MLN8054, an Inhibitor of Aurora A Kinase, Induces Senescence in Human Tumor Cells Both In vitro and In vivo

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Abstract

Aurora A kinase is a serine/threonine protein kinase responsible for regulating several mitotic processes including centrosome separation, spindle assembly, and chromosome segregation. Small molecule inhibitors of Aurora A kinase are being pursued as novel anticancer agents, some of which have entered clinical trials. Despite the progress in developing these agents, terminal outcomes associated with Aurora A inhibition are not fully understood. Although evidence exists that Aurora A inhibition leads to apoptosis, other therapeutically relevant cell fates have not been reported. Here, we used the small molecule inhibitor MLN8054 to show that inhibition of Aurora A induces tumor cell senescence both in vitro and in vivo. Treatment of human tumor cells grown in culture with MLN8054 showed a number of morphologic and biochemical changes associated with senescence. These include increased staining of senescence-associated β-galactosidase, increased nuclear and cell body size, vacuolated cellular morphology, upregulation/stabilization of p53, p21, and hypophosphorylated pRb. To determine if Aurora A inhibition induces senescence in vivo, HCT-116 xenograft–bearing animals were dosed orally with MLN8054 for 3 weeks. In the MLN8054-treated animals, increased senescence-associated β-galactosidase activity was detected in tissue sections starting on day 15. In addition, DNA and tubulin staining of tumor tissue showed a significant increase in nuclear and cell body area, consistent with a senescent phenotype. Taken together, this data shows that senescence is a terminal outcome of Aurora A inhibition and supports the evaluation of senescence biomarkers in clinic samples. Mol Cancer Res; 8(3); 373–84. ©2010 AACR.

Introduction

Accelerated senescence is an irreversible terminal growth arrest that occurs as a result of cellular stress or DNA damage (1). Senescent cells can be identified by their hallmark phenotype of an enlarged cellular size, increased number of vacuoles, and the presence of senescence-associated β-galactosidase (SA-β-gal) activity (2). The onset of senescence is associated with increased levels of the tumor suppressor proteins p53 and p21 (3). In addition, the tumor suppressor Rb protein is hypophosphorylated in senescent cells (3). A wide variety of anticancer agents have been shown to induce accelerated senescence in tumor cells (reviewed in refs. 1, 4). Of these agents, the antimitotics have been shown to be both strong (discodermalide; ref. 5) and weak (taxol, vincristine; refs. 4, 6) inducers of senescence. The role senescence plays in therapeutic outcome is controversial; however, preclinical studies have shown that the immune system is capable of eradicating solid tumors by removal of senescent tumor cells (7).

Aurora kinases are a family of serine/threonine mitotic kinases responsible for regulating a diverse set of mitotic processes ranging from centrosome separation, spindle assembly, checkpoint activation, and monitoring of kinetochore-microtubule connections (8). Aurora A kinase maps to the 20q13.2 amplicon, a chromosomal region that is frequently amplified in tumors and is associated with poor prognosis in patients with breast and colon cancer (9, 10). Aurora A kinase localizes to the centrosomes and spindle poles, and is overexpressed in many primary tumors. Cells overexpressing Aurora A result in defects in the mitotic spindle and chromosome alignment, and experience transient delays in mitosis followed by inaccurate chromosome segregation (11, 12). Inhibition of Aurora A using short interfering RNA (siRNA) or small molecule inhibitors phenocopies many of the effects seen with Aurora A overexpression (13, 14).

MLN8054, a selective inhibitor of Aurora A kinase, has shown similar effects associated with Aurora A knockdown using either siRNA or antibodies directed against Aurora A (14, 15). Moreover, treatment with MLN8054 has been shown to induce apoptosis both in vitro and in xenograft tumor models after repeat dosing (15). In this article, we investigated the ability of MLN8054 to cause an alternative cell fate that has been associated with classic antimitotic agents, i.e., cellular senescence.
Here, we show that inhibition of Aurora A resulted in both apoptosis and senescence in tumor cells. Cultured human tumor cells treated with MLN8054 display the following senescent markers: hypophosphorylated Rb, stabilization and/or increased levels of both p53 and p21, increased cellular and nuclear size, and SA-β-gal staining. Moreover, SA-β-gal staining was observed in HCT-116 (colon) tumor tissue harvested from tumor-bearing mice treated with MLN8054. To our knowledge, this is the first time that Aurora A inhibition has been directly connected to senescence both in vitro and in vivo.

Materials and Methods

Cell Culture and Reagents

HCT-116, A549, DLD-1, NCI-H460, and SW480 cells were obtained from American Type Culture Collection. HCT-116 cells were cultured in McCoy’s 5A medium supplemented with heat-inactivated 10% fetal bovine serum. All other cell lines were cultured in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum. Lysotracker was obtained from Molecular Probes.

Immunoblotting

HCT-116 cells were plated in 10 cm dishes (1.1 × 10⁶ cells/dish) and then incubated for 24–48 h at 37°C. Cells were treated with either doxorubicin (0.1 nmol/L), MLN8054 (0.25, 1, or 4 μmol/L), or bortezomib (0.03 μmol/L) for the indicated times, then treated with either compound or DMSO control (0.2% DMSO). At 48 and 72 h after treatment, floating and adherent cells were collected (trypsin was used to detach the adherent cells), centrifuged, and then counted using trypan blue dye (Life Technologies) exclusion to differentiate live and dead cells according to the instructions of the manufacturer. Prior to compound treatment, pretreatment samples were counted.

Annexin V Fluorescence-Activated Cell Sorting Assays

HCT-116 cells were plated in 12-well plates (1.4 × 10⁵ cells/well) and allowed to incubate at 37°C for 24 h. Cells were treated with compound, as indicated, and incubated at 37°C for 72 h. Floating and adherent cells were collected (trypsin was used to detach the adherent cells), centrifuged, and then scored using Annexin V (Biovision AV-FITC Apoptosis Detection Kit) according to the instructions of the manufacturer. Cells were analyzed by flow cytometry using a Becton Dickinson FACS Calibur.

β-Gal Staining of Cultured Cells

Cells were plated in 12-well plates (1.4 × 10⁵ cells/well) and incubated overnight at 37°C. The next day, cells were pretreated ±100 μmol/L Z-vad-fmk (or DMSO control) in fresh medium for 1 h (37°C) and then challenged with compound as indicated. At 48 and 72 h after treatment, floating and adherent cells were harvested and stained with Annexin V (Biovision AV-FITC Apoptosis Detection Kit) according to the instructions of the manufacturer. Cells were analyzed by flow cytometry using a Becton Dickinson FACSCalibur.

Aurora A Kinase siRNA Experiments

Suspended HCT-116 tumor cells (2 × 10⁵) were transfected as described previously (14) at 0 h and again at 72 h.
Cells were harvested at 24, 72, and 144 h after transfection and processed for Western blot analysis as described previously (14). A monoclonal antibody directed against Aurora A was used to detect Aurora A protein expression (Anti-AIK1; BD Transduction Laboratories). Aurora A Kinase siRNA transfected cells and scrambled siRNA control cells were stained for the presence of β-galactosidase as described above. Images shown were taken as described above using a 20× objective.

**In vitro Crystal Violet Staining**

HCT-116, A549, DLD-1, NCI-H460, and SW480 cells were plated in six-well plates (600 cells/well) and incubated overnight at 37°C. The next day, cells were treated with MLN8054 (0.25, 1, or 4 μmol/L). Cells were treated continuously for 6 or 12 d and then stained with crystal violet, or for 12 d and then allowed to recover for 6 d in drug-free medium and stained on day 18. On the indicated days, cells were fixed using ice-cold methanol and then stained with a 0.5% crystal violet solution to identify the presence of cell colonies. Each well was imaged and the number of colonies was determined using Metamorph (Molecular Devices) software, in which colonies containing less than 20 cells were excluded. Results are reported as the number of colonies ±SD of three separate wells.

**In vivo Efficacy Study**

NCr female nude mice (Charles River) bearing HCT-116 xenograft tumors were dosed orally (p.o.) with vehicle or MLN8054 (30 mg/kg) for 21 d (n = 10 animals/group) using a twice daily dosing schedule (0 and 8 h daily dosing). Tumor growth was measured using vernier calipers and tumor growth inhibition was calculated using the following formula: tumor growth inhibition = 100 - (MTV treated / MTV control) × 100. Additional details have been described previously (15). Statistical significance in the tumor growth trends over time between pairs of treatment groups were assessed using linear mixed effects regression models. These models account for the fact that each animal was measured at multiple time points. A separate model was fit for each
comparison, and the areas under the curve for each treatment group were calculated using the predicted values from the model. The percentage of decrease in areas under the curve relative to the reference group were then calculated. A statistically significant $P$ value suggests that the trends over time for the two treatment groups were different.

**In vivo Immunohistochemistry**

HCT-116 tumor-bearing NCr female nude mice were dosed with MLN8054 at 30 mg/kg using a twice daily dosing (0 and 8 h) schedule. Tumor tissue was harvested at the indicated times and placed in 10% neutral buffered formalin. Immunofluorescence was done on 5-μm paraffin-embedded tumor sections using the Discovery XT automated staining system (Ventana Medical Systems). Sections were deparaffinized, followed by epitope unmasking with cell conditioning 1 solution (Ventana Medical Systems) for 20 min. Tumor sections were stained for pHisH3 (Cell Signaling Technologies) as described previously (15). The DNA stain DAPI (Vector Laboratories, Inc.) was used to estimate the total number of cells/field. One representative tissue section was used for each of the three animals in a treatment group. Images were acquired using a Leica DMLB microscope (Leica Microsystems) with a Photometrics Cool Snap HQ camera. Five images from each slide were captured using a 20× Leica Plan objective (Leica Microsystems) and analyzed on Metamorph image processing software using a custom image processing application module (Molecular Devices). The number of pHisH3-positive cells were counted and averaged in the five fields of view and DAPI staining was used to estimate the total number of cells in the fields. Anti-alpha tubulin antibody (Cell Signaling Technologies, 2125; 0.18 μg/mL) was diluted in Dako diluent and incubated with tissue sections for 1 h at 37°C. Secondary goat anti-rabbit rhodamine red-X conjugate (Jackson ImmunoResearch, 111-295-144; 30 μg/mL) was added for 30 min at room temperature. DAPI vectashield HardSet Medium (Vector Laboratories) was used as a chromatin counter stain. Images were captured with a Nikon Eclipse E800 (20× objective) and analyzed with Metamorph 6.3r7 software (Molecular Devices).

**In vivo β-Gal Staining in HCT-116 Xenograft Tumors**

Throughout the efficacy study satellite tumors were excised and frozen in optimal cutting temperature compound on days 3, 5, 10, 15, and 23. Five-micron tumor sections were stained for SA-β-gal expression using a kit from U.S. Biologicals. Staining was quantified using Metamorph software (Molecular Devices) and represented in the graph as a total percentage of the positive area. Dunnett’s multiple comparison test was used to ascertain statistical significance.

**Results**

**Aurora A Kinase Inhibition Leads to Apoptosis in Cultured Cells**

A previous study showed that MLN8054 selectively inhibits Aurora A over Aurora B at concentrations of ≤1 μmol/L, whereas a 4.0 μmol/L concentration inhibits both Aurora A and B kinases (15). Also in this study, MLN8054 (0.25-4 μmol/L) induced an apoptotic response in the HCT-116 cell line as shown by PARP and caspase-3 cleavage. Consistent with this previous report, HCT-116 cells displayed PARP and caspase-3 cleavage 48 hours after MLN8054 treatment (Fig. 1A). Expanding on this earlier work, the following apoptotic markers were examined: (a) caspase-8, the most proximal caspase in the extrinsic cell death pathway; (b) caspase-9, the most proximal caspase in the intrinsic cell death pathway; and (c) executioner caspase-7. The antibodies directed against caspase-8 and -9 in our studies recognize the full-length caspases, therefore, a diminution in signal is indicative of caspase activation. MLN8054 treatment resulted in no detectable decrease in either caspase-8 or caspase-9 after 48 hours, suggesting
that these caspases were not activated following MLN8054 treatment (Fig. 1A). In contrast, bortezomib treatment (positive control) induced the activation of both caspases-8 and -9. The caspase-7 antibody used in our studies recognized both the uncleaved (full-length) and cleaved forms of caspase-7. Unlike the positive control (bortezomib), MLN8054 did not result in caspase-7 cleavage. Similar results were found at the 72-hour time point (data not shown). Summarizing the Western blot data, MLN8054 treatment results in a modest level of apoptosis.

**FIGURE 3.** MLN8054 modulates the protein levels of p53, p21, and phosphorylated Rb in HCT-116 cells. A, MLN8054 stabilized and/or upregulates p53 and p21. Doxorubicin (0.1 μmol/L) was used as a positive control. B, MLN8054 treatment results in hypophosphorylated Rb for the first 48 h. Longer time points were examined (out to 5 d) but phosphorylated Rb levels dropped below the detection limit. The antibody used in this experiment recognizes only phosphorylated Rb (Ser795). Solid arrows, hypophosphorylated state of Rb.
To better quantify the level of apoptosis associated with MLN8054 treatment, the Annexin V assay was used (reviewed in ref. 16). As a positive control, HCT-116 cells were treated with 0.2 μmol/L of staurosporin for 48 to 72 hours, resulting in 54% to 67% Annexin V staining (Fig. 1B). Treating HCT-116 cells with MLN8054 (0.25-4.0 μmol/L) resulted in 15% to 25% Annexin V-positive staining. Pretreating the cells with the pan spectrum caspase inhibitor, z-VAD-fmk, completely blocked MLN8054-induced Annexin V staining, indicating that the cell death mechanism was indeed caspase-dependent and apoptotic.

Short-term Aurora A Kinase Inhibition Leads to a Cytostatic Effect in Cultured Tumor Cells

The data above indicate that MLN8054 induces an apoptotic response in tissue culture cells. Next, we wanted to determine if the apoptotic response was sufficient to cause a net reduction in cell viability by comparing a pretreatment cell count to a posttreatment cell count. HCT-116 cells were treated with increasing concentrations of MLN8054 (0.1-10.0 μmol/L), and after 72 hours, floating and adherent cells were trypsinized, treated with trypan blue, and counted to determine the number of viable cells. A baseline count was taken just prior to MLN8054 treatment to determine the number of viable cells prior to treatment. Comparing the number of viable cells in the 72-hour MLN8054 treatment to the baseline count, no significant changes were observed (Fig. 2). Control cells which were seeded and left untreated increased in number by 5-fold compared with the baseline count, and bortezomib (0.030 μmol/L) treatment reduced the cell number by greater than half. These data suggest that although MLN8054 induces (albeit minor) apoptosis, this process is insufficient to reduce the total cell population below the baseline within 72 hours. It is possible that the apoptotic cell loss is counterbalanced by low-level cell proliferation, but the above results also suggest that alternate cellular outcomes exist.

Aurora A Kinase Inhibition via MLN8054 Leads to Senescence in Tissue Culture Cells

The above results stimulated our interest in exploring alternate cellular outcomes associated with Aurora A inhibition. Senescence is a terminal cellular outcome resulting in a cytostatic effect. To determine if Aurora A inhibition induced senescence, several biochemical senescent markers were evaluated in MLN8054-treated HCT-116 cells. The tumor suppressors p53, p21, and Rb are particularly important in regulating cellular senescence (17-20). Previous work by Liu et al. (21) showed that Aurora A kinase phosphorylates p53 on Ser215, abrogating p53’s DNA binding and transactivation activity. P53 has been shown to transcriptionally activate the p21 gene (22). Therefore,
we hypothesized that inhibition of Aurora A kinase would interfere with the stability of both p53 and p21. The protein levels of both p53 and p21 were examined in MLN8054-treated HCT-116 cells using immunoblotting. Aurora A kinase inhibition (via MLN8054) resulted in the stabilization and/or upregulation of both p53 and p21 (Fig. 3A) on days 1 to 5. Doxorubicin, a known inducer of senescence, yielded similar effects on both p53 and p21.

Although no direct link has been found between Aurora A kinase and Rb, hypophosphorylated Rb has been associated with SN-38-induced senescence (3). Based on this connection between Rb and senescence, the phosphorylation status of Rb was examined in MLN8054-treated HCT-116 cells. MLN8054 treatment resulted in hypophosphorylation of the Rb protein on days 1 and 2 (Fig. 3B).

Decoy receptor 2 (DcR2) has also been associated with senescence. Using immunohistochemistry, Collado et al. showed an increase in DcR2 staining in senescent adenomas (23). We measured DcR2 levels in MLN8054-treated cells (using immunoblotting) and found no differences in DcR2 expression on days 1 to 5 (data not shown). The tumor suppressor 16INK4a also had a strong connection to senescence (24). However, HCT116 cells have a methylated p16INK4a promoter and are p16INK4a null (25), therefore, p16INK4a levels were not examined. It is interesting to note that our data suggests that senescence could occur in the absence of p16INK4a.

To date, one of the best known markers for senescence is SA-β-gal expression (26). To determine if Aurora kinase inhibition was linked to senescence, SA-β-gal activity was examined in HCT-116 cells treated with MLN8054. Cells were chronically treated for 3 to 17 days and stained for SA-β-gal activity on days 3, 5, 7, 10, 14, and 17. As a positive control for the SA-β-gal activity doxorubicin (0.1 μmol/L), a known inducer of senescence (6), was used. As expected, doxorubicin induced an increase in cellular size and induced SA-β-gal staining (data not shown). Doses of MLN8054 (0.25-1 μmol/L), which selectively inhibit only Aurora A, induced SA-β-gal staining starting on day 5 (Fig. 4A and B). The 4 μmol/L dose of MLN8054, which inhibits both Aurora A and B kinase, also induced SA-β-gal staining beginning on day 5.

After 17 days of MLN8054 treatment, many of the cells remained viable, and the phenotype was characterized by enlarged cellular size and nucleus as well as enlarged cytoplasmic vacuoles (Fig. 4C). These characteristics are all commonly observed in senescent cells (reviewed in ref. 1). Senescent cells typically exhibit an increase in lysosomes, both in size and number (26). After staining the cells with LysoTracker, a dye which specifically accumulates in lysosomes, many of the vacuoles did not stain positive, suggesting that many of these vacuoles were nonlysosomal.

To determine if Aurora A inhibition induces SA-β-gal staining in a variety of other cell lines, SW480, A549, Anchorage-independent growth.
DLD-1, and H460 cells were treated with MLN8054 and then assayed for SA-β-gal activity. Data in Fig. 5 indicate a dose-dependent SA-β-gal staining pattern in all lines examined. In addition, MLN8054-treated cells displayed a large, flat senescent phenotype (data not shown), similar to what was observed in the HCT116. In summary, Aurora A kinase inhibition via MLN8054 triggered the induction of senescence in cultured tumor cells.

**Aurora A Kinase siRNA Phenocopies the Effects Observed Using the Small Molecule Inhibitor MLN8054**

Previously, we used an Aurora A kinase siRNA construct to show that the small molecule inhibitor MLN8054 phenocopied the effects observed using the siRNA construct (14, 15). Using the same siRNA construct (scrambled siRNA was used as control), HCT116 cells were double transfected on days 0 and 3, and SA-β-gal activity was assayed on day 6. Although the transfection efficiency was <100%, Aurora A siRNA significantly knocked down the Aurora A protein levels (Fig. 6A). In addition, Aurora A siRNA treatment resulted in large, flat, vacuolated cells which stained positive for SA-β-gal activity (Fig. 6B). These data indicate that targeting Aurora A kinase with an siRNA construct phenocopied the effects observed using the small molecule inhibitor MLN8054.

**MLN8054 Senescent Cells Do Not Re-enter the Cell Cycle and Divide**

Colony-forming assays were used to determine if MLN8054-induced senescent cells re-enter the cell cycle and divide. Cells were cultured in the presence of MLN8054 for up to 12 days, drug was removed, and then cells were cultured in drug-free medium for 6 additional days. Cells were stained with crystal violet on days 6, 12, and 18 to determine the number of colonies. Data shown in Fig. 7 indicate a reduction in the number of HCT116 and H460 colonies (compared with untreated controls) when cultured in the presence of 250 nmol/L of MLN8054. Few to no colonies were present after treatment with 1 and 4 μmol/L of MLN8054, respectively. The number of colonies present on day 18 inversely correlated with the percentage of senescent cells (SA-β-gal positive), indicating that senescent cells do not re-enter the cell cycle and divide. This correlation was also evident in the A549, DLD-1, and SW480 cell lines (data not shown).

**Aurora A Kinase Inhibition Induces Senescence In vivo**

The HCT-116 xenograft tumor model was used to determine if Aurora A kinase inhibition leads to senescence in vivo. First, we confirmed that dosing MLN8054 (twice daily using 30 mg/kg) in vivo did not inhibit Aurora B kinase activity. Mice bearing HCT116 tumors were treated orally with two 30 mg/kg doses (twice daily schedule) of MLN8054 (8 hours apart), and tumor tissue was analyzed via immunofluorescent chemistry for phosphorylated histone H3 (pHisH3), a direct substrate for Aurora B kinase. Dosing MLN8054 resulted in an increase in pHisH3 staining over time (Fig. 8A), indicating that Aurora B kinase activity was not blocked. Previously, it was shown that a 30 mg/kg dose of MLN8054 inhibited Aurora A kinase activity in the HCT-116 tumor model, as evidenced by a decrease in the direct Aurora A substrate pT288 (15). HCT-116 tumor-bearing mice were treated orally for 21 days with MLN8054 (30 mg/kg dosed on a twice daily...
schedule) to determine the antitumor effect associated with this dose and schedule. Significant tumor growth inhibition was observed ($P = 0.0005$) on day 21 with a tumor growth inhibition rate of 81% (Fig. 8B). Throughout this study, satellite tumor samples were taken (days 3, 5, 10, 15, and 23) and analyzed for the presence of senescence. A marked increase in tumor cell size, DNA content/cell, and SA-β-gal expression was observed in the

FIGURE 7. MLN8054 treatment prevented/inhibited colony formation in both the HCT-116 and H460 cell lines. Cells were treated for up to 12 d (fresh medium and drug added every 2-3 d) with MLN8054 or vehicle. MLN8054 was removed on day 12 and cells were allowed to recover in the presence of drug-free medium for 6 d (day 18). Cells were fixed and stained using a 0.5% crystal violet solution on days 6, 12, and 18 to identify colonies. Each individual well was imaged and the colonies were counted using Metamorph software (Molecular Devices). Graphs: on day 6, only control colonies were counted; on day 18, control colonies could not be counted due to confluency; *, HCT-116 cells treated with $0.25 \mu mol/L$ were too confluent to count on day 18, therefore day 12 counts are shown in the graph. Columns, average of three separate wells; bars, SD.
MLN8054-treated tissues on days 15 and 23 (Fig. 8C and D). These data strongly suggest that Aurora A kinase inhibition leads to senescence as a terminal outcome in an \textit{in vivo} setting.

**Discussion**

Although previous studies by us and others have shown that Aurora A kinase inhibition leads to apoptosis, our expectation, based on the findings from other antimitotic agents, was that alternative terminal outcomes (reviewed in ref. 27) might also be associated with Aurora A kinase inhibition. Moreover, the following observations in solid tumor cells/models led us to hypothesize that Aurora A inhibition leads to outcomes other than apoptosis: (a) data suggest that MLN8054 induced a modest apoptotic response in cultured cells (our report) and in \textit{in vivo} model systems, and (b) in xenograft solid tumor models, treatment with MLN8054 resulted mostly in a tumor-static effect (15). Taken together, these data suggest that Aurora A kinase inhibition leads to terminal outcomes other than apoptosis.

In tissue culture, using a concentration of MLN8054 which selectively inhibits Aurora A kinase activity, we showed that MLN8054 induces senescence as characterized by a senescent-like cellular morphology (enlarged cell body/nucleus and increased vacuolization), SA-β-gal staining, upregulation of p53 and p21, and a hypophosphorylated Rb status. Higher concentrations of MLN8054 (4.0 μmol/L) inhibit both Aurora A and B kinase activity, therefore, it is unknown if the observed senescence at this concentration is a result of inhibiting Aurora A or inhibiting Aurora B kinase. To answer this question, a selective Aurora B kinase inhibitor is needed.

Our findings indicate that MLN8054 selectively inhibits Aurora A kinase activity \textit{in vivo} when dosed at 30 mg/kg. At this dose in HCT116 tumor tissue, MLN8054 has been shown to inhibit Aurora A autophosphorylation...

... the effects of Aurora A overexpression and Aurora A inhibition seem to phenocopy each other. The similarity in effect between Aurora A overexpression and Aurora A inhibition seem to occur by either inhibition of Aurora A, or by overexpression of Aurora A, the latter serving to sequester necessary components of the complex.

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Disclosure of Potential Conflicts of Interest

All authors are employees of Millennium Pharmaceuticals.

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