PRECLINICAL STUDIES

NSC126188, a piperazine alkyl derivative, induces apoptosis via upregulation of RhoB in HeLa cells

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Summary We describe here a piperazine alkyl derivative, NSC126188, which induced apoptosis of HeLa cells by upregulating RhoB expression. NSC126188 caused multi-septation of fission yeast and hypersensitized a Δrho3 mutant, which implicates the involvement of functional human homolog RhoB. The treatment of cells with NSC126188 induced apoptosis and a dramatic increase in RhoB expression. In addition, RhoB knockdown using siRNA rescued cells from apoptosis, indicating a crucial role of RhoB in NSC126188-induced apoptosis. In a reporter assay using luciferase and EGFP under control of the RhoB promoter, NSC126188 increased both luciferase activity and the expression of EGFP, implicating transcriptional activation of RhoB by NSC126188. Furthermore, NSC126188 demonstrated in vivo anti-tumor activity, inhibiting tumor growth by 66.8% in a nude mouse xenograft using PC-3 human prostate cancer cells. These results suggest that NSC126188 is a potential lead compound and that upregulation of RhoB is associated with NSC126188-induced apoptosis.

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Introduction

Rho proteins contain more than 20 members of the Ras super-family, including RhoA, RhoB, RhoC, Rac1, and cdc42 [1], and are known to regulate actin organization, vesicle trafficking, cell proliferation, motility, invasion, and apoptosis [2–5]. RhoB mediates apoptosis of neoplastic cells after DNA damage, and targeted deletion of the *RhoB* gene renders transformed cells resistant to apoptosis [6, 7]. RhoB expression is suppressed during tumor progression in cancer patients [8, 9], and RhoB prevents oncogene proteins such as EGFR, ErbB2, Ras, and Akt from inducing tumor survival, malignant transformation, invasion, and metastasis [10, 11]. RhoB overexpression inhibits oncogenic signaling in cultured human cancer cell lines and tumor growth in nude mice [12]. Therefore, RhoB has become an important target molecule in cancer therapeutics.

RhoB expression is elevated rapidly by many stimuli, including radiation, growth factors, cytokines, and genotoxic stress [13]. The farnesyltransferase inhibitors (FTIs) induce reactive oxygen species (ROS)-mediated RhoB expression by dissociation of HDAC1 and HAT, leading to histone acetylation of the RhoB promoter [20, 30]. RhoB expression has been shown to be repressed by histone deacetylase 1 (HDAC1) and upregulated by the HDAC inhibitor, trapoxin A (TPX), which is mediated by an inverted CCAAT box [15]. Farnesyltransferase inhibitors (FTIs) induce reactive oxygen species (ROS)-mediated RhoB expression, which is critical for apoptosis in transformed murine embryo fibroblasts [7]. These observations suggest that small molecules that increase RhoB expression could therefore be developed as anticancer agents.

The compound NSC126188, 4-hexadecanoyl-1,1-dimethyl-piperazin-1-ium iodide, was identified as one of active compounds in the screening for haploinsufficiency of *Saccharomyces pombe* deletion mutants [16]. Here, we report that NSC126188 increases RhoB expression, resulting in apoptosis of HeLa cells. NSC126188 also demonstrated a total growth inhibition (TGI) of 66.8% in an in vivo mouse xenograft model using the human prostate cancer cell line, PC-3. These results suggest NSC126188 is a potential lead compound in cancer therapy that induces apoptosis through upregulation of RhoB.

Materials and methods

Materials

NSC126188, a 4-hexadecanoyl-1,1-dimethyl-piperazin-1-ium iodide derivative (http://dtp.nci.nih.gov/) was synthesized using standard techniques. The farnesyltransferase inhibitor FTI-277 was purchased from Calbiochem (Darmstadt, Germany). Protease inhibitor cocktail and sulforhodamine B (SRB) were purchased from Roche Applied Science (Indianapolis, IN, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. The annexin V/propidium iodine (PI) double-staining assay kit and mouse monoclonal antibody against cyclin D were obtained from BD Biosciences (San Jose, CA, USA). Mouse monoclonal antibodies against RhoB and p21 and rabbit polyclonal antibody against GFP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against caspase 3, PARP, and β-actin were supplied by Cell Signaling (Danvers, MA, USA). BD pharmingen 1 antibody,

Yeast strains and growth

Fission yeast *S. pombe* deletion mutants were generated from the haploid strain ED665 *h⁻* (ade6-M210 leu1-32

ura4-D18) [17]. The ED6657*h⁻* parent strain and mutants were grown in Edinburgh Minimal Medium (EMM) containing adenine, uracil, and leucine (AUL). To examine the hypersensitivity of the mutants to NSC126188, a spot assay was performed in an plate (EMM + AUL) containing 6.0 μM NSC126188, as described previously [17].

Cell culture

The human cancer cell lines HeLa (human epithelioid cervical carcinoma), NUGC-3 (human gastric cancer), SW-620 (human colon carcinoma), A549 (human non-small cell lung adenocarcinoma), HCT-116 (human colorectal carcinomas), PC-3 (human prostate carcinoma), LOX-IMVI (human skin cancer), ACHN (human renal carcinoma), NCI-H23 (human non-small cell lung adenocarcinoma), and MDA-MB-468 (human breast carcinoma) were used for evaluation of the in vitro cytotoxicity of NSC126188. Rat2 cells (normal rat fibroblast cell line) were used as a control. All cell lines were maintained in RPMI 1,640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C in a humidified incubator containing 5% CO_2. All cell lines were obtained from the Cancer Bank and the Pre-Clinical Research Program at KRIBB (Daejeon, Korea).

Annexin V/PI double staining

An annexin V/propidium iodine (PI) double-staining analysis was performed as described by the manufacturer (BD Biosciences). Cells were treated with NSC126188 for 12 h and washed twice with pre-chilled phosphate-buffered saline (PBS) (4°C). The treated cells were stained with Annexin V-FITC staining buffer and PI solution for 15 min at room temperature and analysed with a FACSCalibur Flow Cytometer (Becton Dickinson, San Jose, CA, USA).

Western blot analysis

The cultured cells were harvested in RIPA buffer containing a protease inhibitor cocktail (1 tablet/50 ml buffer) and collected by gentle scraping. Whole cell lysates were analyzed by Western blot analysis as previously described [18].

Sulforhodamine (SRB) assay

Growth inhibition of cancer cell lines in the presence of NSC126188 was determined using the SRB assay, as previously described [19]. The SRB dye bound to the cell matrix was quantified using a spectrophotometer at 530 nm.

Cloning of RhoB promoter into PGL2

A 595-bp DNA fragment from the RhoB promoter [20] was amplified by PCR, using NCF7 genomic DNA as a template. The oligonucleotide primers used were 5′-ATTGGTACCGG CAGCGGCAGCAGCAGCGCGG-3′ at the 5′ terminus and 5′-ATATAAGCTTAGCAGGAGGGGGCCCGCGAACGC-3′ at the 3′ terminus of the promoter. The PCR product was cloned into a pGEMT vector at *Kpn*I and *Hind*III restriction sites. The 595-bp DNA fragment obtained via digestion with *Kpn*I and *Hind*III was inserted into the pGL2 basic luciferase vector to produce a pGL2-RhoB-luciferase plasmid. In addition, pGL2-RhoB-GFP was generated by replacing the luciferase gene with the EGFP gene in pcDNA-DEST53E.

Luciferase assay

Transactivation of RhoB was determined by a reporter assay using a dual-luciferase reporter assay system (Promega, Madison, WI, USA), as previously described [18]. HeLa cells at 75–90% confluence were transiently co-transfected with the pGL2- RhoB-luciferase plasmid containing the RhoB promoter and pRL-SV40 encoding firefly renilla luciferase. Luciferase activity was integrated over a 10-second period and measured using a luminometer (Victor X Light; Perkin Elmer, Waltham, MA, USA). The results were normalized to the activity of renilla luciferase.

siRNA-induced RhoB knockdown

Knockdown of RhoB expression was performed using siRhoB RNA, as previously described [21]. The siRNAs used were: siScrambled (scrambled siRNA, [22]), 5′-CCUACGCCACCAAUUUCGUTT-3′; siRhoB1, 5′-CCGUCUUCGAGAACUAUGUTT-3′. siRNAs (10 nM) were transfected into HeLa cells using the lipofectamine LTX reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. After 24 h, cells were treated with 3 μM NSC126188 for 36 h to determine the effect of the RhoB knockdown after treatment.

Confocal fluorescence microscopy

Cells were transfected with plasmid pGL-RhoB-EGFP expressing EGFP under control of the RhoB promoter. EGFP expression was observed with a confocal fluorescence microscope (LSM5 Live DuoScan; Carl Zeiss, Stuttgart, Germany) in the presence or absence of NSC126188.

In vivo xenograft assay

The in vivo antitumor activity of NSC126188 was analysed using the human PC-3 prostate carcinoma cell line. PC-3 cells were injected intraperitoneally into 4- to 6-week-old athymic female nude mice to generate tumors (10 nude mice per group,Crj:BALB/c nu/nu; Charles River). NSC126188 (30 mg/kg/day) and adriamycin (2 mg/kg, Q2D) were administered intraperitoneally from the day after transplantation for 3 weeks. Tumor volume (V) was determined using the following equation: $V = (L \times W^2) \times 0.5$, where L is the length of the long side of the tumor and W is the length of the short side. Total growth inhibition was analyzed for statistical significance using Student's *t*-test [23].

Results

NSC126188 causes growth inhibition of cancer cells

The compound NSC126188 (Fig. 1A) was identified as one of 40 active compounds conferring growth inhibition of *S. pombe* mutants [16]. NSC126188, a piperazine alkyl derivative, is structurally unique and novel among anti-cancer compounds. NSC126188 caused growth inhibition of most cancer cell lines tested, with a GI_{50} range of 0.5–3.0 μM (Table 1). Although NSC126188 had a greater effect on cell growth in some cancer cell lines, including NUGC-3, PC-3, A549, and HCT-116, than in others, the differences were not significant.

NSC126188 causes growth defects in fission yeast Δrho3 haploid mutants

NSC126188 induced multi-septation of *S. pombe* haploid cells (Fig. 1Ba). In the cell division cycle of *S. pombe*, septum formation and cytokinesis are regulated both spatially and temporally to achieve proper coordination with mitosis [24]. Therefore, haploid *S. pombe* mutants with deletion of genes involved in mitosis and cytokinesis were selected, and the effect of NSC126188 on growth of these mutants was examined using a spot assay (Fig. 1Bb). *S. pombe* mutants Δrho3 [25], Δlim domain, and Δdma [26] showed hypersensitivity to NSC126188. *S. pombe* Rho proteins such as *rho1, rho2, rho3* [25], and *rho4* [27] have distinct characteristics even though they share sequence homology and common function. Interestingly, the Δrho3 mutant exhibited the most severe growth defect in the presence of NSC126188. The Rho3 protein is localized at the division site during mitosis and septation and interacts with the diaphanous/formin For3 [25], which is homologous to mDia. Rho3 appears to be a functional homolog of human RhoB, which interacts with mDia on the actin coat, and affects membrane trafficking in human cell lines [5]. The hypersensitivity of the Δrho3 mutant to NSC126188 implies that the mode of action of NSC126188 is related to
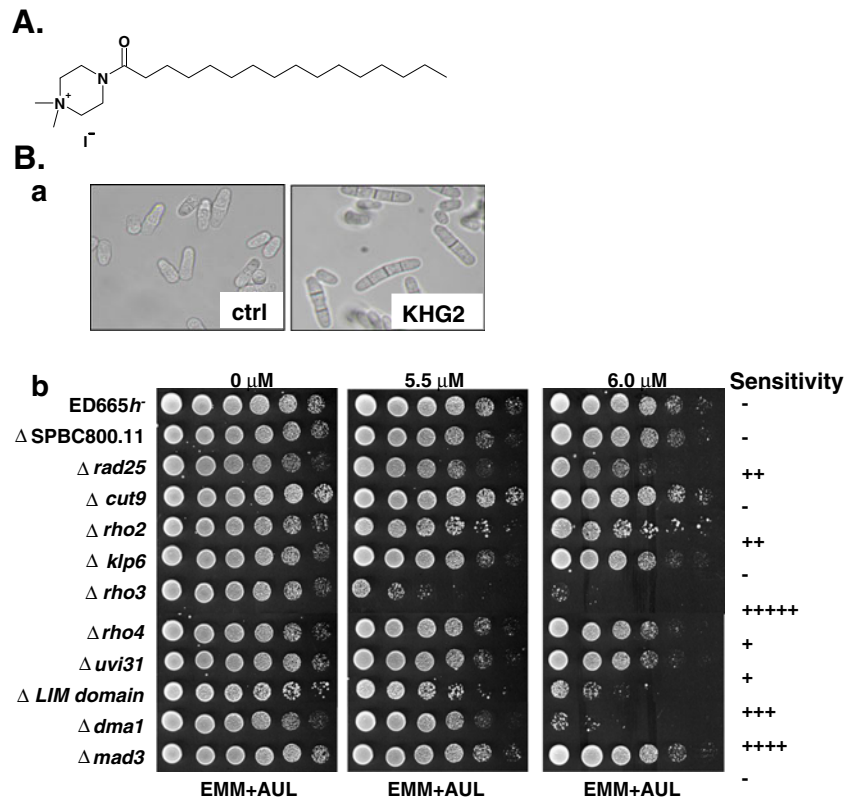
A.

B.

a

ctrl   KHG2

b

	0 μM	5.5 μM	6.0 μM	Sensitivity
ED665h⁻				-
Δ SPBC800.11				-
Δ rad25				++
Δ cut9				-
Δ rho2				++
Δ klp6				-
Δ rho3				+++++
Δ rho4				+
Δ uvi31				+
Δ LIM domain				+++
Δ dma1				++++
Δ mad3				-

EMM+AUL   EMM+AUL   EMM+AUL

Fig. 1 Characteristics of NSC126188. A. Chemical structure of NSC126188, 4-hexadecanoyl-1,1-dimethyl-piperazin-1-ium iodide. B. Effect of NSC126188 on *S. pombe* morphology. (**a**) Morphological change of *S. pombe* in the presence of NSC126188 (6 μM). *S. pombe* haploid ED665 (*h⁻*, *ade6-M210 leu1-32 ura4-D18*) was grown in liquid minimal media (EMM + AUL) containing 6 μM NSC126188 for 16 h. (**b**) Hypersensitivities of *S. pombe* deletion mutants to

NSC126188. The haploid mutants used have deletions in the following chromosomal genes in parentheses: Δ*SPBC800.11* (SPBC800.11), Δ*rad25* (SPAC17A2.13c), Δ*cut9* (SPAC6F12.15c), Δ*rho2* (SPAC16.01), Δ*klp6* (SPBC1685.15c), Δ*rho3* (SPAC23C4.08), Δ*rho4* (SPAC16A10.04), Δ*uvi31* (SPBC16E9.06c), ΔLIM-domain (SPBC4F6.12), Δ*dma1* (SPAC17G8.10c), and Δ*mad3* (SPCC895.02)

RhoB function and suggests that NSC126188 may somehow affect functions regulating RhoB expression in mammalian cells.

NSC126188 induces apoptosis of HeLa cells

To investigate the potential anti-cancer property of NSC126188, HeLa cells were chosen as a model cell line. NSC126188 showed dose-dependent growth inhibition of these cells (Suppl. Fig. 1). Cell death was observed via microscopy by treating cells with 10 μM NSC126188 for 12 h (Suppl. Fig. 2). NSC126188-induced apoptosis of HeLa cells was investigated by Annexin V/PI staining analysis (Fig. 2Aa); Annexin V binds to phosphatidylserine (PS) on the membrane of early apoptotic cells and Propidium iodine (PI) distinguishes between viable and nonviable cells. When cells were treated with 10 μM NSC126188 for 12 h, 21% of early apoptotic cells were stained with Annexin V-FITC antibody (Fig. 2Aa, lower right of scatter-grams, PI⁻, AV⁺). About 51% of the cells seemed to be in late apoptosis, or the transition from the

apoptotic to the necrotic state (Fig. 2Aa, upper right (PI⁺, AV⁺)). The non-treated control cells appeared at the lower left, indicating the live cell population. This result suggests that NSC126188 induces apoptosis of HeLa cells.

PARP cleavage and procaspase 3 cleavage to produce active caspase 3 were detected approximately 12 h after treatment of cells with NSC126188 by Western blot analysis (Fig. 2Ab), indicating that NSC126188 induced apoptosis of HeLa cells. A decrease in cyclin D1 and an increase in p21 were also detected, suggesting an association of them in the growth inhibition and induction of apoptosis in the presence of NSC126188.

NSC126188 induces apoptosis via upregulation of RhoB in HeLa cells

On occasion, deletion and overexpression of a gene in *S. pombe* can show similar physiological effects and phenotypes because *S. pombe* phenotypes for gene regulation is relatively simple [28]. Therefore, NSC126188 inducing growth defects of Δ*rho3* mutant could be associated with

either upregulation or downregulation of RhoB in mammalian cells.

RhoB is typically downregulated in cancer cells [8], and RhoB overexpression is known to cause apoptosis in HeLa cells [21]. In NSC126188-treated cells, a dramatic increase in RhoB expression was detected in a time-and dose-dependent manner (Fig. 2Ba and Bb, respectively), implicating the involvement of RhoB in apoptosis of HeLa cells.

Next, we examined whether siRNA knockdown of RhoB could rescue HeLa cells from apoptosis induced by NSC126188 (Fig. 2C). NSC12688 increased transcription, thus expression, of RhoB in HeLa cells (Fig. 2Ca). RhoB knockdown by siRhoB rescued cells from apoptosis, also shown by both relative survival of cells (Fig. 2Cb) and microscopic observation (Suppl. Fig. 3), in the presence of NSC126188. RhoB knockdown by siRhoB was confirmed by a decrease in both RNA and protein levels (Fig. 2Ca). It

is clear that RhoB knockdown suppressed apoptosis induced by NSC126188, which suggests that NSC126188 induces apoptosis via upregulation of RhoB in HeLa cells.

RhoB plays a crucial role in NSC126188-induced apoptosis in HeLa cells

Because RhoB is regulated by NSC126188 at the level of transcription (Fig. 2Ca) [14, 20], the effect of NSC126188 on RhoB expression was examined in a cell-based reporter assay using plasmids containing the RhoB promoter [20] fused to either the luciferase or the EGFP gene (Suppl. Fig. 4). Activation of the RhoB promoter in the presence of NSC126188 was examined along with FTI-277 [20], a positive control, and NCI compounds NSC608781, NSC20535, and NSC19703, which induce apoptosis of cancer cells [16] (Fig. 3). NSC126188 increased luciferase
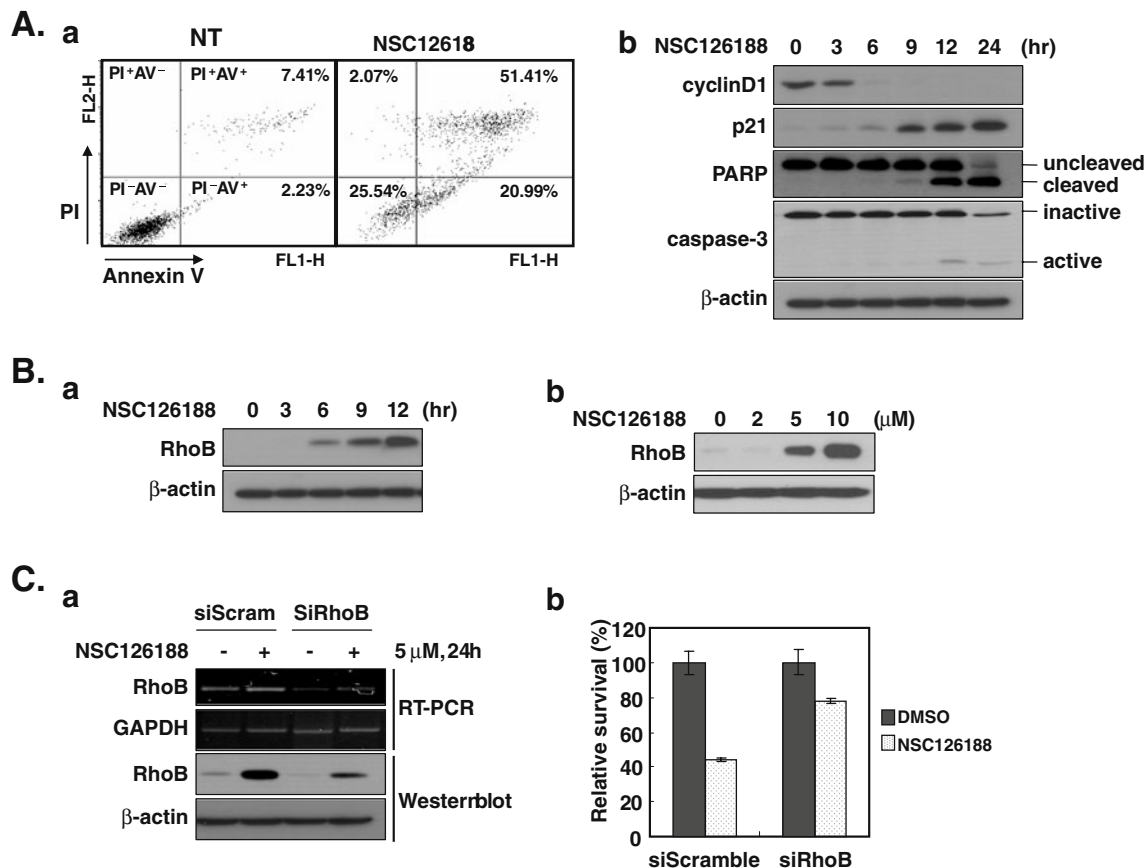

Fig. 2 Induction of apoptosis by NSC126188. A. Apoptosis of HeLa cells induced in the presence of NSC126188. (a) Scatter-grams of Annexin V/PI double staining. (b) Western blot analysis of cells treated with NSC126188. B. Induction of RhoB expression during treatment of HeLa cells with NSC126188. Cells were treated with 10 μM NSC126188 for the designated time (a) and designated concentration for 12 h (b). C. The effect of RhoB knockdown on HeLa cell growth. The HeLa cells were treated with siRhoB (10 nM) for 36 h in the absence or presence of NSC126188 (3 μM). Scrambled siRNA (siScrambled) was used as a negative control. (a) siRNA-induced RhoB knockdown in the presence and absence of NSC126188. (b) Relative survival of cells treated with siRhoB in the presence of NSC126188. Live cells in the presence of NSC126188 were determined by counting trypan blue-stained cells
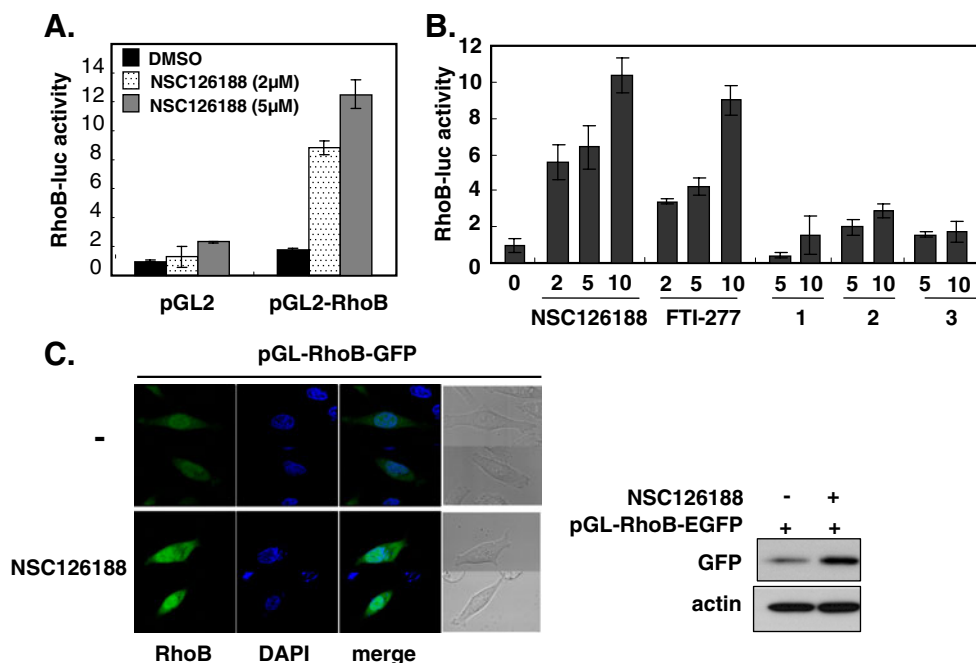
Fig. 3 Detection of RhoB promoter activation by reporter assays. **a**. Activation of RhoB promoter by NSC126188. Luciferase activity was determined in the cells transfected with either the pGL2 vector or pGL-RhoB-Luc in the presence of NSC126188. **b**. Comparison of RhoB promoter activation. Luciferase assay was carried out in the presence of NSC126188, FTI as a positive control, and the following NCI chemicals: 1, NSC608781; 2, NSC205357; and 3, NSC19703. **c**. Confocal microscopic observation of cells transfected with pGL-RhoB-EGFP under control of the RhoB promoter. EGFP expression was observed using a fluorescence microscope in the absence and presence of NSC126188 (5 μM, 12 h). The increase of EGFP protein was also detected by Western blot analysis

activity dramatically in a dose-dependent manner (Fig. 3A and B), indicating transcriptional activation of RhoB in HeLa cells, similar to the increase in luciferase activity by FTI-277 (Fig. 3B). The NCI compounds, NSC608781, NSC20535, and NSC19703, did not increase luciferase activity, indicating they induce apoptosis without upregulation of RhoB (Fig. 3B). In addition, when EGFP expression was under control of the RhoB promoter, stronger EGFP fluorescence was observed in the presence

of NSC126188 (Fig. 3C). Western blot analysis also revealed increased EGFP expression in cells treated with NSC126188, further demonstrating activation of the RhoB promoter under these conditions. This result suggests that RhoB plays a crucial role in NSC126188-induced apoptosis, but not in the apoptosis induced by the NCI compounds used. The mechanism of RhoB transcriptional activation by NSC126188 is not clear and a more detailed promoter study is warranted to better understand such activation.

Fig. 4 In vivo anti-tumor activity of NSC126188. **a**. Upregulation of RhoB in PC-3 cells by NSC126188. Cells were treated with 5 μM NSC126188 for the designated time. **b**. In vivo mice xenograft. PC-3 cells were injected intraperitoneally to generate tumors in 6 nude mice per group. Either NSC126188 (30 mg/kg/day) or adriamycin (2 mg/kg, Q2D) was administered intraperitoneally from the day after transplantation for 3 weeks. (**a**) tumor weight (mg). (**b**) Pictures of excised tumors after in vivo xenograft
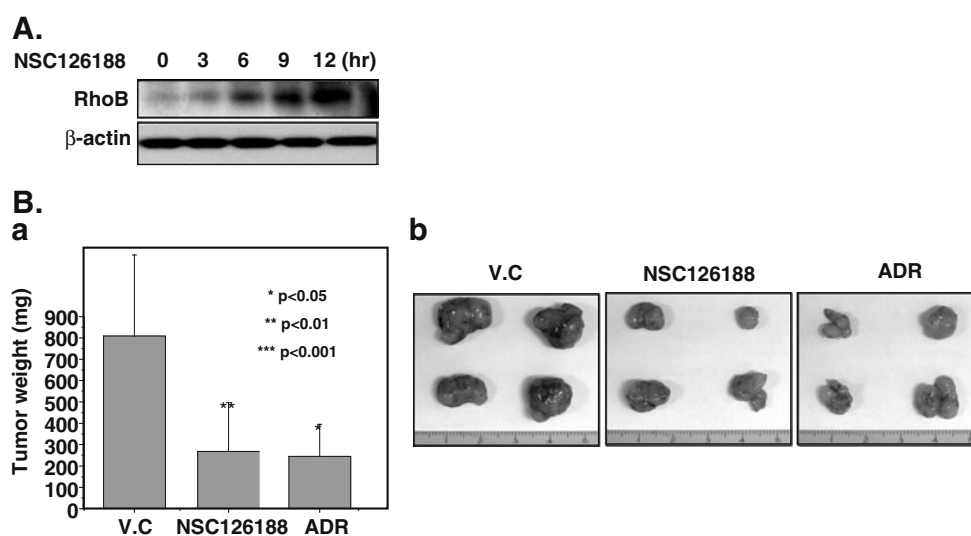
KHG2 exhibits in vivo antitumor efficacy in human prostate cancer xenografts

To investigate in vivo anti-tumor activity of NSC126188 using the human prostate cancer cell line, PC-3, we treated these cells with NSC126188 and found increased RhoB expression during treatment (Fig. 4A). In vivo efficacy of NSC126188 was then examined using a mouse xenograft model (Fig. 4B). At the end of the experimental period, NSC126188 produced a 66.8% TGI, whereas adriamycin, a well-known anticancer drug, produced 69.8% TGI (Fig. 4B). NSC126188 did not cause significant loss of body weight, whereas more weight loss was observed in the group treated with adriamycin (Suppl. Fig. 5). Additionally, NSC126188 caused no adverse effects, such as skin ulcers or other severe symptoms. This result suggests that NSC126188 could be a potential anti-cancer compound.

Discussion

Based on the study of *S. pombe* deletion mutants, RhoB was considered to be associated with growth inhibition of cancer cells in the presence of NSC126188. HeLa cells treated with NSC126188 exhibited a dramatic increase in RhoB expression during apoptosis. RhoB knockdown suppressed the cell death caused by NSC126188, indicating a critical role of RhoB in apoptosis.

RhoB is an attractive molecule as an anti-cancer therapeutic despite a lack of information on the mechanism of apoptosis induction. RhoB induction by γ-radiation occurred at the transcriptional level by the JNK pathway and contributes to the early apoptotic response to ionizing radiation in Jurkat cells [31]. The Dia proteins mDia1 and mDia2 are important effecter molecules for RhoB function, and it has been suggested that the RhoB-mDia pathway is critical for FTI-induced cell death [29]. Also, RhoB was found to interact with TNFAIP1 to induce apoptosis via SAPK/JNK signaling in HeLa cells [21]. Studies on isoprenyl modification of RhoB and the association of RhoB with effector molecules are needed to understand the mechanism of apoptosis by RhoB. In addition, the mechanism of RhoB-induced apoptosis by NSC126188 should be further investigated.

The mechanism of transcriptional regulation of RhoB has been studied. The farnesyltransferase inhibitors induce reactive oxygen species (ROS)-mediated RhoB expression by dissociation of HDAC1 and HAT, leading to histone acetylation of the RhoB promoter [20, 30]. The involvement of ATF-2 with NF-YA, the CCAAT-binding factor, was also shown to be important for the transcriptional activation of the RhoB gene via the CCAAT box under genotoxic conditions [14]. It is not clear how NSC126188 activate RhoB expression. A signal transduction analysis and detailed promoter study should be performed to elucidate transcriptional activation of RhoB expression by NSC126188.

In conclusion, NSC126188 treatment resulted in a growth defect in the *S. pombe* Δ*rho3* mutant, which implicates the involvement of RhoB, a functional human homolog of Rho3. NSC126188 treatment increased the expression of RhoB, which is associated with the induction of apoptosis of HeLa cells. NSC126188 also demonstrated in vivo anti-tumor activity of 66.8% TGI in nude mice xenografts using the human prostate cancer cell line, PC-3. These results suggest that NSC126188 is a potential lead compound for cancer therapy and upregulation of RhoB is associated with NSC126188-induced apoptosis.

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