# Therapeutic targeting of nuclear receptor corepressor misfolding in acute promyelocytic leukemia cells with genistein

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## Abstract

**We have recently reported that PML-RAR–induced misfolding of the N-CoR protein could be reversed by retinoic acid (RA), a therapeutic agent that promotes differentiation of acute promyelocytic leukemia (APL) cells. This finding suggests a role of misfolded N-CoR in the differentiation arrest of APL cells and highlights its significance as a potential molecular target in protein conformation–based therapy for APL. Based on this hypothesis, we investigated the therapeutic potential of several protein conformation modifiers on APL-derived cell lines NB4 and NB4-R1. Through a small-scale screening of these selected compounds, we identified genistein as a potent inhibitor of growth of both RA-sensitive and RA-resistant APL cells. Genistein inhibited the growth of NB4 cells through its collective regulatory effects on cell cycle progression, differentiation, and apoptosis. Genistein-induced apoptosis of NB4 cells was mediated by activation of caspase-9 and caspase-3 and was associated with a decrease in mitochondrial transmembrane potential and cytosolic release of cytochrome *c*. Genistein promoted differentiation of both RA-sensitive and RA-resistant NB4 cells and induced cell cycle arrest by blocking the $G_2$-M transition. Genistein up-regulated the expression of PML and N-CoR proteins, promoted degradation of PML-RAR, and reorganized the microspeckled distribution of PML oncogenic domains to a normal dot-like pattern in NB4 cells. Moreover, genistein significantly reversed the PML-RAR–induced misfolding of N-CoR protein by possibly inhibiting the selective phosphorylation-dependent binding of N-CoR to PML-RAR. These findings identify genistein as a potent modifier of N-CoR protein conformation and highlights its therapeutic potential in both RA-sensitive and RA-resistant APL cells. [Mol Cancer Ther 2007;6(8):2240–8]**

## Introduction

Acute promyelocytic leukemia (APL) is caused by PML-RAR, a fusion protein resulting from a unique translocation involving the *PML* gene located on chromosome 15 and the *RAR* gene located on chromosome 17 (1, 2). The growth suppressive function of PML is linked to its localization to the nuclear dot-like structures known as PML oncogenic domains (POD). The normal dot-like distribution of PML is converted to a predominantly microspeckled-like distribution pattern in APL cells, and retinoic acid (RA)-induced differentiation of APL cells is always preceded by reorganization of PODs to a dot-like pattern. Although RA effectively promotes clinical remissions in ~90% of APL patients, such remission, however, is usually short-lived and is always followed by complications, such as RA resistance and relapse of the disease (3–5). It is widely believed that N-CoR recruited by PML-RAR contributes to the differentiation arrest of APL cells through actively repressing the RA responsive genes essential for the maturation of promyelocytic cells, and that dissociation of N-CoR from PML-RAR triggered by RA relieves this repression, ultimately leading to activation of RA responsive genes and differentiation of APL cells (6–9). However, recent findings from our laboratory suggest an unexpected role of N-CoR protein in APL pathogenesis. We found that PML-RAR–induced accumulation of misfolded N-CoR in the endoplasmic reticulum (ER) triggers ER stress and unfolded protein response, which ultimately leads to the induction of APL cell-specific proteases that cleave N-CoR protein (10–12). We have also shown that RA, at a concentration which usually promotes differentiation of APL cells, significantly inhibits the misfolding of N-CoR protein (11).

It has been shown that RA-induced disassociation of N-CoR from the RAR moiety of PML-RAR is the key event that defines the RA sensitivity of APL cells, and that impaired dissociation of N-CoR from the mutant PML-RAR or PLZF-RAR contributes to RA resistance (6–8). Interestingly, our finding suggested that misfolding of N-CoR protein depends on the nature of its association with

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**Note:** D.S. Nin and J.H. Fong contributed equally to this work.

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PML-RAR protein as well. Through mutational analysis of PML-RAR, we showed that simultaneous association of N-CoR with both the coiled coil and RAR domains of PML-RAR is required to induce misfolding in N-CoR protein (11). Together, these findings suggest a possible link between N-CoR misfolding and differentiation arrest of APL cells and highlight the fact that targeting the molecular mechanism underlying N-CoR misfolding could represent an attractive therapeutic strategy for APL. Therefore, in an attempt to identify agents capable of arresting the growth of APL cells through a mechanism which would involve inhibition of N-CoR misfolding, we tested several compounds known for their inhibitory effect on the process that promotes protein misfolding or ER stress. Of the several compounds tested, we identified genistein, an isoflavonoid from soy protein, as a potent inhibitor of growth of both RA-sensitive and RA-resistant APL cell types. Besides being an inhibitor of receptor tyrosine kinase activity, genistein is also known to be a potent inhibitor of ER stress in mammalian cells (13, 14). Recently, genistein was reported to be a potent inhibitor of transthyretin misfolding, a protein involved in the pathogenesis of transthyretin amyloidosis (15). In agreement with these findings, here we report that genistein inhibits PML-RAR–induced misfolding and insoluble aggregation of N-CoR protein and promotes growth arrest in both RA-sensitive and RA-resistant APL cells through a collective regulatory effect on cell cycle progression, apoptosis, and differentiation.

## Materials and Methods

### Cell Culture and Reagents

The RA-sensitive APL cell line NB4 and its RA resistant variant NB4-R1 were generous gifts from Dr. Homma (Japan) and Dr. Lanotte (France), respectively. Genistein and daidzein were purchased from Sigma (St. Louis) and dissolved in DMSO. RA was purchased from Sigma and dissolved in 100% ethanol. The antibodies caspase-3 (antibodies recognizing the precursor or the processed form) and caspase-9 (same antibody recognizing both the precursor and the processed forms) were purchased from PharMingen. The N-CoR (C-20), PML, and RAR antibodies were purchased from Santa Cruz Biotechnology and were used as described previously (10–12).

### Morphologic Analysis by Wright-Giemsa Staining

Cell morphology of either genistein or vehicle-treated NB4 or NB4-R1 cells was evaluated by Wright-Giemsa staining of the cells on cytospun slide preparations, and cell viability was assessed by trypan blue dye exclusion. The morphologic features that were considered consistent with apoptosis are cell shrinkage, nuclear condensation, nuclear fragmentation, and formation of apoptotic bodies.

### Assay for Cellular Differentiation

NB4 or NB4-R1 cells were grown separately with various concentrations of genistein (6.25–50 μmol/L), RA (1 μmol/L), or vehicle in RPMI medium supplemented with 10% fetal bovine serum. After 72 h, cells were collected, washed twice with PBS + 0.5% bovine serum albumin, and incubated for 60 min on ice in 500 μL of PBS + 0.5% bovine serum albumin with PE-conjugated monoclonal mouse anti-human CD11b or CD14 antibodies and vitamin D3 or control IgG (DAKO). Antibody-conjugated cells were then washed with PBS + 0.5% bovine serum albumin and analyzed by fluorescence-activated cell sorting (NUMI core facility, National University of Singapore).

### Immunofluorescence Staining

NB4 cells smeared onto glass slides through cytospan centrifugation were fixed with 100% cold methanol or 4% paraformaldehyde. The smeared cells were then stained with anti-PML or anti–N-CoR antibodies (Santa Cruz) and FITC or rhodamine-conjugated secondary antibodies. DNA was stained with 4′,6-diamidino-2-phenylindole (Invitrogen-Molecular Probe). Florescence signals were captured with confocal microscopy.

### Limited Proteinase K Digestion of N-CoR Protein

293T cells were transfected with Flag-tagged N-CoR and empty vector or HA-tagged PML-RARα plasmid using the Fugene transfection reagent. After 8 h of incubation, transfected cells were treated with vehicle or genistein (50 μmol/L) for an additional 40 h. Harvested cells were sonicated in Tris-lysis buffer [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, 0.5% deoxycholate], and aliquots of extract were incubated with proteinase K for 30 min on ice. The degradation products were resolved by SDS-PAGE, followed by Western blotting with anti-Flag antibody and Coomassie blue staining.

### Coimmunoprecipitation and Protein Solubility Assays

293T cells cultured in 10-cm discs were transfected with Flag-tagged N-CoR and HA-tagged PML-RAR using the Fugene transfection reagent. After 24 h of incubation, transfected cells were treated with DMSO or genistein for 0, 6, 12, or 24 h and cytosolic extracts were prepared in hypotonic buffer [20 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 10 mmol/L NaCl, 0.1% NP40, 1 mmol/L NaF, 1 mmol/L $Na_3VO_4$, 20 mmol/L β-glycerolphosphate, 1.5 mg/mL IAA, protease inhibitor cocktail]. PML-RAR was immunoprecipitated from cytosolic extracts with anti-HA antibody, and amount of coprecipitated N-CoR was determined in Western blotting assay with anti-Flag antibody. For the detection of PML-RAR phosphorylation, 293T cells transfected with HA-tagged PML-RAR and treated with vehicle or genistein were lysed by sonication in buffer [500 mmol/L of NaCl, 50 mmol/L of Tris-HCl (pH 8), 0.5% NP40, 0.5% SDS, 0.5% deoxycholate, 1 mmol/L EDTA, 0.8 mmol/L of phenylmethylsulfonyl fluoride, 1 mmol/L DTT, 1 mmol/L NaF, 1 mmol/L $Na_3VO_4$, 20 mmol/L β-glycerolphosphate, 1.5 mg/mL of IAA, and complete protease inhibitors cocktail]. PML-RAR was then immunoprecipitated with anti-HA antibody and probed with anti–phospho-Ser/Thr antibody MPM2 (Upstate) and anti-phosphotyrosine antibody PY20 (BD Bioscience). N-CoR solubility assay was done as described previously (11).

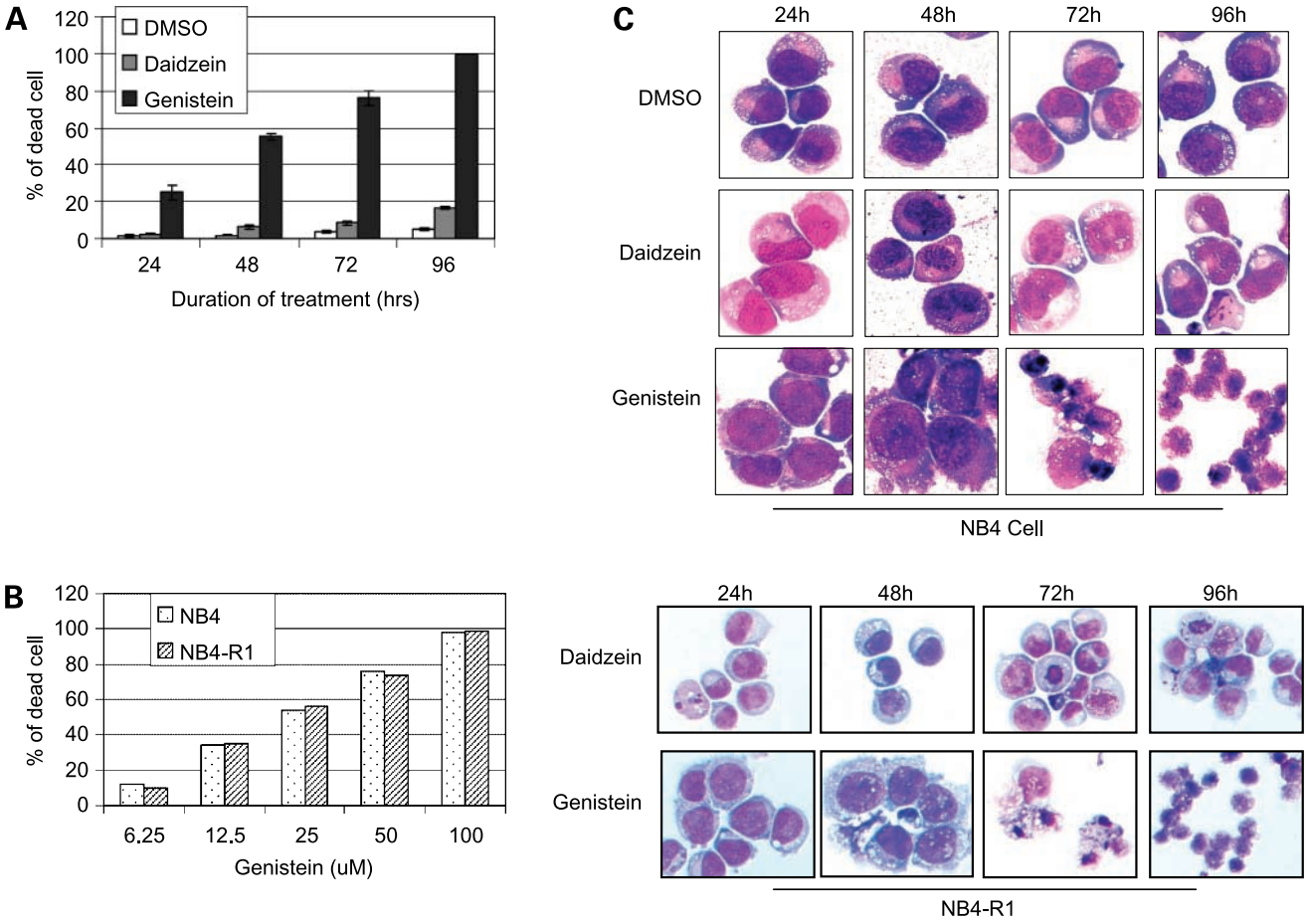


**Figure 1.** Genistein inhibits the growth of RA-sensitive (NB4) and RA-resistance (NB4-R1) APL cells. **A,** selective cytocidal effect of genistein on NB4 cells. NB4 cells were treated with vehicle, genistein (50 μmol/L) or daidzein (50 μmol/L) for a total duration of 72 h. Cell viability was assessed by trypan blue dye exclusion. *Columns,* mean of at least three different experiments; *bars,* SD. **B,** to assess the dose-dependent effect of genistein, NB4 or NB4-R1 cells were treated with various concentration of genistein (6.25 – 100 μmol/L) for 72 h, and cell viability was assayed by trypan blue dye exclusion. **C,** morphologic changes characteristic of apoptosis in genistein-treated NB4 and NB4-R1 cells. NB4 or NB4-R1 cells treated with vehicle, 50 μmol/L daidzain or 50 μmol/L genistein, for the duration (hr) as mentioned were spun on the slides and stained with Wright-Giemsa.

## Results

### Genistein Promotes Apoptosis of RA-Sensitive and RA-Resistant APL Cells

To test the therapeutic potential of genistein on APL cells, we analyzed its effect on the growth and viability of the following two types of APL-derived cell lines: the NB4 cells which are sensitive to RA and its RA-resistant variant NB4-R1 (16–18). NB4 and NB4-R1 cells were cultured with genistein or daidzein, an analogue of genistein which lacks the anti-tyrosine kinase activity found in genistein (19), and the viability of cells was determined by trypan blue dye exclusion and morphologic analysis by Wright-Giemsa staining. Genistein significantly increased the number of trypan blue–positive (dead) cells in a time-dependent manner, whereas daidzein had minimal effects at the same concentration (Fig. 1A). Next, we tested the dose-dependent effect of genistein on the growth and viability of NB4 and NB4-R1 cells. Cells were treated with genistein at concentrations ranging from 6.25 μmol/L to 100 μmol/L for 72 h. Genistein treatment, as expected, increased the number of trypan blue–positive cells in a dose-dependent manner in both types of cells (Fig. 1B). In addition, several well-characterized morphologic features suggestive of apoptosis, such as condensed chromatin, fragmented nuclei, and apoptotic bodies, were visible in genistein-treated cells when morphology of these cells was analyzed by Giemsa staining (Fig. 1C). The proapoptotic effect of genistein on APL cells was further confirmed by a DNA fragmentation assay (Supplementary Fig. S1)[6] and Annexin V assay (Supplementary Fig. S2).[6] When NB4 or NB4-R1 cells were exposed to 50 μmol/L genistein, a time-dependent increase in the number of Annexin V–positive

[6] Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

cells was observed (Supplementary Fig. S2A).[6] Similarly, a dose-dependent increase in the number of Annexin V–positive cells was observed when NB4 cells were treated with genistein in concentration ranging from 6.25 to 100 μmol/L (Supplementary Fig. S2B).[6]

### Genistein-Induced Apoptosis of APL Cells Is Mediated by a Loss of Mitochondrial Transmembrane Potential and Activation of Caspase-9 and Caspase-3

Selective activation of certain caspases, especially caspase-3 and caspase-9, are observed in cells undergoing apoptotic changes after drug treatment (20, 21). The caspase cascade in drug-induced apoptosis is initiated with the cytosolic release of cytochrome *c* and the sequential activation of caspase-9 and caspase-3 through proteolytic processing. In an apoptotic cell, the 48-kDa precursor form of caspase-9 is processed to a 35-kDa activated form. This activated caspase-9 then cleaves procaspase-3 to multiple smaller activated forms. We examined the effect of genistein on the activation of these two caspases in NB4 cells. When the status of caspase-9 in untreated NB4 cells was analyzed, a ~35 kDa fragment, along with 48-kDa precursor form of caspase-9, was detected (Supplementary Fig. S3A).[6] This apparent *de novo* processing of caspase-9 in NB4 cells seems to be specific to APL cells, as no processing was observed in several non-APL cell lines that were tested as controls (data not shown). When NB4 cells were treated with genistein, relative amounts of ~35-kDa fragment of caspase-9 was significantly increased (Supplementary Fig. S3A).[6] However, treatment of NB4 cells with 1 μmol/L RA, the dose usually required for the differentiation of NB4 cell, abrogated the processing of caspase-9 (Supplementary Fig. S3A).[6] In contrast to caspase-9, the processing of caspase-3 to the activated forms was observed only after 48 h of treatment with genistein (Supplementary Fig. S3B).[6] In apoptotic cells, alteration of mitochondrial transmembrane potential correlates with the cytosolic release of cytochrome *c* and the activation of caspase cascade. Therefore, we tested the effect of genistein on the mitochondrial

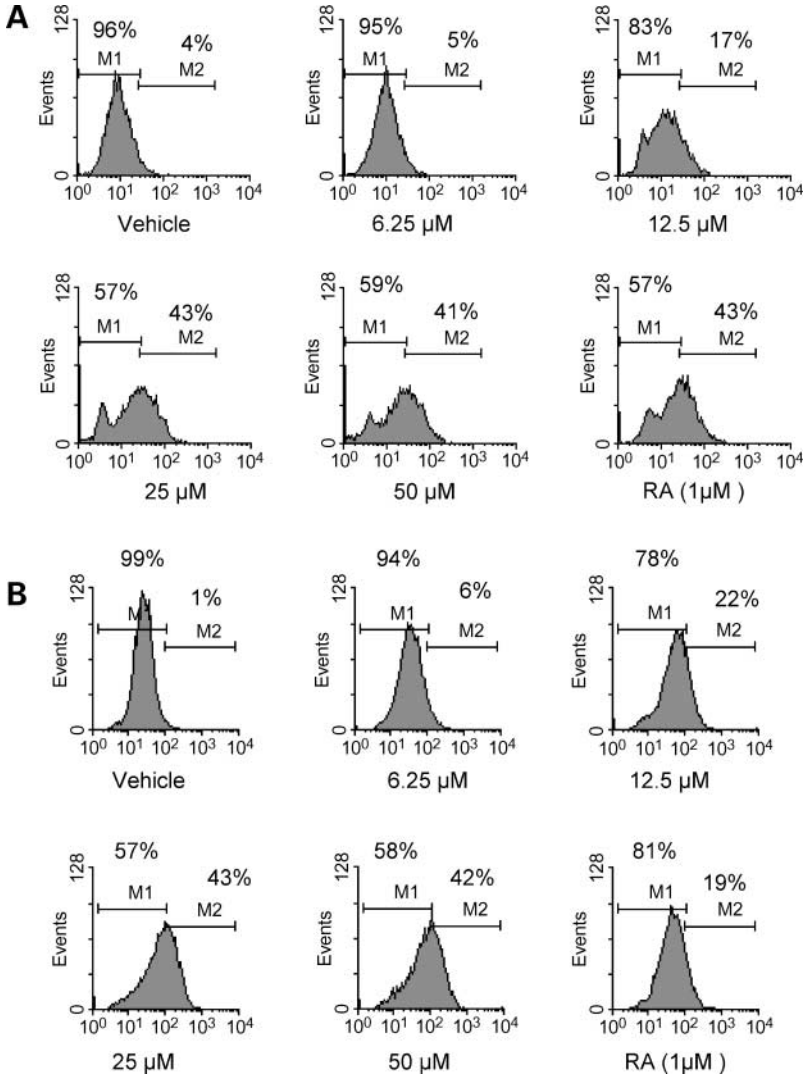


**Figure 2.** Genistein promotes differentiation of RA-sensitive and RA-resistant NB4 cells. NB4 (**A**) or NB-R1 (**B**) cells treated with vehicle, genistein (6.25 – 50 μmol/L) or RA (1 μmol/L) for a total duration of 72 h, were stained with PE-conjugated anti-CD11b antibody and analyzed by flow cytometry. M2 reflects the percentage of differentiated cells that were CD11b-positive. A dose-dependent increase in the number of CD11b positive cells was observed in both types of cell population after genistein treatment.

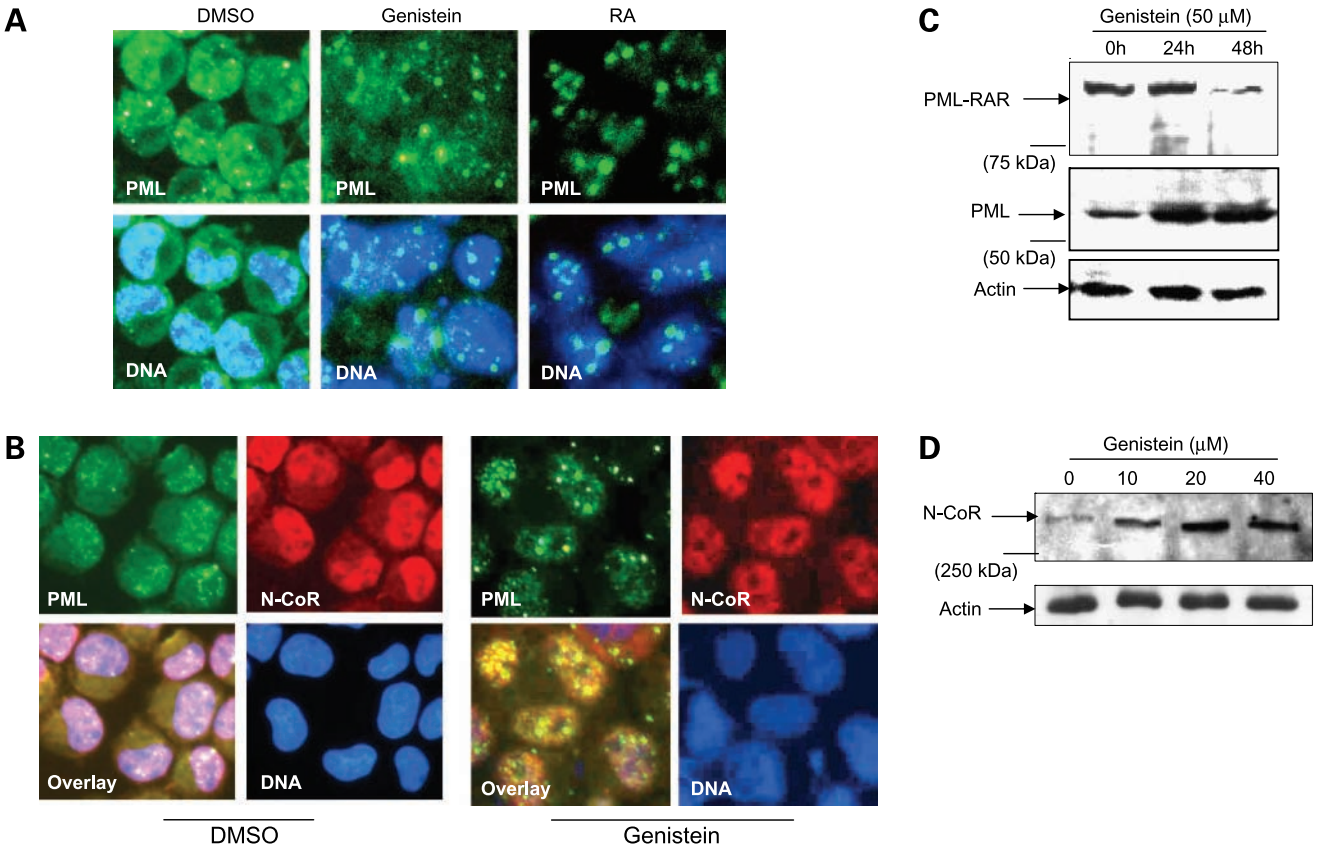


**Figure 3.** Genistein promotes reorganization of PODs to a dot-like pattern. **A,** genistein converts microspeckled-like distribution of PODs to dot-like patterns. NB4 cells treated with vehicle, 25 μmol/L genistein for 48 h or 1 μmol/L RA for 72 h, were stained with an anti-PML and secondary mouse FITC-conjugated antibodies. DNA was stained with 4′,6-diamidino-2-phenylindole. **B,** genistein promotes partial colocalization of PML and N-CoR in PODs. NB4 cells treated with DMSO or 25 μmol/L genistein for 48 h were stained with anti-PML (*green*) and anti–N-CoR (*red*) antibodies and florescence label secondary antibodies. **C,** genistein up-regulates PML while promoting degradation of PML-RAR in NB4 cells. Whole-cell extracts of NB4 cells treated with 50 μmol/L genistein for the duration as mentioned were resolved and stained with anti-PML or anti-RAR antibodies. **D,** genistein up-regulates N-CoR protein expression in NB4 cells. NB4 cells were treated for 48 h with various concentration of genistein as mentioned and N-CoR protein level in whole-cell extract was measure in Western blotting assay using anti–N-CoR antibody.

transmembrane potential and the cytosolic release of cytochrome *c* in NB4 cells. We used rhodamine 123, a lipophilic fluorochrome, to measure the effect of genistein on the mitochondrial transmembrane potential. Genistein activated the release of cytochrome *c* from the mitochondria to the cytosol within 24 h of treatment (Supplementary Figs. S3C and D)[6] while also lowering the mitochondrial transmembrane potential of NB4 cell in dose and time-dependent manners (Supplementary Figs. S3E and F).[6]

### Genistein Promotes Cell Cycle Arrest and Differentiation of RA-Sensitive and RA-Resistant NB4 Cells

Next we investigated the effect of genistein on the cell cycle progression of NB4 cells. NB4 cells were treated with vehicle or genistein at various concentrations for a total duration of 24 h and were subjected to DNA content analysis. Genistein, at concentrations ranging from 25 μmol/L to 50 μmol/L, increased the number of cells arrested at the $G_2$-M phase of cell cycle (Supplementary Fig. S4).[6] Concomitantly, it reduced the number of cells in the $G_1$ phase but did not affect the cell number of cells in the S phase. It has been shown that arsenic trioxide induces apoptosis of APL cells when used at relatively higher doses, whereas lower doses promote differentiation (22, 23). Moreover, arsenic trioxide was found to induce differentiation of both RA-sensitive and RA-resistant APL cells (24, 25). To test whether genistein possesses a similar differentiation-inducing capacity as observed with arsenic trioxide, we investigated the effect of genistein on cell surface expression of two well-characterized myeloid maturation markers, CD11b and CD14. The percentage of CD11b-positive NB4 or NB4-R1 cells was increased in a dose-dependent manner when these cells were treated with genistein at concentrations ranging from 12.5 to 50 μmol/L for a total duration of 72 h (Fig. 2A and B). The maximum effect (43%) was observed when genistein was used at a concentration of 25 μmol/L. The differentiation-inducing capacity of genistein was comparable with that of RA in RA-sensitive NB4 cells (Fig. 2A). Genistein did not exhibit any demonstrable effect on CD14 expression (data not shown).

### Genistein Promotes Reorganization of PODs to Normal Dot-Like Pattern

Among the multiple proteins closely associated with PODs, PML is regarded as the principal structural and functional component of PODs. N-CoR, which is not a bona fide component of PODs, exhibited a significant level of colocalization with PML in PODs of RA-treated NB4 cells (11). Although the real significance of this colocalization in the differentiation of NB4 cells is not known, it is tempting to speculate that a natively folded N-CoR localized in the PODs might be essential for some of the growth suppressive function of PML. Therefore, in an attempt to investigate whether genistein-induced differentiation of NB4 cells involves the reorganization of PODs to a dot-like pattern as observed with RA, we analyzed the subcellular distribution of PML and N-CoR proteins in genistein-treated NB4 cells. The diffused microspeckled-like distribution of PML observed in NB4 cells (Fig. 3A, DMSO panels) was converted to a more centralized dot-like distribution after genistein treatment (Fig. 3A, genistein panels). The effect of genistein on reorganization of PODs was very similar to that of RA (Fig. 3A, RA panels). Similarly, the diffused nucleocytoplasmic distribution of N-CoR (Fig. 3B, DMSO panels) was changed to a predominantly nuclear distribution pattern and exhibited significant colocalization with PML after genistein treatment (Fig. 3B, genistein panels). To obtain further insights into the mechanism of genistein-induced reorganization of PODs, we analyzed the effects of genistein on the overall expression of PML, N-CoR, and PML-RAR proteins in NB4 cells. As shown in Fig. 3C, genistein promoted degradation of PML-RAR while up-regulating the expression of PML. As observed with PML, expression of N-CoR was also significantly up-regulated in NB4 cells treated with genistein (Fig. 3D). PML is involved in both caspase-dependent, as well as caspase-independent, apoptosis pathways (26–28). Up-regulation of PML by genistein suggests that its proapoptotic effect might be mediated through the activation of PML-dependent apoptosis pathways.

### Genistein Inhibits PML-RAR–induced Misfolding of N-CoR Protein

To delineate the molecular mechanism underlying genistein-induced inhibition of N-CoR misfolding, we first analyzed the effect of genistein on the conformation of N-CoR protein through limited protease digestion and detergent solubility assays. Natively folded N-CoR (N-CoR expressed with empty vector in 293T cells) was sensitive to proteinase K (Fig. 4A, *lanes 1–3*), whereas misfolded N-CoR (N-CoR coexpressed with PML-RAR in 293T cells) exhibited resistance to proteinase K digestion (Fig. 4A, *lanes 4–6*). When 293T cells expressing misfolded N-CoR were treated with genistein, their sensitivity to proteinase K was significantly restored (Fig. 4A, *lanes 7–9*). Moreover, natively folded N-CoR was predominantly soluble in detergent (Fig. 4B, *top, lane 2*), whereas misfolded N-CoR was largely insoluble (Fig. 4B, *top, lane 3*). However, when 293T cells expressing insoluble N-CoR were treated with genistein, solubility of N-CoR protein was significantly improved (Fig. 4B, *top, lane 6*). Under the same conditions, the relative solubility or insolubility of b-actin was not affected by genistein (Fig. 4B, *bottom*). Similarly, genistein significantly enhanced the solubility of endogenous N-CoR protein of NB4 cells and inhibited its cleavage (Fig. 4C).

### Genistein Inhibits the Binding of N-CoR to PML-RAR and Abrogates Serine/Threonine Phosphorylation of PML-RAR

We have shown that aberrant association of N-CoR with PML-RAR in the cytosol contributed to the misfolding of N-CoR protein (11, 12). Therefore, we investigated whether genistein-induced inhibition of N-CoR misfolding results from a possible inhibition of N-CoR binding to PML-RAR

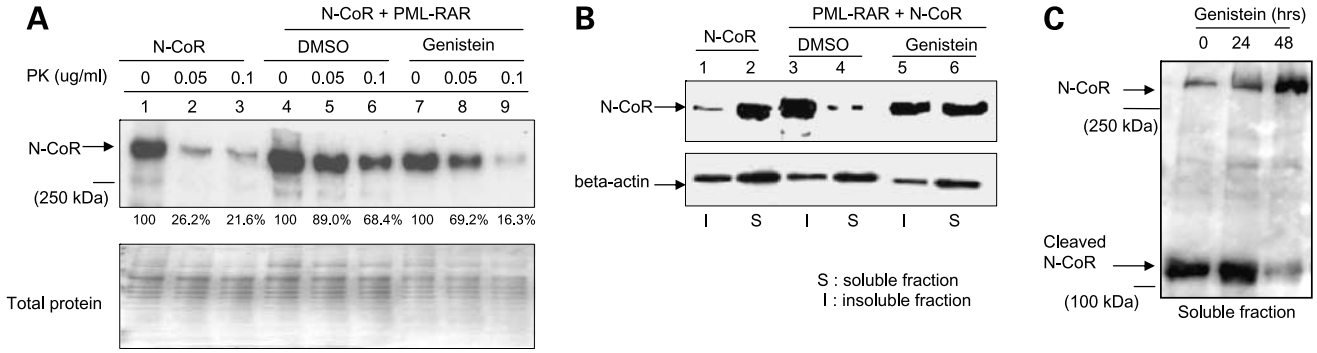


**Figure 4.** Genistein inhibits misfolding of N-CoR protein. **A,** genistein restores protease sensitivity of N-CoR protein. Extracts of 293T cells transfected with Flag-tagged N-CoR alone or with HA-tagged PML-RAR and treated with DMSO or genistein (50 μmol/L) were subjected to limited protease digestion with proteinase K (*PK*). After digestion, proteinase K sensitivity of N-CoR was determined by Western blotting assay using anti-Flag antibody (*top*). *Numbers,* remaining N-CoR protein as percent of respective input (untreated) N-CoR protein. As a loading control, total protein in each lane was resolved by SDS-PAGE and stained with coomassie blue (*bottom*). **B,** genistein inhibits PML-RAR–induced insolubility of N-CoR protein. 293T cells transfected with Flag-tagged N-CoR alone or with HA-tagged PML-RAR were treated with DMSO or genistein as indicated. Transfected cells were passively lysed and the soluble (*S*) and insoluble (*I*) fractions were separated by high-speed centrifugation. Levels of N-CoR and b-actin proteins in each fraction was determined in Western blotting assay using anti-Flag and anti–b-actin antibodies, respectively. **C,** genistein enhances the solubility of N-CoR protein of NB4 cells. NB4 cells untreated or treated with genistein (50 μmol/L) were passively lysed, and the N-CoR protein level in soluble fraction was determined by Western blotting assay using an anti–N-CoR antibody.

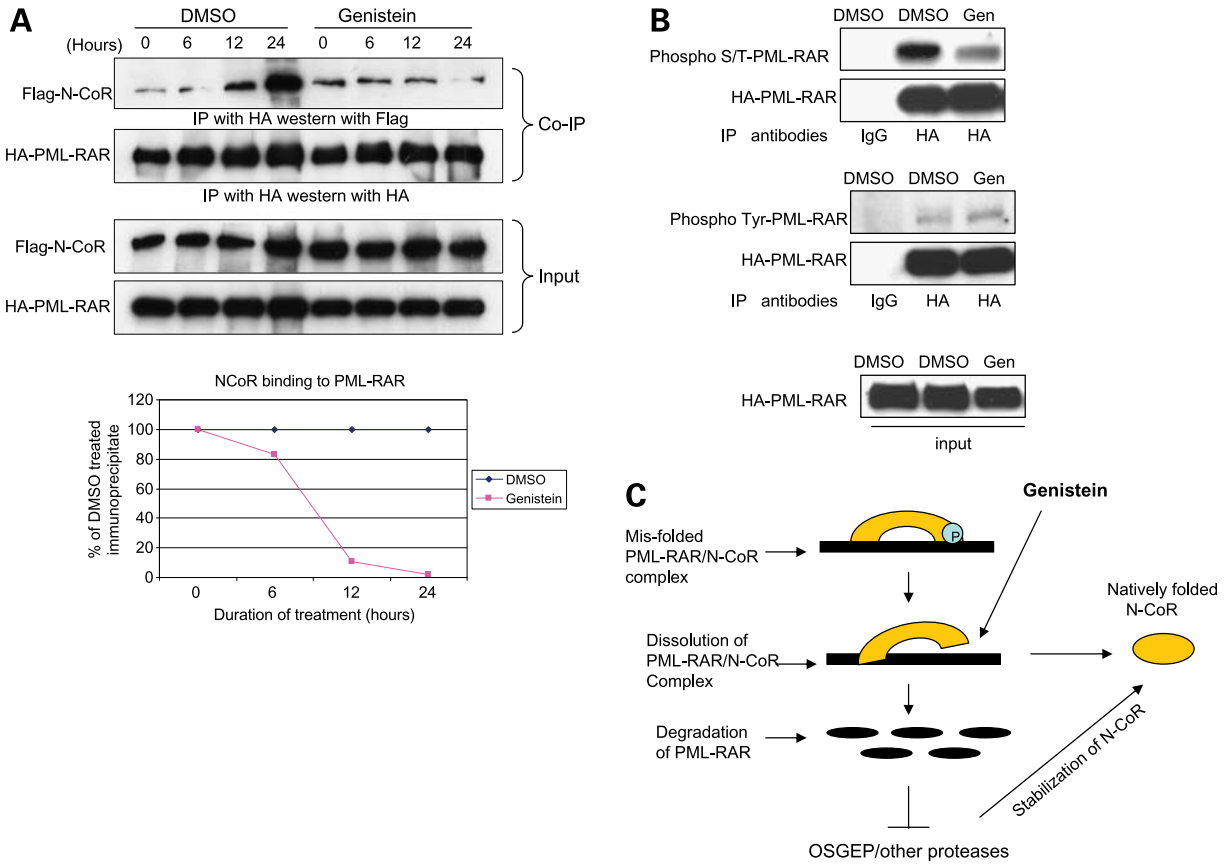


**Figure 5.** Genistein inhibits the binding of N-CoR to PML-RAR and abrogates serine/threonine phosphorylation of PML-RAR. **A,** 293T cells were transfected with Flag-tagged N-CoR and HA-tagged PML-RAR and treated with DMSO or genistein (50 μmol/L) for the duration (in hr) as indicated. PML-RAR from the cytosolic extracts of 293T cells was then immunoprecipitated with anti-HA antibody, and amount of coprecipitated N-CoR was determined with anti-Flag antibody (*first panel from top*). The membrane was then reprobed with anti-HA antibody to determine the amount of immunoprecipitated PML-RAR (*second panel*). Levels of Flag-N-CoR and HA – PML-RAR proteins at each time point were determined by immunoblotting an aliquot of each extract with respective antibodies (*third* and *fourth panels*). The density of coprecipitated N-CoR protein at each time point was measured, and the values were plotted in graph as percent of DMSO control. **B,** genistein inhibits the serine/threonine phosphorylation of PML-RAR. Whole-cell extracts of 293T cells transfected with HA-tagged PML-RAR and treated with DMSO or genistein (50 μmol/L) were immunoprecipitated with anti-HA antibody and blotted with anti-phosphoserine/threonine (*S/T*) or phosphotyrosine (*Tyr*) antibodies as indicated (*top* and *middle*). Each membrane was then reprobed with anti-HA antibody to determine the amount of precipitated PML-RAR (HA – PML-RAR; *top* and *middle*). The antibody used in immunoprecipitation is indicated under individual lanes. Amount of PML-RAR protein in each extract was determined by blotting an aliquot with anti-HA antibody (*bottom*). **C,** the cartoon illustrates the mode of action of genistein. Genistein-induced inhibition of serine/threonine phosphorylation of PML-RAR (*P*) disrupts the physical association of N-CoR and PML-RAR proteins, which leads to the destabilization of PML-RAR, and facilitates the release and refolding of N-CoR protein. Dissolution of misfolded N-CoR/PML-RAR protein complex would also lead to the attenuation of ER stress, promoting down-regulation of APL cell – specific proteases and stabilization of the N-CoR protein.

in the cytosol. The effect of genistein on the binding of N-CoR to PML-RAR was analyzed in a time-dependent coimmunoprecipitation assay using cytosolic extracts of 293T cells transfected with Flag-tagged N-CoR and HA-tagged PML-RAR plasmids. PML-RAR was immunoprecipitated with anti-HA antibody, and amount of coprecipitated N-CoR at each time point was determined by blotting the immunocomplex with an anti-Flag antibody. Genistein significantly inhibited the binding of N-CoR protein to PML-RAR under a condition when the steady-state level of both proteins was more or less stable (Fig. 5A). It has been shown that phosphorylation of the so-called N-CoR box located on the RAR domain, which facilitates the binding of N-CoR to PML-RAR, is critical for the capacity of PML-RAR to induce N-CoR misfolding and to block the differentiation of promyelocytic cells (8, 11). To test whether genistein-induced inhibition of N-CoR binding to PML-RAR results from an inhibition of PML-RAR phosphorylation, we tested the phosphorylation status of PML-RAR before and after genistein treatment. The high level of serine/threonine phosphorylation of PML-RAR observed in DMSO-treated cells was significantly abrogated by genistein (Fig. 5B, *top*). Surprisingly, there was no apparent decrease in the level of tyrosine phosphorylation of PML-RAR after genistein treatment (Fig. 5B, *middle*). This finding suggests that genistein-induced inhibition of serine/threonine phosphorylation may have contributed to the dissociation of N-CoR from PML-RAR.

## Inhibition of N-CoR Cleavage by Genistein Is Mediated by Down-regulation of APL Cell–Specific Proteases

Proteolytic processing of PML-RAR by neutrophil elastase, proteinase 3, and cathepsin G, as well as cleavage of misfolded N-CoR by OGSEP, has been implicated in the pathogenesis of APL (12, 29). Expression of some of these APL-specific proteases, such as OSGEP and cathepsin G, are indeed restricted to NB4 cells, as their expression was not detected in the non–APL cell line HL-60 (Supplementary Fig. S5A).[6] To get more insight into the mechanism that leads to the stabilization of N-CoR protein in NB4 cells after genistein treatment, we analyzed the effects of genistein, RA, or AEBSF on the expression and enzymatic activity of these APL cell–specific proteases in NB4 cells. As observed with RA and AEBSF, genistein also inhibited the cleavage of endogenous N-CoR protein of NB4 cells, which resulted in stabilization of full-length N-CoR protein (Supplementary Fig. S5B).[6] To investigate whether stabilization of N-CoR results from a direct inhibition of OSGEP activity or it is an indirect outcome of down-regulation of OSGEP expression, we analyzed the activity and expression of OSGEP and other proteases in NB4 cells treated with genistein, RA, or AEBSF. As expected, very little N-CoR cleaving activity was detected in the extract of AEBSF-treated NB4 cells (Supplementary Fig. S5C, *lane 5*).[6] On the other hand, extracts of genistein or RA-treated NB4 cells exhibited some degree of N-CoR cleaving activity (Supplementary Fig. S5C, *lanes 3* and *4*).[6] Interestingly, when expression of individual APL cell–specific protease genes in AEBSF or genistein-treated NB4 cells was analyzed, down-regulation of all three protease, including OSGEP, was observed only in genistein-treated cells (Supplementary Fig. S5D).[6] These findings suggest that genistein-induced inhibition of N-CoR cleavage results from a possible down-regulation of OSGEP expression rather than a direct inhibition of its proteolytic activity as observed with AEBSF.

Based on the findings described in this report and previously, the possible mechanism underlying the inhibition of N-CoR misfolding and cleavage by genistein is highlighted in Fig. 5C. Genistein-induced inhibition of PML-RAR phosphorylation, along with subsequent degradation of PML-RAR in NB4 cells, may facilitate the release of N-CoR molecule from the misfolded N-CoR/PML-RAR protein complex and promote its refolding to the native conformation. Dissolution of misfolded N-CoR/PML-RAR protein complex would also lead to the attenuation of ER stress, promoting down-regulation of APL cell–specific proteases and stabilization of the N-CoR protein.

## Discussion

Although we have presented evidence suggesting that genistein-induced growth arrest of APL cells may result from a combination of multiple growth inhibitory mechanisms, such as apoptosis, differentiation, and cell cycle arrest, it is not clear which of these three mechanisms would actually require the function of a natively folded and soluble N-CoR protein. We have previously reported that retinoic acid enhances the solubility of N-CoR protein, suggesting a possible link between a soluble N-CoR protein and differentiation of APL cells (11). In light of that finding, it is likely that a soluble and natively folded N-CoR contributes to the differentiation of genistein-treated NB4 cells as well. However, we are not sure what role, if any, native N-CoR plays in genistein-induced apoptosis or cell cycle arrest of APL cells. A natively folded and soluble N-CoR reorganized in PODs after genistein treatment may cooperate with PML to activate various apoptosis pathways, in which PML is involved. Nevertheless, it is also possible that genistein-induced apoptosis or cell cycle arrest of APL cells is mediated through a mechanism which is entirely independent of N-CoR function.

N-CoR protein is thought to be involved in PML-RAR–induced repression of RAR target genes, which contributes to the differentiation arrest of promyelocytic cells (6–9). Therefore, a direct role of N-CoR in the differentiation of APL cells, as claimed in this report, may sound rather paradoxical and contradictory. It is likely that under physiologic concentrations of RA, N-CoR may cooperate with PML-RAR and repress the RAR target genes that are essential for myeloid maturation. However, under much higher pharmacologic concentrations of RA, N-CoR may dissociate completely from the PML-RAR, thus relieving the repression and allowing the reexpression of the same target genes. In this context, effect of genistein on N-CoR function seems to be similar to that of RA at pharmacologic concentration.

Flow cytometric analysis of NB4 cells treated with various concentrations of genistein revealed a dose-dependent increase in the percentage of cells present in $G_2$-M phase of cell cycle and a corresponding reduction in number of cells present in $G_0$-$G_1$ phase. We did not observe any significant increase in S-phase population upon genistein treatment. The absence of blockage in S-phase entry suggests that genistein may not inhibit the various kinases involved in $G_1$-S transition. Rather, accumulation of cells in $G_2$-M phase after genistein treatment points to a premitotic arrest that may have progressed through the S phase without interruption.

A critical role of serine/threonine phosphorylation of PML-RAR in its interaction with N-CoR and in the blockage of differentiation of promyelocytic cells has already been documented (8, 11). Based on our evidence, this serine/threonine phosphorylation of PML-RAR also seems to be critical for N-CoR misfolding. Although genistein is known more for its selective anti–tyrosine kinase activity, its role in the inhibition of serine phosphorylation of ERK/MSK1 molecules has recently been documented (30, 31). Genistein-induced inhibition of PML-RAR phosphorylation may induce a conformational change in PML-RAR protein, which will block its interaction with newly synthesized N-CoR protein as well as promote dissociation of N-CoR protein from existing

N-CoR/PML-RAR protein complex. Dissolution of N-CoR/PML-RAR complex would destabilize PML-RAR, ultimately leading to the release and refolding of N-CoR protein to the native conformation.

In this report, we have presented evidence suggesting that genistein-induced inhibition of N-CoR misfolding could be exploited as an effective therapeutic strategy for the treatment of APL, including the RA-resistant subtype. Correction of conformational defects in misfolded proteins through small molecule–mediated stabilization of native state is being increasingly used as a therapeutic strategy for a number of protein aggregation diseases, including amyotrophic lateral sclerosis and amyloidosis (32). Our results further highlight the potential of genistein as a small molecular inhibitor of protein misfolding in the treatment of various conformational and protein aggregation diseases, including APL.

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