Model of Inhibition of the NPM-ALK Kinase Activity by Herbimycin A

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ABSTRACT
Anaplastic large cell lymphoma (ALCL) exhibiting the t(2;5) translocation is characterized by the resulting expression of the oncogenic fusion protein nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) gene product. The ALK domain of NPM-ALK contains kinase activity, which is responsible for the autophosphorylation of tyrosine residues of the oncogenic protein and phosphorylation of SH2-protein substrates. Herbimycin A is a general protein tyrosine kinase inhibitor active as an antiproliferative compound against different types of mammalian cells. Herbimycin A inhibited the NPM-ALK-associated autophosphorylating activity in an in vitro cell-free kinase assay. The inhibition was specific when tested against other kinase inhibitors and extended to other cell lines derived from t(2;5)-ALCL. SUDHL-1 cells showed increasing percentage of cells in G1 after 18 h of incubation with a dose of herbimycin A. NPM-ALK, Akt, and pAkt were down-regulated after 24 h of incubation with herbimycin A. Apoptosis was observed only if the dose of inhibitor was given every 12 h for prolonged time. Our results show that herbimycin A interferes with NPM-ALK and Akt pathways in SUDHL-1 cells. It seems that prolonged inhibition of these biochemical pathways may lead to cell cycle arrest and apoptosis. This study supports the idea of investigating protein kinase inhibitors as therapeutic compounds for t(2;5)-ALCL.

INTRODUCTION
ALCL exhibits as a separate clinical entity is described by the revised European American classification of lymphoma classification (1). ALK-positive lymphoma or “ALKoma” has been recently defined as a lymphoma of T- or null-cell origin (2). This lymphoma is associated with the t(2;5)(p23;q35) translocation that results in the expression of an oncogenic fusion protein encompassing the NH2 terminus of the NPM fused to the cytoplasmic region of the ALK (3). Variants of the t(2;5) translocation, derived from the fusion of ALK to partners other than NPM, are present in a minority of ALKomas (4). It has been estimated that “ALKoma” accounts for 50–60% of the primary systemic ALCL as defined by the expression of an ALK fusion protein. NPM-ALK is present in ~72–85% of cases, and a fusion protein containing ALK and other gene products accounts for the remaining of the case (4). The loss of the membrane component of ALK and its replacement by NPM results in dysregulation of the kinase domain. Mutations within the NPM regulate dimerization of the NPM-ALK molecules inducing conformational changes in the ALK component resulting in a constitutive activation of the kinase domain (5). NPM-ALK protein shows oncogenic activity both in vitro, when expressed in transfected NIH-3T3 and Fr3T3 cells, and in vivo in BALB/cByJ mice transplanted with murine bone marrow expressing retrovirally induced NPM-ALK (5, 6). Recent studies have shown that NPM-ALK mediates its mitogenicity by targeting phospholipase C-γ (7). In the current study, we assess the in vitro effects of HA, a tyrosine kinase inhibitor, in an attempt to investigate protein kinase inhibitors as therapeutic compounds for t(2;5)-ALCL.

MATERIALS AND METHODS
Cell Culture. SUDHL-1, SUPM2, and KARPAS 299 cells are derived from human ALCL with the t(2;5) translocation. SUDHL-1 and SUPM2 cells were kindly provided by Dr. S. Morris, St. Jude Children’s Research Hospital, as described previously (8). KARPAS 299 cells were purchased from DSMZ, Berlin, Germany. Cells were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 15% heat-inactivated fetal bovine serum as described previously (9).

NPM-ALK-associated Autophosphorylation Assay and Inhibitors. Cells were harvested and lysed as described previously (10). Briefly, monoclonal antibody ALK1 was used to immunoprecipitate NPM-ALK from 500 μg of cell proteins for each sample as described previously (10, 11). The immunoprecipitate was incubated with a mixture of kinase buffer and γ32PATP as described previously (10). The samples were loaded on 7.5% PAGE gel and stained with Coomassie to assess equal load of proteins. Gel was dried and exposed to film for autoradiography (10). Inhibitors HA, genistein (tyrosine kinase inhibitor), PD98059 (mitogen-activated protein kinase pathway inhibitor), DAPI, 4′,6-diamidino-2-phenylindole; PI3K, phosphatidylinositol 3′-kinase.
were expressed as mean ± SD. On the basis of these initial results, cells were analyzed in duplicate for changes in their cell cycle using an Epics Altra cytometer (Coulter Reagents Kits). Histograms of the percentage of cells in the G1, S, and G2/M phases from each test condition were generated. Results were expressed as mean ± SD. To assess cells for apoptosis, SUDHL-1 cells were either incubated for 24 h in culture media either with one dose of HA (250 ng/ml) initially given or with a dose repeated every 12 h. To assess cells for apoptosis, SUDHL-1 cells were incubated for 24 h in culture media either with one dose of HA (250 ng/ml) initially given or with a dose repeated every 12 h. To assess cells for apoptosis, SUDHL-1 cells were incubated for 24 h in culture media either with one dose of HA (250 ng/ml) initially given or with a dose repeated every 12 h. To assess cells for apoptosis, SUDHL-1 cells were incubated for 24 h in culture media either with one dose of HA (250 ng/ml) initially given or with a dose repeated every 12 h. To assess cells for apoptosis, SUDHL-1 cells were incubated for 24 h in culture media either with one dose of HA (250 ng/ml) initially given or with a dose repeated every 12 h. To assess cells for apoptosis, SUDHL-1 cells were incubated for 24 h in culture media either with one dose of HA (250 ng/ml) initially given or with a dose repeated every 12 h.

RESULTS

HA Inhibits the Autophosphorylation-associated Activity of NPM-ALK in an in Vitro Cell-free Kinase Assay. It has been shown that the NPM-ALK-associated kinase activity involves autophosphorylation of tyrosine residues in the fusion protein (10, 11). We and other investigators have shown recently the relevance of the phosphorylation level of NPM-ALK in regulating its functional activity (10, 12). In the current study, the phosphorylating activity of NPM-ALK derived from a fixed amount of total cell proteins (500 μg) per sample and assessed by the level of uptake of γ32PATP was notably reduced after 6 h of incubation with 435 nm (250 ng/ml) HA (Fig. 1A, Lanes 3 and 4). We also evaluated the inhibitory activity of lower or higher than 435 nm concentrations of HA on the NPM-ALK autophosphorylating activity. Although lower concentrations than 435 nm were associated with inhibitory activity of the compound, the data suggested that a concentration of 435 nm exerted the maximum effect (Fig. 1B). The level of inhibition of the oncogenic protein-associated autophosphorylating activity was not affected by increasing the concentration of the inhibitor (Fig. 1C). To assess whether the inhibition of the autophosphorylating activity associated with NPM-ALK was specifically related to HA, we investigated the effects of several kinase inhibitors on the same activity. HA notably reduced the NPM-ALK-associated kinase activity, as shown by the faint band of phosphorylated NPM-ALK present in the assay compared with the controls (Fig. 2A, Lane 3). The concentration of the other inhibitors used in the study was derived as an average from concentrations used in the literature for different mammalian cells. Although we only tested one concentration for each other inhibitor, HA was the only compound showing inhibition at the dose or time tested.

We also tested the inhibitory activity of HA on NPM-ALK-associated autophosphorylating activity derived from other cell
lines with t(2;5) translocation. NPM-ALK proteins immunoprecipitated from a fixed amount of cell proteins (500 µg) derived from SUPM2 and KARPAS 299 showed inhibition of their associate autophosphorylating activity after 6 h of incubation with 435 nM HA (Fig. 2B). Although NPM-ALK derived from SUDHL-1 cells showed a higher autophosphorylating activity compared with proteins derived from SUPM2 and KARPAS 299 cells, the level of inhibition was similar in all cell lines tested (Fig. 2B, Lanes 2, 4, and 6).

Effects of HA on Cell Cycle in SUDHL-1 Cells. We assessed the response to HA by analyzing the DNA content of the SUDHL-1 cells 18 and 24 h after the inhibitor was given. The percentage of cells in G1 phase increased to 76.5%, whereas the percentage of cells in S phase decreased to 16% after 18 h of incubation with 435 nM HA (Fig. 3). The percentage of cells in G1 phase declined, however, to 56%, whereas the percentage of cells in S phase rose to 24.5% after 24 h of incubation with the same concentration of inhibitor. These data suggested that although a single dose of HA was able to induce G1 arrest within the initial 18 h of incubation, this was not sufficient to maintain the effect over the time (Fig. 3).

HA Reduces NPM-ALK and Akt/pAkt Levels in SUDHL-1 Cells. Recent studies have shown a discrepancy between the potent effect of benzoquinone ansamycins HA, geldanamycin, and its analogue 17-allylamino-17-demethoxygeldanamycin in inhibiting kinase activity in a cell culture model and the weakness as an inhibitor when used in an *in vitro* cell-free kinase assay (13–15). Hsp-90 has been identified as a benzoquinone-binding protein responsible for the destabilization of v-src kinase and reduced levels in the cells (13). Hsp90-mediated effect of geldanamycin and 17-allylamino-17-demethoxygeldanamycin has been recently shown to be responsible for the reduced levels of Bcr-Abl oncogenic protein in a cell culture model of chronic myelogenous leukemia (14, 15). The reduced levels of Bcr-Abl protein were associated with apoptosis in the cell lines tested (14, 15). On the basis of these previous studies, we examined the effects of different concentrations of HA on the levels of NPM-ALK and Akt/pAkt in SUDHL-1 cells after 24 h of incubation. Recent studies have shown that NPM-ALK regulates the Akt/pAkt pathway (16, 17). NPM-ALK protein was absent in SUDHL-1 cells after 24 h of incubation with HA (Fig. 4, *NPM-ALK panel, Lanes 2 and 3*). The levels of Akt were also decreased with disappearance of the phosphorylated active pAkt (Fig. 4, *Akt and pAkt panels, Lanes 2 and 3*). The maximum effect of HA on down-regulation of NPM-ALK/Akt/pAkt was observed at a lower dose of HA required for the maximum inhibitory effect in the *in vitro* cell-free kinase assay of NPM-ALK (261 versus 435 nM).

Prolonged Incubation with HA Induces Cell Cycle Arrest Associated with Increasing Levels of Cell Debris in SUDHL-1 Cells. On the basis of these preliminary results, we tested the response of SUDHL-1 cells to the same dose of HA
(435 nM = 250 ng/ml) given in culture every 12 h for a total of 48 h. The controls included cells that received only one dose of HA and were analyzed after 12 and 36 h or cells grown in culture for 48 h without HA. The G0-G1 phase was at the lowest level (range 10.7–13.2%) in cells after 36 and 48 h, when HA was given every 12 h compared with the controls (40.6%; Fig. 5, top and mid panels, histograms to the left). The cell cycle profile of cells receiving HA every 12 h was completely shifted to the left of where the G1 peak usually stands, mainly represented by cell debris (Fig. 5, top and mid panels histograms in log scale to the right). The magnitude of the sub-G1 area on a log scale represents an indirect sign of cell death. Cells receiving the inhibitor every 12 h for 48 or 36 h had the highest level of cell debris compared with the controls (Fig. 5, top and mid panels). Although G0-G1 phase in cells after 12 or 36 h of incubation with HA was lower than the controls (22 and 22.1% versus 40.6%), this was higher than in cells receiving the inhibitor every 12 h for 36 and 48 h (13.2 and 10.7%, respectively; Fig. 5, HA36h0 and HA12h0). This difference was even more pronounced if we considered that a large fraction of cells in G1 derived from culture receiving a dose of HA every 12 h for 36 or 48 h was represented by disrupted cells. Lastly, cells grown in culture for 24 h and receiving HA every 12 h presented a more pronounced left shift of the sub-G1 on the log scale of the histograms compared with that derived from cells after 12 h of incubation with the inhibitor (Fig. 5, bottom panel).

HA Induces Apoptosis in SUDHL-1 Cells. To investigate whether the increasing level of cell debris was related to apoptosis or not, we stained the nuclei of cells with DAPI after incubation with either one dose of HA or a dose repeated every 12 h for 24 h. Cells treated with HA showed a brighter fluorescence of the nuclei and increased number of fragmented cells compared with the untreated controls, when observed under fluorescence microscope (Fig. 6A). Cytospins derived from cells receiving the inhibitor every 12 h presented a higher level of fragmented cells on cytospins as compared with the cells re-
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Fig. 6 A. DAPI staining of the nuclei of SUDHL-1 cells. Cells were spun on slides, fixed with methanol, stained with DAPI, and viewed under the fluorescence microscope. The intensity of the fluorescence of the nucleus stained with DAPI is correlated with the content of fragmented DNA. The most representative slides (×200) derived from cells grown in medium without HA for 24 h (left top panel), cells grown in medium with DMSO for 24 h (right top panel), cells grown in medium with an initial dose of HA (250 ng/ml) for 24 h (left bottom panel), and cells derived from culture receiving a dose of inhibitor every 12 h for 24 h (right bottom panel) are shown. B. Western blot analysis of PARP cleavage. SUDHL-1 cells were incubated for 18 (Lane 2) and 24 (Lane 3) h with a dose of HA (250 ng/ml) repeated every 12 h. Cells grown in medium without inhibitor for 24 h (Lane 1) are shown as controls. Cells lysates were run on 7.5% SDS-PAGE gel and immunoblotted with polyclonal antibody anti-PARP. The band represents M, 85,000 main cleavage product of PARP. β-actin blot derived from the cells at each time of incubation is shown as a marker of protein load.

receiving only one dose of the compound (Fig. 6A, bottom panels). These data clearly showed that apoptosis was the final event in cells arrested in G1 after exposure to HA. On the basis of the observation that a repeated dose of HA was more efficient than a single dose in inducing apoptosis by prolonging G1 arrest, we investigated SUDHL-1 cells treated with a dose of inhibitor every 12 h after 18 and 24 h. During apoptosis, caspases 3 and 7 cleave PARP to yield an M, 85,000 and an M, 25,000 fragment. PARP cleavage is considered an apoptosis-associated marker, and the polyclonal antibody anti-PARP, used in this study, specifically detects the M, 85,000 fragment. We showed by Western blot analysis that the M, 85,000 fragment of PARP appears in cells receiving a dose of HA every 12 h after 18 or 24 h of incubation with the inhibitor (Fig. 6B, 2 and 3) as compared with the controls (Fig. 6B, 1). These data were in agreement with the increasing amount of cell debris attributable to apoptosis as shown by flow cytometry analysis or by the fragmented cells observed on cytospins.

DISCUSSION

Protein tyrosine kinase substrates are involved in cellular signaling pathways, and they mediate their effects by phosphorylating domains such as the Src homology (SH2)-domain (16). The importance of the signaling pathways triggered by the phosphorylation of NPM-ALK in cell proliferation and survival has been well established (18). Although SH2 proteins such as SHC, Grb2, and IRS-1, have been found to interact with NPM-ALK, phospholipase C-γ is the only NPM-ALK SH2-protein substrate that has been shown to mediate its mitogenicity (7). It has been shown recently that NPM-ALK constitutively activates the PI3K/Akt antiapoptotic signaling pathway and that this activation plays an important role in the NPM-ALK mitogenicity (10, 16, 17). These observations prompted us to investigate the effects of tyrosine kinase inhibitors on the activity of NPM-ALK. HA is a benzoquinone ansamycin that belongs to a class of antibiotics initially isolated from actinomycete broth (19). Because HA was initially found to reverse the phenotype of Rous sarcoma virus-transformed fibroblasts, it has become a frequently used tyrosine kinase inhibitor (20). Recent studies have demonstrated that the chaperone protein Hsp90 plays a major role in mediating the in vivo inhibitory effect of the benzoquinone ansamycins in cell culture models (13–15). Hsp90, which binds to several kinases, is displaced by the ansamycins causing destabilization of the kinase leading to increased catabolism (15). It has been shown that reduced levels of the Bcr-Abl kinase are associated with increased apoptosis in cells expressing the oncogenic proteins (14, 15). Our study shows that HA is an effective inhibitor of the kinase activity of the oncogenic protein NPM-ALK as illustrated by its reduced autophosphorylation in the in vitro cell-free kinase assay. We also demonstrate that HA reduces the levels of NPM-ALK and Akt/pAkt in SUDHL-1 cells at a lower dose than that required to inhibit the kinase activity. We and other investigators have shown previously that NPM-ALK is a key protein in the proliferation of cells (5, 7, 10, 16, 17, 21, 22). NPM-ALK also appears to play a major role in the control of apoptosis (10, 16, 17). Our findings show for the first time how the inhibition or the down-regulation of the oncogenic fusion protein NPM-ALK that causes t(2;5)-ALCL leads to G1 arrest and apoptosis. The inhibition of the tyrosine phosphorylation cascade seems to trigger the interruption of redundant antiapoptotic signaling pathways, such as the Akt pathway, that leads to apoptosis as an ultimate effect. On the other hand, the reduced levels of NPM-ALK and Akt/pAkt may exert the same apoptotic effect. These data are in agreement with our previous observations (10). Further investigation will be necessary to define other target signaling pathways and the mechanisms involved beside the ones examined in the current study. This effort will also help in understanding the discrepancy between the maximum doses and the effects observed in our study. A study published during the preparation of our manuscript has shown that HA has both a time- and dose-dependent apoptotic effect on cell lines derived from t(2;5)-ALCL (23). This study also shows that HA-induced apoptosis is associated with caspase-3 activation. Our data are in agreement with the findings of the other study. In conclusion, our study further supports the idea to investigate the use of tyrosine kinase inhibitors as therapeutic tools for the treatment
of ALCL caused by chromosomal translocation-gene products involving ALK protein.

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REFERENCES


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