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ORIGINAL ARTICLE

Antitumor effect of novel small chemical inhibitors of Snail-p53 binding in K-Ras-mutated cancer cells

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p53 is frequently mutated by genetic alternation or suppressed by various kinds of cellular signaling pathways in human cancers. Recently, we have revealed that p53 is suppressed and eliminated from cells by direct binding with oncogenic K-Ras-induced Snail. On the basis of the fact, we generated specific inhibitors against p53-Snail binding (GN25 and GN29). These chemicals can induce p53 expression and functions in K-Ras-mutated cells. However, it does not show cytotoxic effect on normal cells or K-Ras-wild-type cells. Moreover, GN25 can selectively activate wild-type p53 in $p53^{WT/MT}$ cancer cells. But single allelic mt p53 containing cell line, Panc-1, does not respond to our chemical. *In vivo* xenograft test also supports the antitumor effect of GN25 in K-Ras-mutated cell lines. These results suggest that our compounds are strong candidate for anticancer drug against K-Ras-initiated human cancers including pancreatic and lung cancers.
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Introduction

p53 is the strongest tumor suppressor gene, which induce cell death, growth arrest and suppress metastasis in response to various kinds of cellular stresses including oncogene activation, DNA damage, hypoxia and so on (Levine, 1997; Weinberg, 2006). Thus, reactivation of p53 seems to be the most promising strategy for cancer therapy. Indeed, intact p53 blocks the oncogene-induced cell transformation (Serrano *et al*., 1997) and activation of p53 leads to the tumor regression in H-Ras-driven mouse tumor models (Ventura *et al*., 2007; Xue *et al*., 2007). However, cellular functions of p53 are completely or partially suppressed in many kinds of human cancers by signaling distortions, including overexpression of MDM2 or loss of p14/AFR expression or by genetic mutation itself (Sherr, 2006; Brooks and Gu, 2006). Indeed, the half of human cancers possess mutated p53 (Levine, 1997; Weinberg, 2006). In general, genetic mutation of p53 occurred in single allele, whereas the function of wild-type p53 is masked by mutant p53, the so-called dominant-negative mutation (Levine, 1997; Brooks and Gu, 2006; Weinberg, 2006).

Recently, we have revealed the novel p53 inhibition mechanism in K-Ras-activated cancer cells (Lee *et al*., 2009b, c). Oncogenic K-Ras suppresses the p53 function through induction of Snail, which binds to and eliminates p53 through exocytosis. Thus, tumor suppressive function of p53 is impaired in Snail-overexpressed or K-Ras-activated cells. We have also observed the restoration of p53 functions by blocking the interaction of p53 and Snail or by Snail knockdown in K-Ras-mutated cancer cells (Lee *et al*., 2009c). These results strongly suggest that specific inhibitor against p53-Snail binding would be used for anticancer drug against K-Ras-mutated or Snail-overexpressed cancers such as pancreatic, colon and lung cancers (Downward, 1998; Leslie *et al*., 2003; Deramaudt and Rustgi, 2005).

To realize our hypothesis, we have screened the chemical library using enzyme-linked immunosorbent assay (ELISA) system that has been established previously (Lee *et al*., 2009c). Indeed, in our previous experiment, we have isolated three kinds of compounds as the inhibitors of p53-Snail binding in natural compound library (Lee *et al*., 2009a, c). Although these chemicals can activate p53 in K-Ras-mutated cells, they also show the side effects such as induction of p-GSK3β in high concentration and the low biological activity in low concentration (Lee *et al*., 2009c). To obtain more specific chemical inhibitor, we have performed the chemical screening and characterized their effect in this study. Moreover, in this study, we have tested the *in vivo* effect of our chemical. Considering our results, these chemicals seem to be strong anticancer drug candidates for K-Ras-mutated human cancers such as pancreatic, colon or lung cancer.

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Result

Isolation of specific inhibitors of Snail-p53 binding

To isolate more effective chemicals, we performed the previously established ELISA-based chemical screening using about 500 kinds of chemicals (Lee *et al*., 2009c), and found that a group of chemicals showed the inhibitory effect on p53-Snail binding (Supplementary Figure S1A and B). Because our chemical libraries are arranged according to structural similarity, this result strongly suggests that similar structural motif of chemical is responsible for inhibition of p53-Snail binding. To verify the effect of compounds, we treated the candidate chemicals and measured the expression of p53 and its target genes (*p21* and *PUMA*) in K-Ras-mutated cells through western blot (WB) analysis. As shown in Supplementary Figure S1C, two chemicals (GN051011, B3 and GN051224e, C2) could induce the expression of p53, PUMA and p21 more effectively than other chemicals did (Supplementary Figure S1C).

So we checked the effect of two chemicals on the binding between p53 and Snail by glutathione *S*-transferase (GST) pull-down assay and found that they blocked the interaction of p53–Snail (Figure 1a). Because our candidate chemicals possess the very similar structure (Supplementary Figure S1D), we used B3 as the standard chemical for further experiment. From the cell proliferation assay using B3, we obtained that the proliferation of A549 cells (oncogenic K-Ras/wt-p53), but not of MKN45 (wt-K-Ras/wt-p53), was obviously reduced in response to B3 (Supplementary Figure S1E). This result is consistent with our previous result that blocking of Snail-p53 binding can activate p53 and suppress cell proliferation in K-Ras-mutated cells (Lee *et al*., 2009a, c). B3 could also induce the p53 and p21 expression in time- and dose-dependent manner in HCT116 (oncogenic K-Ras/wt-p53; Figures 1b and c), but not in MKN45 (Figure 1b and data not shown). Finally, we monitored the cell viability using MTT assay and found that B3 reduced about 20% of viability in A549 but not in MKN45 (Supplementary Figure S1E). However, p53 expression was restored at 12 h (Figure 1c), suggesting that chemical seems to be unstable compound.

Chemical modification

To overcome the problems of B3 (short working duration), we modified the backbone molecule of B3 and C2 (Supplementary Figure S2). Our first approach was to make 2-substituted thio-1,4-naphthoquinone (2-thio-NQ) analogs (GN1–GN10) instead of 2-thio-DMNQ moiety as shown in Supplementary Figure S2A. The second approach was to replace sulfur atom of 2-thio-DMNQ analogues with nitrogen atom, 2-amino-DMNQ (GN11–GN20) as shown in Supplementary Figure S2B. The third approach was to synthesize 2-thio-DMNQ analogs (GN21–GN26) with hydrophilic functional groups such as OH or COOH to improve solubility in water as shown in Supplementary Figure S2B. The final approach was to introduce amide functional group (GN28, GN30 and GN31) or ester functional group (GN29) at terminal carboxylic group of GN26 or GN25 to find out the structural requirement for the inhibitory in interaction between Snail and p53 as shown in Supplementary Figure S2C. Also, the sulfur atom of GN29 was oxidized to sulfoxide group as shown in Supplementary Figure S2C. From the effort of our modification, we generated 31 kinds of modified chemicals (GN1–GN31). Using WB analysis and ELISA assay, we isolated two kinds of chemicals (GN25 and GN29; Figure 1d), which could induce p53 and p21 expression, similar to Nutlin-3 (Nut-3; well-known p53 inducer by masking the MDM2; Vassilev *et al*., 2004), and block the p53-Snail binding (Supplementary Figure S3A and B). As shown in Figure 1d, these chemicals possess common motif and the modifying side chain. Although other kinds of chemicals (such as GN19, GN20, GN28 and GN31) also induced p53 strongly, they showed the cytotoxity in K-Ras wild-type cells (data not shown). So, we excluded them from our candidates.

Inhibitors activate p53 in K-Ras-dependent manner

To confirm the effect of these chemicals, we treated K-Ras-transformed mouse embryonic fibroblast (MEF) (established by single oncogenic K-Ras; Lee *et al*., 2009c) or N-Ras/Myc-transformed MEF cells with these chemicals. K-Ras-transformed cells were very sensitive to GN25 and GN29, but GN29 induced cell death in them (Figure 1e, Supplementary Figure S4A and B). In contrast, N-Ras/Myc-transformed cells were resistant to them. This result implies that biological activity of our chemical is dependent on K-Ras status. We could also observe the induction of p53 in response to GN25 and GN29 in both cell lines. Induction of p53 and p21 was more obviously detected in K-Ras/MEF than in N-Ras/Myc-MEF or nontransformed MEF (Figure 1f), which was consistent with our expectation. Next, we checked the effect of GN25 in H-Ras/Myc-transformed MEF. Consistent with our hypothesis, GN25 did not suppress the viability in this kind of cells (Figure 1g), indicating that anti-proliferating ability of our chemicals is fully dependent on K-Ras mutation. We could also confirm the K-Ras dependency of GN25 in si-K-Ras-transfected cells, where GN25 did not induce p53 expression (Supplementary Figure S4C).

p53 activation in cancer cell line by the chemicals

To explore the antitumor effect of our chemicals in human cancer cells, we treated our chemicals to K-Ras-mutated A549, HCT116 cell lines and MKN45. Although GN25 and GN29 could induce p53, GN25 was more effective in induction of p53 (Figure 2a). It resulted from the chemical's property that GN25, containing hydrophilic carboxylic group, is water and DNSO soluble, whereas GN29, containing isobutyl ester group, is only dimethyl sulfoxide soluble. Moreover, induction of p53 was not detected in MKN45, indicating that p53 induction is achieved in K-Ras-dependent manner (Figure 2a). However, Nut-3 could induce p53

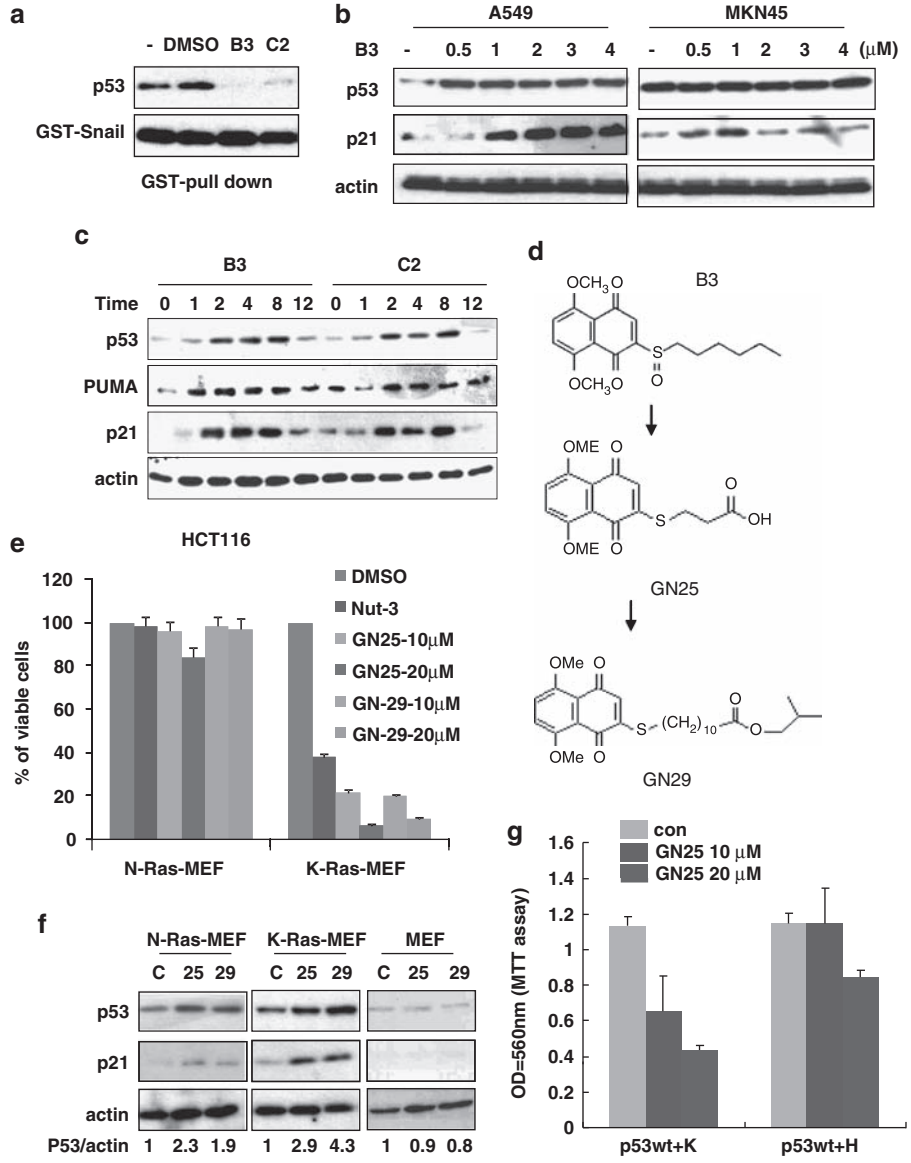


Figure 1 Screening and isolation of specific p53-Snail binding inhibitor. (**a**) Two chemicals block the binding between p53 and Snail in GST pull-down assay. 0.1 mM (final concentration) of each chemical was used for GST pull-down assay. (**b**) Dose-dependent induction of p53 by B3. In A549, B3 could induce p53 and p21 expression. However, in MKN45, this chemical did not induce the expression of p53 or p21. Each cell line was incubated with indicating dosage of B3 for 4 h. Actin was used for loading control. (**c**) B3 and C2 chemicals can induce p53 and its target genes in HCT116. The cells were incubated with 1 μM of chemicals for indicating times (h) and used for WB analysis. (**d**) Simple scheme for production of GN25 and GN29. (**e**) Cell viability assay shows the sensitivity of K-Ras-transformed cells. Viable cells were determined by Trypan blue dye exclusion assay. N-Ras/Myc-MEF did not show the obvious response to our chemicals. (**f**) Induction of p53 and p2 by GN25 and GN29 treatment is detected only in K-Ras-MEF, but not in wild-type or N-Ras/Myc transformed cells. Nontransformed, N-Ras/Myc and K-Ras MEFs were incubated with 5 μM of GN25 or GN29 for 4 h. (**g**) H-Ras/Myc-transformed MEF cells are also resistant to anti-proliferating activity of GN25. H-Ras/Myc-transformed cells were incubated with indicated concentration of GN25 for 12 h and measured the viability through MTT assay.

in all of tested cell lines (Figure 2a). Induction of p53 and p21 expression by GN chemicals in A549 was as strong as that by DNA-damaging agents, adriamycin and etoposide (Supplementary Figure S4D). We could also observe oncogenic K-Ras-dependent induction of p21 in response to GN25 (Supplementary Figure S5A). We next checked the effect of these chemicals on cell proliferation by Trypan blue staining and cell counting assay. Compared to MKN45, cell proliferation of HCT116 and A549 was obviously suppressed by GN25 and GN29 (Figures 2b and c). Their anti-proliferating effect was stronger than Nut-3's effect (Figures 2b and c). Considering the molecular weight of this chemical (GN25; MW = 332), effective dosage of GN chemicals (weight/volume) seemed similar to that of Nut-3 (MW = 581). Next, we checked the dosage effect of GN25 and found that p53 and p21 inductions were increased from 5 μm at 24 h (Figure 2d). This result

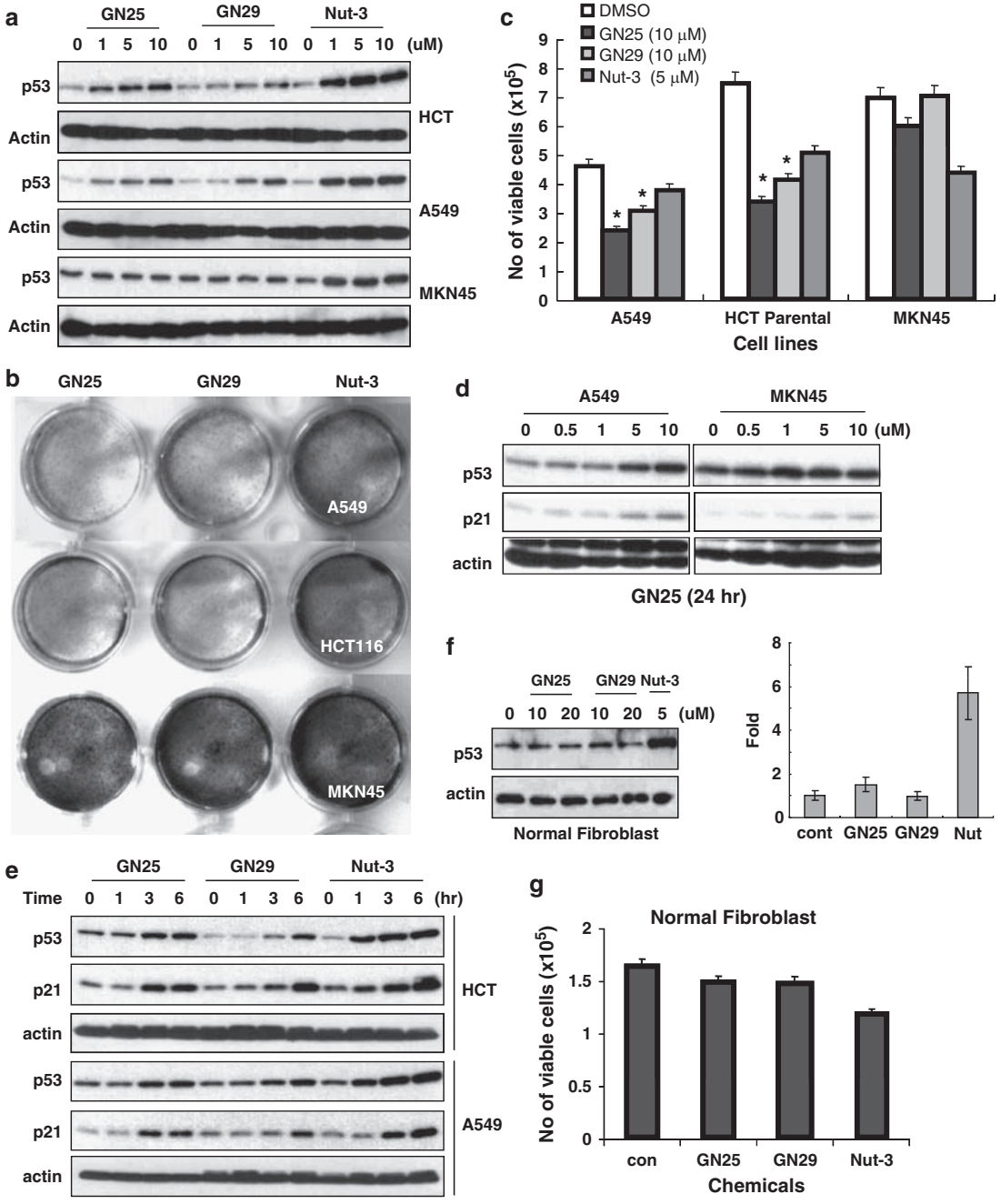


Figure 2 Induction of p53 by our chemicals in K-Ras-mutated cancer cell lines. (**a**) p53 expression is increased by GN25 and GN29 in K-Ras-mutated cell lines but not in MKN-45. Nut-3 was used for positive control for induction of p53. Chemicals were treated with indicated concentration for 6 h. (**b**) Different viability in response to chemicals. Each cell line was incubated with GN25 (10 μM), GN29 (10 μM) and Nut-3 (5 μM) for 24 h. After removing the floating cells, attached viable cells were fixed with 2% paraformaldehyde (PFA) and stained with TB. (**c**) Oncogenic K-Ras-dependent reduction of cell viability by GN25 and GN29. Cells were incubated with GN25, GN29 or Nut-3 for 24 h and its viability was determined by cell counting. Asterisk indicates statistical significance ($P<0.05$; Student's *t*-test). (**d**) Dose-dependent induction of p53 by GN25 is detected only in A549 but not in MKN45. Each cell line was incubated with indicated concentration of GN25 for 24 h and analyzed their expression of p53 and p21 through WB analysis. (**e**) Time-dependent induction of p53 and p21. HCT116 and A549 were incubated with 5 μM of GN chemicals or 2.5 μM of Nut-3 for indicated time. (**f**) GN chemicals do not induce p53 expression in normal fibroblast (GM 00038; 9-year-old female). Normal cells were incubated with indicated concentration of chemicals for 6 h. Comparing induction of p53 with Nut-3, GN chemicals did not induce p53 expression. Graph in right panel show the quantitative result from three repeated experiments. (**g**) GN25 and GN29 do not decrease cell viability in normal cell. Normal human fibroblast was treated with 10 μM of GN25 for 24 h and the viability was measured by cell counting.

indicated that GN25 and GN29 overcome the problem of B3, short working duration. To confirm this, we measured the time-dependent induction of p53 and p21 in response to GN25 and GN29 (Figure 2e and Supplementary Figure S5B). Induction of p53 by GN chemical was delayed than that by Nut-3. It resulted

from difference of working mechanisms that Nut-3 blocks the interaction between p53 and MDM2 (Vassilev *et al*., 2004), whereas GN chemicals block the interaction between Snail and p53.

Next, we examined the effect of GN25 and GN29 on normal fibroblasts. As expected, Nut-3 induced the p53 expression, whereas GN25 and GN29 did not induce the p53 expression significantly in normal cell (Figure 2f). Consistent with p53 expression pattern, GN chemicals did not suppress the proliferation of normal fibroblast (Figure 3g). We also checked the effect of GN25 under Snail knockdown condition to reveal that the portion of p53 induced by GN chemical is consistent with eliminated p53 by Snail. As consistent with previous literatures (Kajita *et al*., 2004; Lee *et al*., 2009b, c), si-Snail induced p53 expression in A549 (Supplementary Figure S5C). However, GN25 did not show the synergistic or additional effect on p53 expression under Snail knockdown condition (Supplementary Figure S5C). This result means that GN25-induced p53 expression may be achieved by blocking the Snail-p53 binding.

Specific inhibition of p53-Snail binding

To investigate the effect of GN25 and GN29 on p53-Snail binding, we performed the GST pull-down assay with cell extracts. Interaction between p53 and Snail was significantly reduced by GN25 and GN29 in assay with capan-1 extract (Figure 3a). However, we did not

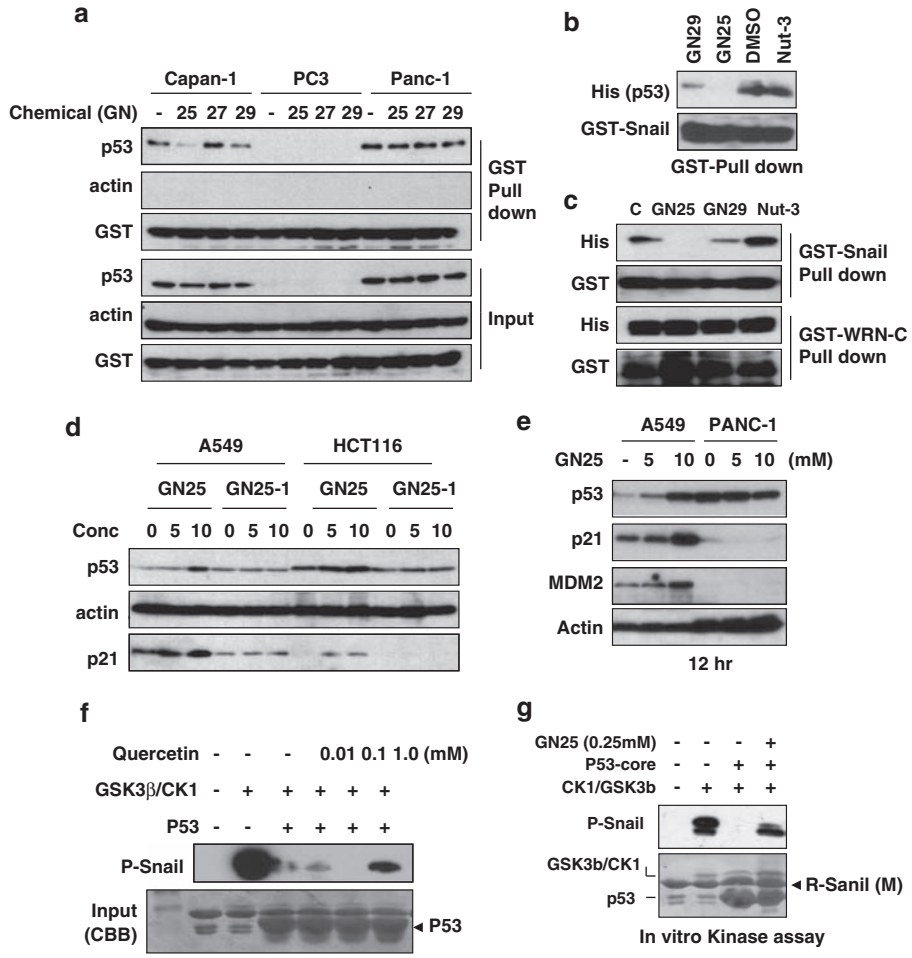


Figure 3 Specific effect of GN chemicals on p53-Snail binding. (**a**) GN25 and GN29 disrupt the interaction of Snail and p53. GST-Snail was incubated with cell lysates with or without GN chemicals (10 μM) for 2 h and associated p53 was determined by WB analysis. GN25 and GN29 did not block the interaction of Snail and p53 in Panc-1. PC3 was used for negative control of binding. To exclude the possibility of nonspecific binding, we used actin for negative (pull down) and positive (input) control. (**b**) *In vitro* GST pull down. His-p53 was incubated with GST-Snail under the presence of GN chemicals. (**c**) GN25 and GN29 do not disrupt the interaction between p53 and WRN-C terminal region. (**d**) Mother structures, conserved in GN25 and GN29, are essential for induction of p53. Nuclear-structure-modified chemical (GN25-1) did not induce p53 and p21 expression in A549 and HCT116. Cells were incubated with each chemical for 6 h. (**e**) Induction of p53 by GN25 is not detected in Panc-1, p53 mutant cell line. A549 and Panc-1 were incubated with GN25 for 12 h. p53 target genes were used for monitoring the activity of p53. (**f**, **g**) GN25 can recover the masking effect of p53 on CK1/GSK3b-mediated Snail phosphorylation. Addition of p53 protein blocked the phosphorylation of Snail, achieved by CK1/GSK3b. However, addition of GN25 (right) and quercetin (left), previously identified as inhibitor of p53-Snail binding, did recover the phosphorylation of Snail.

observe the reduction of the binding affinity between p53 and Snail using the extract of Panc-1 (Figure 3a), which harbors single mutant p53 (Butz *et al*., 2003). We also obtained the similar result from *in vitro* binding assay using recombinant proteins that GN25 and GN29 block the binding between recombinant wild-type p53 and Snail, but not between mutant p53 and Snail (Figure 3b and Supplementary Figure S5D). Next, we checked the effect of these chemicals on p53-WRN binding (Blander *et al*., 1999) as negative control and found that our chemicals did not influence other p53 binding partner (Figure 3c). To test the specificity of our chemical, we generated additional derivative, which possesses modified nuclear structure with the same side chain (GN25-1). This chemical did not induce p53 and p21 (Figure 3d), suggesting that conserved nuclear structure is essential for p53 induction. We also measured the p53 and p21 expression in Panc-1 (single mutated p53 allele) to determine whether induction of p21 is achieved by p53 and also examine the effect of GN25 on mutant p53. However, in this cell, we did not observe the induction of p53, MDM2 and p21 (Figure 3e). This result is consistent with our previous results that GN chemicals did not block the interaction between Snail and p53 in Panc-1 extract (Figure 3a), and between recombinant mutant p53 and Snail binding (Supplementary Figure S5D). To confirm the inhibition effect of GN chemical on p53-Snail binding, we designed following experiment; in previous, we have found that Snail middle region, which is also responsible for binding with p53 (Lee *et al*., 2009c), is phosphorylated by CK1/GSK3β (Cano *et al*., 2000; Yook *et al*., 2006). Thus, addition of recombinant p53 could block the CK1/GSK3β-mediated Snail phosphorylation, whereas p53 did not affect CK1/GSK3β-mediated β-catenin phosphorylation (Piao *et al*., 2008; Ha *et al*., 2004; Supplementary Figure S5E). However, addition of quercetin, specific inhibitor against p53-Snail binding (Lee *et al*., 2009a, c), or GN25 could recover the CK1/GSK3β-mediated Snail phosphorylation in the presence of p53 (Figures 3f and g). These results strongly suggest that GN chemical and quercetin are specific inhibitors against Snail-p53 binding. In addition, our result implies that molecular target of GN chemical may be p53 because GN chemical can discern the mutant p53 and did not block the phosphorylation of Snail.

GN chemicals can activate wild-type p53 in p53$^{WT/MT}$ cells

As we showed in previous result, GN25 and GN29 blocked only the interaction of wild-type p53-Snail binding but not the mutant p53-Snail binding (Figure 3a and Supplementary Figure S5D). We also found that Snail can eliminate mutant p53 (Lee *et al*., 2009b). Thus, we proposed the hypothesis that GN25 and GN29 can activate p53 pathway in p53-mutated cells, because GN chemicals protect only wild-type p53, whereas excess Snail would eliminate mutated p53 more effectively. In fact, GN25 and GN29, but not Nut-3, could suppress the cell viability in capan-1, despite of reduction of p53 expression (Figures 4a and b). TB staining also showed the anti-proliferation effect of GN chemicals (Figure 4c). However, Nut-3 did not suppress the proliferation (Figures 4b and c). We could also observe the induction of p21 in response to GN chemicals but not to adriamycin and Nut-3 in these cell lines (Figure 4d). This result indicates that GN chemicals can overcome the dominant-negative effect of mutant p53 in p53$^{WT/MT}$ status, and activate wild-type allele of p53. We also checked the p53 mRNA through reverse transcription–PCR. However, our chemicals did not alter the transcription of p53 (Figure 4e). Next, we measured the cell viability of capan-1, HCT116 and MKN45 cell lines in response to GN25. Interestingly, viability of capan-1 was more obviously reduced than that of HCT116 ($IC_{50} = 1.25$ vs 7 μM; Figure 4f). In contrast, MKN45 showed resistance to GN25 (Figure 4f). Our result suggests that GN25 and GN29 can work very effective in p53$^{WT/MT}$ cancers.

GN chemical activates wild-type p53 selectively

To confirm the effect of our chemicals under p53$^{WT/MT}$ status, we co-transfected wild-type p53 with p53-GFP in PC-3. Because GFP protein is fused with p53 C terminus, structure of p53-GFP would be different from wild-type p53. When GN25 or GN29 was treated in this cell, we could observe the expression of wild-type p53, which was suppressed by GFP-p53 (Figure 5a). Moreover, although both kinds of p53 were eliminated by oncogenic K-Ras, GN chemical could recover the expression of wild-type p53 but not of GFP-p53 (Figure 5a). However, Nut-3 did not recover the wild-type p53 (Figure 5a). We could also observe the induction of p21, consistent with recovering of wild-type p53. To confirm this, we reperformed the similar experiment using wild-type and p53 R1751H expression vectors. GN25 and GN29 could recover the function of wild-type p53 including induction of p21 and MDM2 (Figure 5b). We could also observe the induction of p21 and reduction of p53 in MDA-MB 468 (p53$^{WT/MT}$ cell line; Kumaravel and Bristow, 2005 by GN25 but not by Nut-3 (Supplementary Figure S5F). To get more evidences, we measured the expression of Snail in GN-treated condition. In p53$^{WT/WT}$ condition such as A549 (diagram of Figure 5c), GN chemicals might protect all of p53 molecules from Snail-mediated exocytosis. Thus, intracellular p53 and Snail expression would be increased (Figure 5c). Indeed, Snail and p53 expression were elevated in response to GN chemicals in A549 (Figure 5c). In contrast, GN chemicals did not induce p53 and Snail expression in p53$^{WT/MT}$ condition such as capan-1, because large amount of mutant p53 still associated with Snail and the complex was eliminated from cells (Figure 5d). Consistent with our hypothesis, in capan-1, Snail expression was not increased by treatment of GN25 or GN29 (Figure 5d). This result implies that mutant p53 may be eliminated by Snail despite of GN chemicals, because it cannot block the interaction between mutant p53 and Snail (Figure 5e). We also checked the effect of GN25 on other pancreatic

cancer cell line, MIA-Paca 2, which also possesses wild/mutant p53 alleles (Yan and Chen, 2009). In this cell line, GN25 suppressed the cell proliferation strongly (Supplementary Figure S5G).

In vivo *antitumoral effect*

To explore *in vivo* effect of GN chemicals, we then performed the xenograft experiment. Because GN29 is water insoluble and less effective than GN25

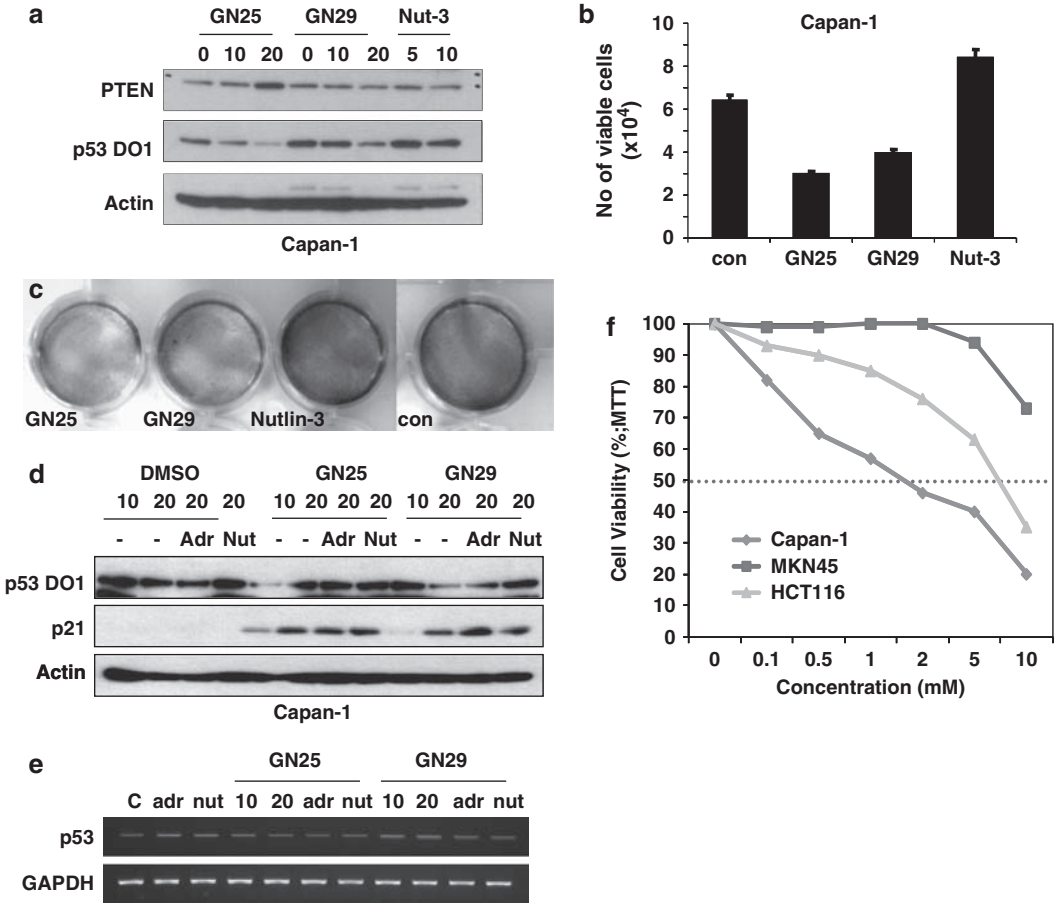
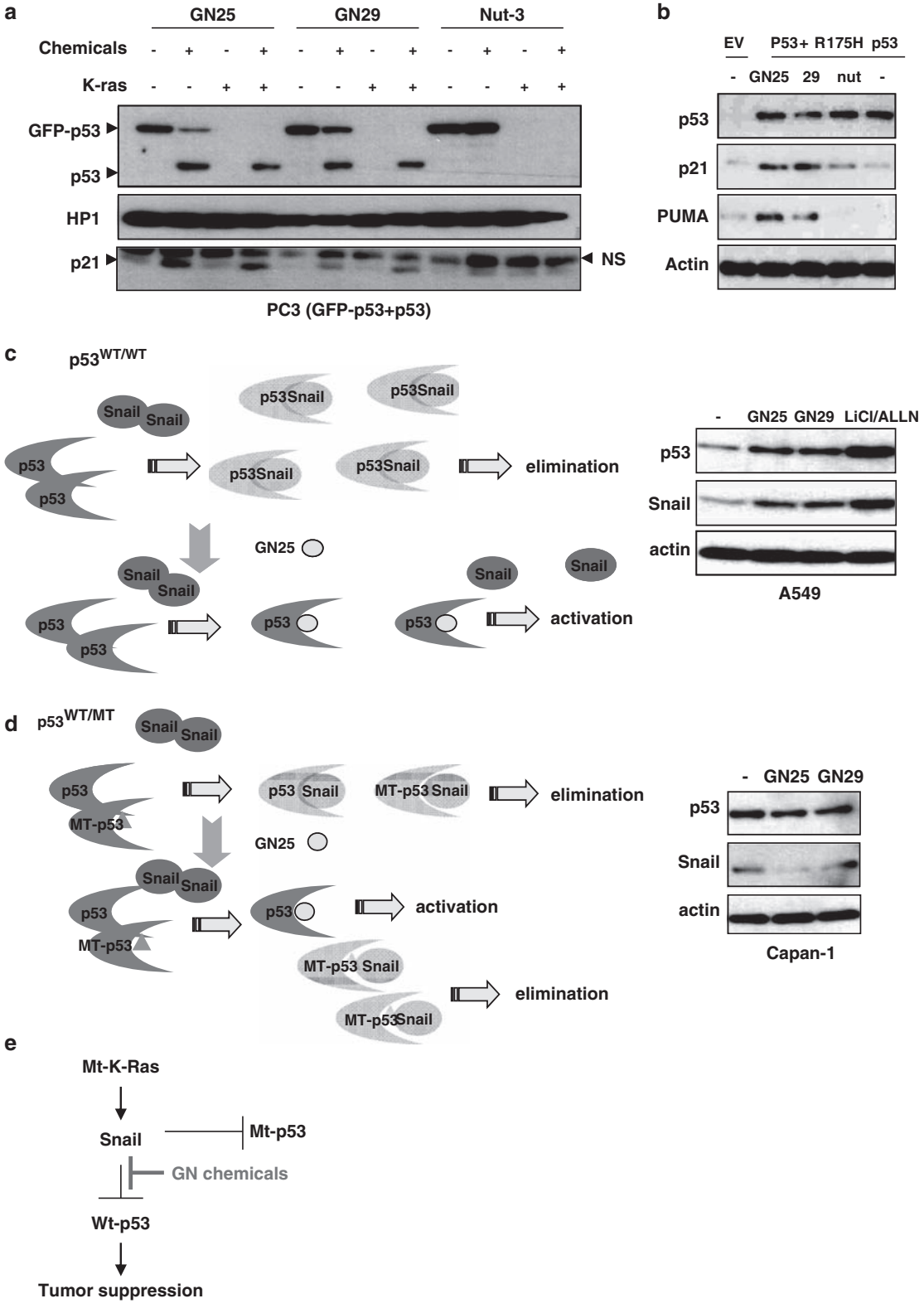


Figure 4 GN chemical can activate wild-type p53. (**a**) In capan-1 (K-Ras-mutated and $p53^{WT/MT}$ cell), GN25 and GN29 suppress p53 expression. However, PTEN, target of p53, is induced by GN25. Capan-1 was incubated with chemicals for 6 h. (**b**) GN25 and GN29 suppress cell viability in capan-1. Cell viability was determined by cell counting after TB staining after incubation with 10 μM of GN chemicals or 5 μM of Nut-3 for 24 h. (**c**) TB staining also shows the reduction of viability in response to GN25 and GN29. However, Nut-3 did not show the cytotoxic effect in this cell line. (**d**) GN25 and GN29 can induce expression of p21. p21 induction, which was not detected by treatment of Adr or Nut-3 alone, occurred by GN25 and GN29 in capan-1. (**e**) GN25 and GN29 cannot alter the transcript of p53. Reverse transcription (RT)–PCR analysis was performed with capan-1 total RNA after treatment of GN25 or GN29 for 6 h. (**f**) Effect of GN25 on cell viability of human cancer cell lines. Cells were incubated with indicated concentration of GN25 for 24 h. Viability was determined by MTT assay.

Figure 5 Specific activation of wild-type p53 in $p53^{WT/MT}$ condition. (**a**) GN25 and GN29 activate wild-type p53 under the presence of mutant p53. In PC-3 (p53 null), co-transfection of wild-type and GFP-fused impaired p53 reduced the expression of wild-type p53. But treatment of GN25 or GN29, but not of Nut-3, could induce wild-type p53 and suppress mutant p53 expression. Consistent with wild-type p53 expression, p21 expression was also induced. NS is nonspecific band. (**b**) GN25 and GN29 can activate wild-type p53 under the presence of mutant p53. PC-3 was co-transfected with wild-type and p53 R175H mutant for 24 h and treated with GN25 and GN29 (10 μM) or Nut-3 (5 μM) for 6 h. Induction of p21 and MDM2 was detected in GN25-treated sample. In fact, because PC-3 harbors mutant PTEN, which can elevate AKT activity and suppress GSK-3b, Snail expression is elevated than other cell lines (our unpublished data). So we can obtain the induction of p53 activity without transfection of oncogenic K-Ras. (**c**) Diagram. When cell contains wild-type p53 in both allele and mt-K-Ras (1), Snail will bind to and eliminate p53. In this condition, GN25 (also GN29) blocks all of Snail-p53 binding. Thus, in cell, p53 and Snail will be elevated. As protein half-life of Snail is very short, sometimes increase of Snail by GN25 is difficult to check. Consistent with the diagram, in A549, GN25 and GN29 can increase p53 as well as Snail expression. LiCl/ALLN was treated for detection of Snail. (**d**) In contrast, if cell contains wild type and mutant, Snail can eliminate both p53 regardless of mutant p53. In this condition, if GN25 is treated, this chemical will block the interaction of wild-type p53-Snail binding but not of mutant p53-Snail binding. Thus, wild-type p53 can be protected from Snail-mediated elimination, whereas mutant p53 will be diminished more effectively due to excess Snail. Thus in this condition, p53 and Snail will be reduced, whereas activity of p53 will be increased. In fact, in capan-1 ($p53^{WT/MT}$ cell), GN chemical did not induce p53 and Snail. However, GN chemical can activate p53 function (Figure 4). (**e**) Simple diagram for working mechanism of GN chemicals.

(Figure 2a), we used GN25 *in vivo* assay. First, we subcutaneously injected capan-1 into nude mice. But this cell line did not form the subcutaneous tumor in our experimental periods. Instead, we tested the toxicity of GN25 using these mice. As shown in Supplementary Figure S6A, our chemical did not induce the loss



of body weight, gross anatomical abnormality (Supplementary Figure S6B) and liver failure (Supplementary Figure S6C). So we next inoculated A549 cells through i.p. injection and monitored the survival and body weight of mice. Similar to previous experiment, injection of GN25 did not alter the rate of weight gain (Supplementary Figure S6A). However, at 10 weeks after A549 cell inoculation, a mouse was dead, but the corpse was too damaged to get reasons. Within 3 weeks, three more mice were dead in phosphate-buffered saline (PBS)-treated group. So we terminated our experiment at 10 weeks after injection of GN25. Survival curve about our i.p. experiment is shown in Figure 6a. Postmortem analysis revealed that dead mice possessed liver, lung or pancreatic tumors (Figure 6b). So we killed the remaining PBS-treated and GN-treated mice. Body weight of mice used in this study was monitored (Supplementary Figure S7). Histological analysis of the samples obtained from three live mice of PBS-treated group showed tumors (abdominal cavity (AC) and pancreas; Figure 6c). The average volume of tumors, detected by autopsy, was 1250 ($\pm$150) mm^3. In contrast, we did not find the tumors in GN-treated group (in both 10 and 20 mg/kg) through gross anatomical analysis. We also performed the histological analysis and found that 3 of 7 mice in 10 mg/kg GN-treated group and 2 of 7 mice in 20 mg/kg GN-treated group possessed the tumor in abdominal cavity (Figure 6c). However, their architectures were severely disrupted and tumors seemed to be regressed (Figure 6c and Supplementary Figure S8). We also analyzed the liver of mice in 10 or 20 mg/kg GN-treated group through basic histology and found that GN25 does not evoke significant toxicity in liver (Supplementary Figure S6C). We also examined the pancreas and kidney. However, we did not observe the severe defect in these organs (data not shown). Summarizing our i.p. experiment, we detected tumors in all of available PBS-treated control group (6/6; one mouse was dead), in 3of 7 mice of 10 mg/kg GN-treated group and 2 of 7 mice of 20 mg/kg GN-treated group (Figure 6d). Moreover, tumors in GN25-treated groups were obviously regressed (Supplementary Figure S8). Our results strongly indicate that GN25 can block the tumor progression and also induce tumor regression.

Discussion

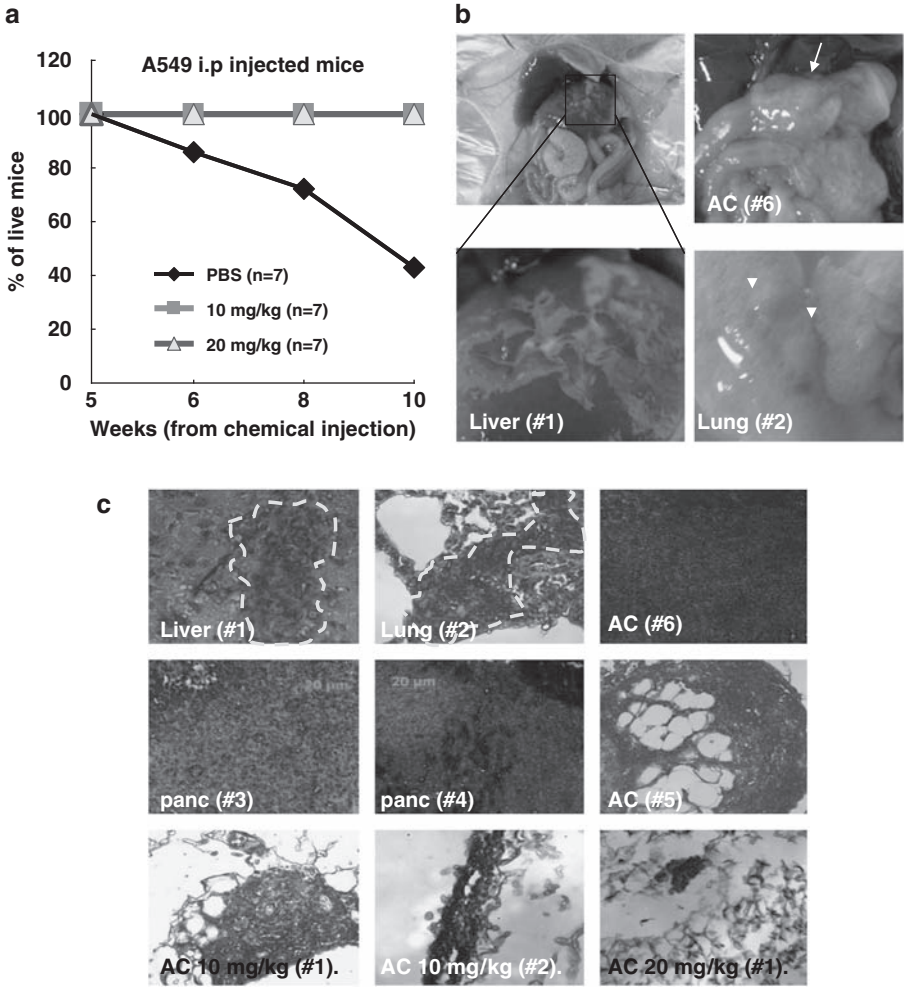
In our previous researches, we have suggested that K-Ras-induced Snail binds to and eliminates p53 from cell. Thus, blocking of p53-Snail binding or elimination of Snail though si-RNA can induce the p53 expression in K-Ras-mutated cancer cells. In this study, we have screened the specific chemical inhibitors against Snail-p53 binding and provide the evidence about antitumoral activity.

Although in previous research, we have revealed that three kinds of natural compounds can induce p53 and block the interaction of p53-Snail binding. However, their biological effects are very diverse and a high-dose treatment can induce unexpected effect. Thus, in this case, we have tried to get more specific inhibitors. As shown in Supplementary Figure S1, a group of chemicals can suppress the interaction of Snail-p53 binding. This result is very interesting and exciting because our library is positioned as according to their chemical structure. Thus, a region shows the similar activity, indicating that certain basic motif is responsible for biological effect. In fact, our GN chemicals share the common motif and disruption of this motif lead to loss of biological activity (Figure 3d and our unpublished data).

More exciting and valuable finding is that our chemicals can activate p53 in $p53^{WT/MT}$ cancer cells (Figure 4). Although genetic mutation of p53 is detected in about half of cancers, because of dominant-negative effect, it generally occurred in single allele (Levine, 1997; Weinberg, 2006). In other words, almost cancer cells possess at least single copy of wild-type p53. Thus, our chemical would be useful for more broad range of cancers. In fact, we could observe the induction of p21 and suppression of cell viability or proliferation in p53-mutated capan-1, MIA-Paca 2 and MDA-MB 468 (Figure 4 and Supplementary Figure S5F and G). Moreover, we can observe the reactivation of wild-type p53 under the presence of mutant p53 in co-transfection experiments (Figure 5).

Considering about binding motif of GN chemical on p53, we observed that GN25 does not disrupt the binding between mutant p53 and Snail (Figure 3a and Supplementary Figure S5D). These results imply that GN chemical would associate though DNA binding domain of p53. Because genetic mutations of p53 are frequently detected in DNA binding domain (mutational hot spot), our chemical may not bind to mutated p53. Instead, Snail-p53 binding seems to be achieved through broader region, so that Snail can associate with mutant p53. Based on these clues, we hypothesize that our chemical binds to DNA binding domain of p53 (perhaps by phenolic ring) and protects the association with Snail (perhaps by sulfur and side chain). However, in mutated p53, GN chemical binding region would be altered. However, to prove this, more accumulated evidence, in particular, structure-based study, should be collected. Indeed, we are preparing the investigation process for chemical-p53 co-complex.

One of unsolved or not fully shown feature is why PC-3 and MDA-MB 468 responded to GN chemicals despite of wild-type K-Ras. About this, very interesting clue is that these cell lines are PTEN deficient (Li *et al*., 1997). This fact suggests that our chemical would be also useful for anticancer drug in PTEN-mutated cancer because loss of PTEN can induce AKT activation, and GSK-3β suppression resulted in Snail expression. In fact, we observed the higher expression of Snail in PC-3 (data not shown). Because large portion of human cancers, in particular pancreatic and lung cancers (Downward, 1998; Deramaudt and Rustgi, 2005), are promoted by K-Ras mutation and only one copy of p53 is mutated, our chemical may be strong candidate against these kinds of cancers.



d

cancer location	Control(n=7)	10 mg/kg(n=7)	20 mg/kg(n=7)
liver	1 (death)	0	0
lung	1 (death)	0	0
pancreas	2 (1/2 death)	0	0
A.C	2 * A mouse expirerd	3 (regression)	2 (regression)

Figure 6 *In vivo* analysis of antitumoral effect of GN25. (**a**) Survival curve of A549 inoculated cells. 1×10^7 cells of A549 were inoculated in nude mouse through i.p. injection. After 4 weeks, PBS, 10 or 20 mg/kg of GN25 was injected through i.p. once a week. From 6 weeks from chemical injection, mice of PBS-treated group were continuously dead. So we terminated experiment at 10 weeks after first chemical injection and killed the remaining mice. In contrast, all of GN-treated group mice lived at the same period. (**b**) Postmortem analysis of mice in PBS-treated group. We could detect the infiltrated cancer cells in liver (#1) or lung (#2) or localized tumor in abdominal cavity (#6) through gross anatomical analysis. (**c**) Histological analysis of tumors. In six PBS-treated mice, tumor cells were detected in liver (#1; dashed line), lung (#2; dashed line), pancreas (#3 and #4) and abdominal cavity (#5 and #6). Because of delayed finding. The first dead mouse was not available for gross anatomical or histological analysis. In contrast, tumors of GN-treated group shrunk and cells did not form the tumor mass. It resulted from regression of tumor. More figures about tumor mass are provided in Supplementary Figure S8. We also examined the liver of GN-treated mice and did not find the severe defect. (**d**) Summary of tumor incidence and location. We could observe the tumors in available six PBS-treated mice. However, in GN-treated groups, 3 of 7 (10 mg/kg) or 2 of 7 (20 mg/kg) mice possessed the regressed tumors.

In summary, GN25 and GN29 are specific inhibitors of p53-Snail binding and induce p53 expression in K-Ras-dependent manner (Figure 5f). Moreover, our chemicals can activate wild-type p53 in $p53^{WT/MT}$ cancer cells. *In vivo* analysis, we could also obtain the evidence about strong antitumoral effect against K-Ras-mutated cancer cells. Our result would be useful for development of rational therapeutic drug against K-Ras-mutated or Snail-overexpressed cancers including pancreatic, lung and colon cancers.

Materials and methods

Cell lines and reagents
Cell lines used in this study were maintained in RPMI-1640 or Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum, 1% antibiotics) at 37 °C chambers, supplied with 5% CO_2. Transformed cells by oncogenic K-Ras or N-Ras were established by transfection with K-Ras (12 V) or N-Ras (12 V)/Myc into MEF cells, obtained from C57/BL6 mice. After 2-month selection by G418 (400 μg/ml), established cell lines were used for study. Normal human fibroblast (GM 00038; 9-year-old female) was obtained from the Coriell Cell Repositories (Coreill Cell, Camden, NJ, USA) and maintained in MEM medium (15% fetal bovine serum, without antibiotics). Antibodies used in this study, except for PUMA (Calbiochem, San Diego, CA, USA), were purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA). Other general chemicals used in this study were provided by Sigma (St Louis, MO, USA).

ELISA
To isolate Snail-p53 binding inhibitor, we generated ELISA system. We immobilized His-p53 (93-292) on 96-well plate using 0.5% paraformaldehyde. After drying and washing, we incubated GST-Snail with 0.1 mM of chemicals (final concentration). After 1 h incubation, 96-well plates were washed with TBST and incubated with anti-GST-Ab (1:10 000, 45 min) and anti-mouse-IgG-HRP (1:50 000, 30 min). After washing twice, plates were incubated with TMB solution (Calbiochem) and Stop solution (1 N H_2SO_4). Using the ELISA reader, we determined the value. More detail information about this ELISA system could be obtained from our previous literature (Lee *et al*., 2009c).

Measurement of cell viability and proliferation
To examine the cell viability, we performed the Trypan blue staining, cell counting and MTT assay. For the measurement of overall cell proliferation, we washed the floating cells and fixed with 1% paraformaldehyde for 20 min. After washing with PBS twice, we stained cells with 0.05% Trypan blue and visualized them by washing with PBS. For cell counting, we collected cells with medium and stained with Trypan blue for 10 min at room temperature. Using hemocytometer, we determined the survival cells. For MTT assay, we incubated cells with 0.5 mg/ml of MTT solution for 4 h at 37 °C. After removing excess solution, we dissolved the precipitated materials in 200 μl dimethyl sulfoxide and quantified by measuring absorbance at 540 nm.

WB analysis and GST pull-down assay
For monitoring the expression of proteins and *in vitro* biding, we performed the general WB procedure and GST pull-down assay (Lee *et al*., 2009c).

In vitro *kinase assay*
To address the effect of our chemical on p53-Snail binding, we first checked the phosphorylation of Snail by CK1/GSK3b. To phosphorylate recombinant Snail proteins, we incubated 15 μg of each GST-fused Snail fragments (91–112) for 4 h at 37 °C with 0.2 μg of recombinant GSK3β and 0.2 μg of recombinant CK1ε kinase domain in 25 μl of 50 mM Tris (pH 8.0) buffer containing 1 mM ATP, 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. Phosphorylated Snail was detected by WB analysis with anti-phospho-β-catenin (S33/37/T41) antibody (Cell Signaling, Danver, MA, USA), which can recognize p-Snail Ser96 as well as p-β-catenin. Under the same condition, we added indicated concentration of quercetin or GN25 and measured the p-Snail with or without recombinant p53.

In vivo *analysis*
Athymic mice were purchased from Lab Animal Co. Ltd (Seoul, Korea) and maintained under temperature- and light-controlled conditions (20–23 °C, 12 h/12 h light/dark cycle) and provided autoclaved food and water *ad libitum*. After adaptation for 2 weeks, athymic mice ($n = 21$) were inoculated with 1×10^7 cells of A549 per mouse by i.p. injection (A549). After 4 weeks for adaptation, each group was divided into three subgroups and PBS, 10 or 20 mg/kg of GN25 was administrated intraperitoneally once a week for 10 weeks. Animal experiment was performed after obtaining approval from and following guidelines of the institute of animal care committee of Pusan National University. Histological tissue analysis was performed through basic procedure (Park *et al*., 2005).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)