SNCG is rich in aspartic and glutamic acid residues, and is responsible for the flexible nature of this region. Furthermore, the tail region SNCG has a chaperone activity (34) similar to that of tubulin (35). The C-terminal tail region of SNCG was found to be placed near the GIG (146–148) motif of the BubR1. GIG motif was found to be crucial for interactions with Cdc20 protein. This motif was also conserved in the yeast Mad3 protein, indicating its important role in the interaction with Cdc20 (36, 37). This finding supports the suggestion that both Cdc20 and SNCG may interact with the same region of BubR1.

**Structural changes induced by the interaction of SNCG on BubR1**

Comparing the superimposition of the native and the docked structures of BubR1, we found significant structural variations in three regions with the RMSD of about 1.33 Å (Fig. 2B). First, the displacement in the position of the residues in the loop (Gln145-Ala152) connecting the TPR motif 2 and 3 was observed (Fig. 2C). The functionally conserved GIG (Gly146-Gly148) motif is located within this region. Interaction of the residues Gln 145, Gly 146, and Gly148 of BubR1 with SNCG residues had imposed a considerable difference in the position of the residues located in this region. Second, a structural transition and deviation was observed in the loop (Gln110-Pro119), which connects the TPR motifs 1 and 2 of BubR1 (Fig. 2D). The transition in the secondary structure (coil to helix) of residues Lys113-Trp116 of this loop was a predominant change observed in the docked BubR1 complex. Third, a huge displacement in the position of N-terminal α helix (Arg60-Glu65) and the loop (Ile66-Pro74) connecting the N-terminal α helix with TPR motif 1 (Pro74-Ala108) was observed in the docked BubR1 complex (Fig. 2E). Importantly, the presence of two KEN boxes accounts for the binding of BubR1 with Cdc20, of which the first box is placed at the N-terminal helix of BubR1 (37, 38). Interaction of SNCG at this region and the subsequent structural deviation might account for the observed functional differences of BubR1, which might affect its binding to other key members in the SAC, such as Cdc20.

The molecular modeling study indicates that the C-terminal AA 106–127 of SNCG interacts with BubR1. To confirm this specific C-terminal interaction with BubR1, we constructed a deletion mutant of SNCG in which the C-terminal region of 106–127 was deleted. Although the wild-type SNCG (F-SNCG) was able to bind to BubR1, C-terminal–deleted SNCG (D-SNCG) failed to bind to BubR1 under the same conditions (Fig. 3A). These data are consistent with the molecular modeling analysis, which indicate that the C-terminal AA 106–127 of SNCG binds to BubR1. We also determined whether SNCG-induced drug resistance to docetaxel is mediated by its C-terminal interaction with BubR1. Although the F-SNCG–transfected MCF-7 cells (MCF-S6) were resistant to docetaxel treatment, two clones of D-SNCG–transfected cells (SNCG-D2 and SNCG-D6) failed to convey resistance to docetaxel treatment; the sensitivity of D-SNCG–transfected MCF-7 cells to docetaxel-induced apoptosis is similar to that of parental MCF-7 cells and the control clone MCF-neo1 cells (Fig. 3B). We determined the tumor growth of MCF-7, F-SNCG stable-transfected MCF-56, and D-SNCG stable–transfected SNCG-D2 cells in response to docetaxel.
Figure 1. SNCG renders a drug resistance. A, effect of SNCG on docetaxel resistance in breast cancer cells. SNCG-negative MCF-7 and MCF-neo1 (vector transfect clone), SNCG stably transfected MCF-S2 and MCF-S6 cells (top); SNCG-negative MDA-MB-435 and neo-435-1 (vector-transfected clone), and SNCG stably transfected SNCG-435-3 cells (bottom) were treated with or without 100 nmol/L docetaxel for 2 days and apoptotic cells were determined. The numbers represent means $\pm$ SD of three cultures. Statistical comparisons of treated versus control indicate $P < 0.001$. B, effects on apoptotic biomarkers. MCF-7 and MCF-S6 cells were treated with or without docetaxel (100 nmol/L, 48 hours). Equal amount of total cellular protein was subjected to Western blot analyses of PARP, cleaved PARP, and actin. C and D, effects of knockdown SNCG expression on SKBR-3 (C) and MDA-MB-231 (D) cells on docetaxel resistance. Inserts indicate the reduced SNCG expression in the siRNA knockdown cells. Cells were treated and analyzed as described for MCF-7 and MDA-MB-435 cells. E, effects of SNCG on nocodazole resistance. MDA-MB-435 and SNCG-435-3 cells were treated with nocodazole (0.5 μmol/L, 30 hours) followed by analysis of apoptosis. F and H, SNCG renders tumor resistance to docetaxel. Tumor growths of MDA-MB-435 (F) and MCF-7 (H) xenografts in response to docetaxel treatment. Tumor cell injection and estrogen supplement (for MCF-7 cells) have been described in Materials and Methods. Mice bearing established tumors were treated with either vehicle control or docetaxel (25 mg/kg, i.p., one time per 5 days for 25 days). All mice were sacrificed at day 25 following the first drug treatment. (Continued on the following page.)
by siRNA, the interactions between endogenous BubR1 Cdc20 and anti-Mad2 (Fig. 4A, Top). Similarly, when treated with docetaxel before immunoprecipitation with anti-Mad2, MCF-S6 cells that had been synchronized and treated with docetaxel before immunoprecipitation with anti-Mad2 with SNCG-BubR1 binding inhibits the physical interaction of BubR1 with Cdc20 and Mad2. SNCG binds to BubR1 in MCF-S6 cells, and this SNCG-BubR1 binding inhibits the physical interaction between BubR1 and Cdc20 and Mad2. Although the full-length SNCG was able to reduce the BubR1 interaction with Cdc20, D-SNCG failed to affect the interactions between BubR1 and Cdc20 (Fig. 4C). These data are consistent with the molecular modeling analysis, which indicate that the C-terminal of SNCG binds to BubR1, causes a structure change of functionally conserved GIG motif, and thus attenuates the BubR1 interaction with other key SAC proteins like Cdc20.

The kinase activity of BubR1 is essential for checkpoint signaling. Recently, activated BubR1 was shown to phosphorylate specific targets, including itself, and to induce mitotic cell death. We, therefore, analyzed BubR1 activity after the induction of mitotic arrest with docetaxel in the presence or absence of SNCG. When we examined the effect of SNCG on the ability of BubR1 to phosphorylate itself and histone H3 in vitro, a decrease in SAC kinase activity was observed in SNCG-positive clone MCF-S6 cells, as evidenced by reduced phosphorylation of BubR1 and phospho-histone H3 (Fig. 4D). To confirm that this reduced phosphorylation is mediated by BubR1, we immunoprecipitated BubR1 from docetaxel-arrested both control MCF-7 and MCF-S6 cells and determined the in vitro ability of precipitated BubR1 to phosphorylate histone H1. As demonstrated in Fig. 4E, expression of SNCG in MCF-S6 cells significantly reduced the kinase activity of BubR1 to phosphorylate histone H1. These data indicate that SNCG/BubR1 binding reduces BubR1 kinase activity and thus attenuates mitotic cell death.

To confirm that the reduced BubR1 function due to its interaction with SNCG contributes to decreased docetaxel-induced mitotic arrested cells, we overexpressed BubR1 in MCF7-neo1 and MCF7-S6 cells by transient transfection of pCS2-BubR1. Figure 4F shows that exogenous expression of BubR1 did not significantly alter the responses to the docetaxel-induced mitotic arrest in MCF7-neo1 clone. In contrast, enforced BubR1 overexpression in MCF7-S6 cells significantly reversed SNCG-mediated resistance to docetaxel-induced mitotic arrest and increased the mitotic index. These data provide additional evidence that links the impaired mitotic checkpoint function to the decreased BubR1 function by SNCG expression.

SNCG compromises mitotic checkpoint function

Prolonged mitotic arrest by microtubule disruption triggers apoptosis in the cells with normal mitotic checkpoint functions. Disruption of BubR1 function, e.g., by

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<th>Cell lines</th>
<th>SNCG expression status</th>
<th>Cytotoxicity IC50</th>
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<tr>
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NOTE: Stable SNCG-transfected MCF-7 (MCF-S6), MDA-MB-435 (SNCG-435-3), and SNCG knockdown T47D (AS-3) cells were previously established. All six cell lines were treated with docetaxel (ranging from 10 to 250 nmol/L) for 24 hours. Cytotoxicity was measured by the MTT assay. The IC50 values were defined as the concentration of drug eliciting 50% cell killing and determined by regression analysis.

(Continued) Statistical comparison for tumor sizes in docetaxel-treated SNCG-negative MDA-MD-435 and 435-neo-1 mice relative to mice in other groups indicates $P < 0.01$. Statistical comparison for tumor sizes in docetaxel-treated MCF-7 mice relative to mice in other groups indicates $P < 0.005$. G and I, effects of SNCG on apoptosis of MDA-MB-435 (G) and MCF-7 (I) tumor xenografts. Histologic sections of tumors from docetaxel-treated or control mice were analyzed for apoptosis (TUNEL assay). Statistical analysis showed that docetaxel-treated SNCG-expressing tumor tissues had a significantly lower percentage of TUNEL-positive cells than did docetaxel-treated SNCG-negative tumor tissues ($P < 0.01$).
SNCG, compromises checkpoint function, and thus leads to a reduced mitotic arrest. Antagonism of docetaxel cytotoxicity by SNCG is likely to result in compromising checkpoint function and a reduced mitotic arrest in response to the drug. We determined mitotic indices of several breast cancer cell lines, which have been alternated for exogenous expression of SNCG or genetically knockdown endogenous SNCG. In MDA-MB-435 and control-transfected neo-435-1 cells, more than 57% of the entire population was in the mitotic stage following 20 hours of docetaxel treatment. This compared with just 3.3% mitotic cells following vehicle treatment (Fig. 5A). In contrast, forced expression of SNCG in SNCG-435-3 cells significantly reduced the percentage of mitotic cells to <13% after docetaxel treatment. The effect of SNCG on antimitotic arrest was also observed in SNCG stably transfected MCF-7 cells (Fig. 5B). Under the same treatment condition, <10% of MCF-S6 cells were arrested at the mitotic phase, whereas cells that do not express SNCG (MCF-7 and control vector–transfected MCF-neo1) cells were predominantly in the mitotic stages (Fig. 5B). The effect of SNCG expression on mitotic arrest in response to docetaxel treatment was further demonstrated by inhibiting endogenous SNCG expression in SKBR3 and MDA-MB-231 cells. Docetaxel treatment caused a significant increase in mitotic arrest in SNCG-knockdown MDA-MB-231 (Fig. 5C) and SKBR3 (Fig. 5D) cells. In contrast, expression of endogenous SNCG partially prevented

Figure 2. Structure analyses of the in silico interaction between BubR1 with C-terminal region of SNCG. A, docked interacting conformation of BubR1 [determined by X-ray crystallography (36)] with the in silico predicted structure of SNCG-C terminal region (blue). A1 and A2, the interaction mode between the residues of BubR1 (yellow) involved in binding with SNCG (sky blue), which influence the displacement in the GIG motif-1 (A1) and those that cause the structural transition 1 (A2). B, structural superimposition of native BubR1 X-ray crystallographic structure (green) with the structure obtained after docking with C-terminal region of SNCG (chocolate brown). C, displacement in the position of GIG motif of BubR1 (green) compared with that of native BubR1 structure (red). D, structural change occurred in the BubR1 [Gln110-Pro119; blue] compared with that of native BubR1 structure (green). E, structural displacement occurred in the region [Pro74-Ala108] of BubR1 (violet purple) with native BubR1 structure (lemon green).
docetaxel-induced mitotic arrest. These results, together, clearly demonstrate that the normal mitotic checkpoint function that is required for cells to be arrested at the mitotic phase by antimicrotubule agents is generally impaired in SNCG-expressing cells.

**SNCG predicts docetaxel resistance in neoadjuvant treatment**

Because SNCG compromises BubR1 and SAC function, overrides docetaxel-induced mitotic checkpoint function, and renders resistance to docetaxel-mediated apoptosis both in cells and in tumor xenografts, we determined the clinical relevance of SNCG expression in predicting patient response to docetaxel-based therapy in a neoadjuvant trial, which consists of three cycles of standard neoadjuvant TE treatment (docetaxel 75 mg/m² 1 hour infusion and epirubine 60 mg/m² i.v.). In this pilot trial, 30 patients were chosen for enrollment, and among them 60% (18 of 30) cases were positive for SNCG expression. SNCG expression was analyzed by immunohistochemistry and defined as percentage of tumor cells showing immunoreactivity as follows: SNCG⁻, <10%; SNCG⁺, 10%–20%; SNCG++⁺, 20%–50%; and SNCG+++, 50%–75%. Figure 6A and B shows representatives of SNCG⁻ and SNCG+++++ tumors. Because of the limited patient number and for the purpose of statistical analysis, 18 patients had ≥SNCG++ tumors and were regarded as SNCG-positive and 12 patients had < SNCG++ tumors and were regarded as SNCG-negative. None of these patients had received prior therapy for cancer. The age of patients was not significantly different in SNCG⁻ group (51.56 ± 9.61 cm) versus SNCG⁻ group (53.42 ± 10.90 cm; P = 0.626). According to World Health Organization (WHO) Response Evaluation Criteria, 18 patients achieved partial response (PR), and among them, 11 patients with PR were in SNCG⁻ group and 7 in SNCG⁻ group. Twelve patients showed stable disease and no patients had progressive disease.
Although the sizes of the tumors were reduced significantly after three cycles of therapy in both SNCG+ and SNCG− groups, for patients in the SNCG+ groups, tumor sizes were reduced moderately (5.94 vs. 4.17 cm; \( P = 0.00 \)); the tumor size reduction in the SNCG− group was reduced more greatly (6.17 vs. 3.00 cm; \( P = 0.000 \)).

The average tumor size in SNCG− group was significantly smaller than that in the SNCG+ group (Table 2).

There were no significant differences in the expression of ER, PR, p53, and Her2 before and after the chemotherapy in both groups. Consistent with the observed tumor regression data, the proliferation rate (Ki67 expression) was significantly reduced in SNCG− group (\( P = 0.001 \)) after chemotherapy, but not in the SNCG+ group (\( P = 0.052 \)). We also analyzed the apoptosis index. The apoptosis in SNCG+ group was significantly induced with a median increase from 11.83\% to 25.92\% (\( P = 0.021 \)). However, apoptosis was only increased slightly but not significant in SNCG− tumors (8.17\% vs. 10.56\%; \( P = 0.411 \)). Although pretreatment apoptotic index was lower for SNCG-positive tumors.
(8.17%) versus SNCG-negative tumors (11.83%), this was not statistically significant. The average apoptotic index in SNCG$^{-}$ group was significantly higher than that in SNCG$^{+}$ group. Figure 6C shows the changes of apoptotic index of individual tumors before and after chemotherapy; among them there were two tumors with apoptotic index increased more than 30% post chemotherapy in the SNCG$^{-}$ group.

Discussion

Antimicrotubule drugs, e.g., docetaxel, the most widely prescribed drug class in oncology, are a mainstay of metastatic cancer treatment during adjuvant therapy and to prevent metastases in postsurgery patients with cancer. Antimicrotubule drugs disrupt cellular microtubule networks and are thought to induce mitotic catastrophe, which activates SAC, resulting in cells arrested in the mitotic phase and leading to apoptosis. If SAC is compromised in cancer cells, the cells will escape the mitotic arrest without undergoing apoptosis. From four different lines of investigation, we provide conclusive experimental evidence that indicate that SNCG renders a resistance to docetaxel treatment. First, we demonstrated that exogenous expression of SNCG in MCF-7 or MDA-MB-435 cells markedly decreased docetaxel-induced apoptosis as compared with their respective control neo clones. Conversely, knocking down the endogenous expression of SNCG in SKBR3, T47D, and MDA-MB-231 cells increased apoptosis in response to docetaxel treatment. Second, tumor xenograft studies further demonstrate that stable SNCG–transfected MDA-MB-435 and MCF-7 cells were less sensitive to docetaxel-induced antitumor effect as compared with SNCG-negative parental and their control neo cells. Third, expression of SNCG in the patients with primary breast cancer indicates a poor response to docetaxel-based chemotherapy in a pilot neoadjuvant trial. Fourth, expression of SNCG inhibits SAC function and mitotic arrest. Although the expression of SNCG prevents docetaxel-induced mitotic arrest, knockdown endogenous SNCG enhances the mitotic arrest in response to docetaxel treatment.

Because SNCG expression overrides the mitotic checkpoint control and confers the cellular resistance to antimicrotubule drug-caused apoptosis, this suggests that the normal mitotic checkpoint function that is required for cells to be arrested at the mitotic phase and the sequent apoptosis by docetaxel is generally impaired in SNCG-expressing cells. Several key proteins for SAC function have been identified, which include BubR1, Mad2, centromere-associated protein E (CENP-E), Cdc20, and others (39). BubR1 kinase is a key regulator of the SAC. The importance of BubR1 in the SAC is reflected by the observation that BubR1 forms the mitotic checkpoint complex (MCC) composed of Mad2, Bub3, BubR1, and Cdc20 and inactivates the APC/C–Cdc20 complex (40). Insufficient BubR1 function may induce a perturbed SAC function, and thus leads to the misaligned chromosomes and high aneuploidy. A complete lack of SAC is incompatible with viability in higher eukaryotes. However, the SAC is not "all or none." It can be compromised to certain degrees as indicated by the fact that the mice heterozygous for the essential components of SAC, e.g., BubR1 or Cdc20, are viable despite clear defects in the checkpoint function (33). Studies have shown that disruption of BubR1 activity results in a loss of checkpoint control, chromosomal instability, and/or early onset of malignancy (41). Previous studies using yeast two-hybrid screening revealed an interaction of SNCG with BubR1 (30).

Figure 5. SNCG renders lower mitotic index. Cells from MCF-7 (A), MDA-MB-435 (B), SKBR3 (C), and MDA-MB-231 (D) were cultured in cover slips inserted into wells of 24-well culture plates and treated with DMSO or 80 nmol/L docetaxel for 20 hours. Data are expressed as percentage of MPM-2–reactive cells in the propidium iodide (PI)–positive population as described in Fig. 4. The numbers represent means ± SD of three cultures.
Because SNCG compromises SAC function and renders a resistance to antimicrotubule drug, we reason that SNCG–BubR1 interaction may contribute to the inhibition of SAC as a result of nonmutational inactivation. To study the molecular mechanism through which SNCG interacts with BubR1, we performed docking analysis of the interaction between SNCG and BubR1. BubR1 is an essential mitotic checkpoint protein with at least two functions: as an active kinase at unattached kinetochores and as a cytosolic inhibitor of APC/C–Cdc20 complex. It is established that N-terminal Cdc20 binding domain of BubR1 is essential for all of these functions (37). We provide evidence that the C-terminal of SNCG binds directly to the N-terminal TPR motifs 2 and 3 of BubR1, which is critical for binding to Cdc20 (42). The chaperone-like activity of SNCG has been demonstrated in the cell-free system by assaying the aggregation of thermally denatured proteins (43). All three members in synuclein family share an extensive sequence homology. The most conserved regions are in the N-terminal portion of the protein. In contrast, the C-terminal region (AA86-127) of SNCG is quite different from synucleinα and β. A unique feature of chaperone proteins is a flexible hydrophilic tail region in the C-terminal. Nuclear magnetic resonance spectroscopy has revealed that proteins with chaperone-like activities have unstructured flexible solvent-exposed C-terminal extensions (44). The polar and flexible C-terminal tail is thought to promote the interaction of the chaperone with the hydrophobic region in the denatured protein and thus play a critical role in substrate–chaperone association. Indeed, previous reports indicated that the C-terminus of SNCG is particularly important in performing the protein binding and chaperone function (34, 44). Consistent with the molecular modeling analysis, our studies using SNCG deletion mutants indicate that the C-terminal AA 106–127 is responsible for its specific binding to BubR1 and such binding is able to reduce the BubR1 interaction with Cdc20. Furthermore, we also demonstrated that C-terminal of SNCG is required to mediate drug resistance to docetaxel.

Because SNCG specifically interacts with the N-terminal Cdc20 binding domain of BubR1, which is essential for its kinase activity and inhibition of Cdc20, we reason that SNCG expression may compromise the mitotic checkpoint control by inhibiting the normal function of BubR1. We hypothesize that expression of SNCG leads to a weakened SAC activation; this compromised SAC response is mediated by at least two categories: (i) binding to and inhibiting BubR1 kinase activity; and (ii) SNCG–BubR1 interaction may attenuate or prevent the physical interaction of BubR1 with other key factors in SAC such as Mad2 and Cdc20 in a competitive manner. Both Mad2 and BubR1 bind directly to Cdc20 and inhibit APC/C activity in vitro (40). However, combined BubR1 and Mad2 are much more potent APC/CCdc20 inhibitors than the individual proteins (45). This, together with the discovery of an in vivo MCC consisting of Mad2, BubR1, Bub3, and Cdc20 and the demonstration that this complex strongly inhibits APC/C activity in vitro (40) led to the speculation that BubR1-Mad2-Cdc20 complexes to form the ultimate cytosolic APC/C inhibitor (37). Mad2 and BubR1 both regulate the timing of mitosis in a kinetochore-independent fashion. It has been proposed that this timing function requires BubR1 binding to Cdc20 and Mad2 at the onset of mitosis when checkpoint proteins have yet to assemble (46). Consistent with molecular structure study (47), we demonstrated that SNCG-BubR1
interaction inhibits BubR1 phosphorylation, the kinase activity, and the ability to bind to Cdc20 and Mad2. These studies suggest that SNCG expression compromises the mitotic checkpoint control by inhibiting the normal function of BubR1. As the action of antimicrotubule drugs relies heavily on the normal function of mitotic checkpoint machinery, in which BubR1 is a critical component, one of the key mechanisms underlying antimicrotubule drug resistance involves acquired inactivation of SAC. In this regard, SNCG binds to the N-terminal TPR motif of BubR1, inhibits BubR1 activity, prevents the formation of BubR1-Mad2-Cdc20 complexes, and thus results in an insufficiency of BubR1-related SAC function. Cancer cells, in the presence of SNCG, have a reduced ability to initiate and maintain proper mitotic arrest, which leads to antimicrotubule drug resistance by premature exit, before cells initiate apoptosis. The inhibitory effect of SNCG on BubR1 function may explain the induced resistance against antimicrotubule drugs in breast (48), prostate (49), and lung (50) cancers. However, the relationship between antimicrotubule drug resistance and SNCG is likely to be complex. Cancer cells resistant to antimicrotubule drugs may have alterations in tubulin dynamics. In this regard, SNCG may act as a functional microtubule-associated protein, affect microtubule properties, and thus may lead to antimicrotubule drug resistance.

Currently, there are no clinically verified factors that can be used to predict docetaxel resistance. These studies suggest that SNCG can be used as biomarker for docetaxel resistance. Interestingly, although the SNCG gene does not have a signal peptide, suggesting it is not a secreted protein, a secreted form of SNCG can be detected in sera of pancreatic (22) and colon (21) cancer and urine samples of bladder cancer (23). In these studies, SNCG protein is abnormally detected in a high percentage in sera (20, 22) or urine samples (23) of patients with cancer but rarely expressed in healthy controls. Identification of cellular factors, particularly a soluble serum factor, that are associated with the sensitivity to docetaxel-based treatment and development of a predictive/prognostic marker to identify responders and nonresponders would have great clinical implications. The possibility of predicting that patients may not respond to docetaxel by measuring a single serum biomarker might be quite compelling. Biomarker analysis is rarely simple or restricted to one protein. It might be necessary to put SNCG expression in context with other possible biomarkers. Nonetheless, our study will potentially lead to a new molecular profile of the tumor for the optimal patient selection for antimicrotubule drugs and a new strategy of combining SNCG targeting with docetaxel as a novel advantageous approach for the treatment of cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support
This study was supported in part by grants 81230054 and 91029739 from the State Key Program of the National Natural Science Foundation of China, a grant from the National Key Technology R&D Program for the 12th 5-year Plan of China (2013BAI01B06), and a grant from the State Key Laboratory of Reproductive Medicine. This study was also supported in

| Table 2. Changes of tumor size, Ki67 expression, and apoptotic index |
|---------------------|---------------------|-------|
|                     | Prechemotherapy     | Postchemotherapy |
| Size (cm)           |                     | t     | P    |
| SNCG⁺               | 5.94 ± 1.76         | 4.17 ± 1.10 | 5.97 | 0.000 |
| SNCG⁻               | 6.17 ± 1.64         | 3.00 ± 0.95 | 7.82 | 0.000 |
| t                   | −0.347              | 3.000  |      |
| P                   | 0.731               | 0.006  |      |
| Ki67 (%)            |                     |       |      |
| SNCG⁺               | 26.67 ± 18.55       | 20.56 ± 11.49 | 0.09 | 0.052 |
| SNCG⁻               | 32.50 ± 14.85       | 14.17 ± 9.73 | 4.26 | 0.001 |
| t                   | −0.910              | 1.582  |      |
| P                   | 0.370               | 0.125  |      |
| Apoptotic index (%) |                     |       |      |
| SNCG⁺               | 8.17 ± 6.64         | 10.56 ± 8.12 | −0.84 | 0.411 |
| SNCG⁻               | 11.83 ± 8.79        | 25.92 ± 19.62 | −2.69 | 0.021 |
| t                   | −1.301              | −2.569 |      |
| P                   | 0.204               | 0.023  |      |
part by a grant 81102016 from the National Natural Science Foundation of China and a Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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Mol Cancer Ther; 13(3) March 2014

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Synuclein γ Compromises Spindle Assembly Checkpoint and Renders Resistance to Antimicrotubule Drugs

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doi:10.1158/1535-7163.MCT-13-0671

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