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Short communication

Mechanisms of apoptosis by the tyrphostin AG957 in hematopoietic cells

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Abstract

AG957 (NSC 654705) is a tyrphostin tyrosine kinase inhibitor that has been demonstrated previously to induce growth arrest in chronic myelogenous leukemia cells by inhibiting $p210^{bcr-abl}$ kinase activity and by stabilizing the association of $p210^{bcr-abl}$ kinase with its signaling adaptor molecules, Shc and Grb2. In previous studies, it has been demonstrated that AG957-associated down-regulation of bcr–abl activates the cytochrome *c*/Apaf-1/caspase-9 pathway and induces apoptosis in chronic myelogenous leukemia blasts and progenitor cells. While AG957 has been purported to have specificity for the $p210^{bcr-abl}$ kinase, antiproliferative effects of AG957 in normal T-lymphocytes and bcr–abl negative leukemia cells suggest that other targets, such as c-CBL, may be substrates. In this study, we explored the mechanisms of AG957-mediated growth inhibition and apoptosis in the $p210^{bcr-abl}$ negative leukemia cell lines Nalm-6 and Jurkat, and demonstrate that AG957-mediated apoptosis is associated with altered phosphorylation of Akt and BAD, which destabilizes the Bcl-xL/BAD complex and releases the block to apoptosis. We, therefore, propose that AG957 induces apoptosis in bcr–abl negative hematopoietic cells by affecting the phosphorylation state of phosphatidylinositol-3 kinase/Akt.

Keywords: BAD; Akt; Tyrphostins; Leukemia; Apoptosis

1. Introduction

AG957 (NSC 654705) is a tyrphostin tyrosine kinase inhibitor modeled after erbstatin, a naturally occurring compound. While some tyrosine kinase inhibitors, such as quercetin and genistein, are ATP antagonists, tyrphostins were designed to be antagonists of specific protein substrates. Prior studies demonstrated that certain tyrphostins were capable of inducing growth arrest of K562 chronic myelogenous leukemia cells, which express the $p210^{bcr-abl}$ tyrosine kinase oncogene, and that this effect was mediated in the case of AG957 and other tyrphostins by inhibition of $p210^{bcr-abl}$ kinase activity [1,2]. This effect is mediated, in part, by alterations in the association of $p210^{bcr-abl}$ with its signaling adaptor molecules, Shc and Grb2. In other studies, AG957 has been demonstrated to inhibit tyrosine-mediated signaling of kinases other than $p210^{bcr-abl}$ [3]. Growth inhibition induced by 25 μM AG957 in the Jurkat T-lymphoblastic leukemia cell line was associated with inhibition of p38 mitogen-activated protein (MAP) kinase and with activation and a decrease in the anti-CD3-stimulated phosphorylation of $p120^{cbl}$. Additionally, growth inhibition was associated with the appearance of higher molecular weight immunoreactive species ascribed to the covalent modification of target molecules through cross-linking.

In addition to inhibiting growth, tyrphostins have been demonstrated to induce apoptosis in HL60 cells and to eradicate pre-B acute lymphocytic leukemia engrafted into SCID mice. Palumbo *et al.* [4] demonstrated that the tyrphostin AG17 induces apoptosis and inhibition of Cdk2 activity in a Bcl-2-expressing immunoblastic lymphoma cell line, OCI-Ly8. While Bcl-2 and Cdk2 proteins were not altered with AG17, Cdk2 activity and p16 and p21 protein levels were reduced markedly.

We have demonstrated that AG957 induces apoptosis in bcr–abl positive and negative leukemia and lymphoma cell lines and in selected epithelial cancer cell lines. To elucidate the mechanism of induction of apoptosis, we evaluated the effects of AG957 on known early apoptosis regulatory elements in leukemia cells, including p53, Bcl-2 and related proteins, and BAD/BAX. In the present study, we demonstrated that AG957 causes alterations in

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the phosphorylation of the Akt kinase and BAD without affecting the phosphorylation status of Bcl-2 or BAX. This is a novel mechanism for the induction of apoptosis by tyrphostins in hematopoietic cells.

2. Materials and methods

2.1. Cell lines and culture conditions

The human leukemia cell lines Nalm-6 (pre-B leukemia), Jurkat (T-cell leukemia), and K562 (chronic myelogenous leukemia) were obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL of penicillin, and 75 U/mL of streptomycin at 37° in 5% CO_2. Cell culture medium, sera, and antibiotics were obtained from Life Technologies.

2.2. Induction and assessment of apoptosis

Cells were seeded in 75 mm^2 flasks at a semi-confluent density of 5×10^5 cells/mL and incubated in the presence and absence of 50 μM tyrphostin AG957 (NSC 654705; obtained from the NCI) for 24–48 hr. Morphological changes were assessed by immobilization of the cells onto glass slides by cytospin (Shandon) at 500 *g* for 10 min at 4°. The cells were fixed and permeabilized by immersion in methanol:acetone (1:1) for 10 min at −20° and then were air-dried. Slides were immersed in DAPI stain (1 μg/mL) for 30 min and washed three times with PBS. Slides were mounted with glycerol and viewed using a Nikon Optiphot 22 fluorescence microscope (Nikon Instruments) equipped with a mercury lamp and an excitation filter for UV (365 nm). Images were recorded using photographic attachments (Microflex-UFX-DX), on Kodak ASA200 color film. Figures were scanned using a Microtek ScanMaker II XE and assembled using Adobe Photoshop.

Cells evaluated for DNA fragmentation were harvested by centrifugation (1000 *g*) for 5 min at room temperature, lysed in DNA lysis buffer (0.5% Triton X-100, 25 mM Tris–HCl, pH 7.4, 25 mM EDTA) for 30 min at 4°, further processed by incubation with 100 μg/mL of proteinase K, 150 mM NaCl, and 0.2% (w/v) SDS, and incubated at 50° for 2 hr. Nucleic acid was extracted twice with phenol/chloroform and treated with 1 mg/mL of DNase-free RNase A for 1 hr at 37°. DNA samples (10 μg per lane) were loaded on a 1.8% agarose gel (with 0.5 μg/mL of ethidium bromide) and separated by electrophoresis using 1 × TBE buffer (89 mM Tris–HCl, 2 mM EDTA, pH 8.0, 89 mM boric acid). Gels were run at 50 V for 5 hr, viewed on a UV transilluminator, and photographed.

To further demonstrate apoptosis, we assessed Annexin-V staining (Oncogene Research Products). Briefly, cells were exposed to 50 μM AG957 at different time points, harvested by centrifugation (500 *g* at 37° for up to 48 hr), and stained with Annexin-V fluorescein isothiocyanate (FITC) and propidium iodide for 30 min. Cells were washed three times with PBS and analyzed by flow cytometry using a Becton Dickinson FACSCAN. Apoptotic cells were differentiated from necrotic cells by evaluating propidium iodide staining.

2.3. Immunoprecipitation

Cells were stimulated by 50 μM AG957 for 24–48 hr and then lysed with lysis buffer (50 mM Tris–HCl, pH 8.2, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, 5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride) on ice for 30 min. Cell lysates were collected and precipitated with primary antibody (Santa Cruz Biotechnology) overnight at 4°. Immunocomplexes were collected by centrifugation (10,000 *g*, 4°), washed three times in Nonidet P-40 buffer (50 mM Tris–HCl, pH 8.2, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40), and analyzed on SDS-PAGE gels.

2.4. Western blot analyses

Cell lysates from apoptotic and non-apoptotic cells were separated on 15% SDS-PAGE gels and electrotransferred onto PVDF membranes (Millipore) for 1 hr at 4° with constant voltage (100 V). Membranes were blocked with 5% non-fat milk (Carnation), resuspended in PBST (0.1% Tween-20, 10 mM phosphate buffer, pH 7.4, 2.7 mM potassium chloride, 137 mM sodium chloride) for 1 hr, followed by incubation with primary antibody (1:5000 dilution) for 1 hr at room temperature. The blots were washed three times with PBST, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Life Science), and then developed using enhanced chemiluminescence (NEN Life Science). Images were developed on Kodak XAR film.

3. Results

The Jurkat T-lymphoblastoid cell line was exposed to 25–50 μM AG957 for 6–48 hr. At 24 hr, DNA fragmentation and morphologic features of apoptosis were noted, as shown in Fig. 1. To examine early events in apoptosis, we examined, by flow cytometry, Annexin-V staining in the Jurkat cells and saw an increase at 8 hr after exposure, as shown in Fig. 2. Caspase activation, which occurs later in the apoptotic cascade, was determined based upon poly-(ADP-ribose) polymerase (PARP) degradation and occurred as early as 6 hr after exposure, as shown in Fig. 3.

We next analyzed alterations in p53 expression in Nalm-6 cells, the pre-B cell line, since p53 is up-regulated early in apoptosis and is believed to be regulated by the phos-

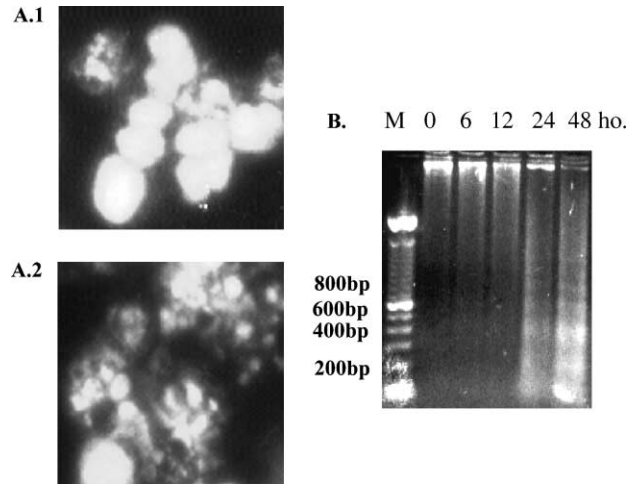

Fig. 1. Induction of apoptosis by the tyrphostin AG957. (A) Apoptotic morphological changes in Jurkat cells. Panels represent fluorescence microscopy of DAPI-stained Jurkat cells before (panel A.1) and after a 24 hr (panel A.2) exposure to 50 μM AG957. (B) Agarose gel detection of internucleosomal DNA fragmentation from Jurkat cells exposed to 50 μM AG957 for 6–48 hr.

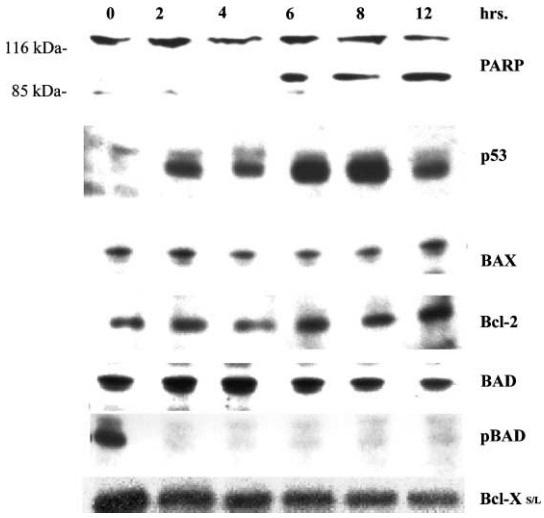

Fig. 3. Western blot analysis of early effectors of apoptosis. Jurkat and Nalm-6 cells were exposed to 50 μM AG957 at different time points (0–12 hr). Whole cell lysates in SDS-loading buffer were separated on 12% SDS-PAGE gels, transblotted, and probed with polyclonal antibodies to PARP, p53, BAX, Bcl-2, BAD, pBAD, and Bcl-$X_{S/L}$. Results are an autoradiograph of blots developed *via* chemiluminescence.

phorylation of p21. Expression of p53 increased, suggesting that there was no effect of AG957 on p21 function, as was demonstrated previously with AG17 [4].

Since AG957 has been demonstrated to modulate the phosphorylation state of a number of tyrosine kinases and cell cycle regulatory proteins, we examined Bcl-2 and its regulatory companion proteins, BAX and BAD. As shown in Figs. 3 and 4, Bcl-2, Bcl-$X_{S/L}$, and BAX expression were unchanged after exposure to AG957 in the setting of induction of apoptosis in the cells. However, while BAD protein also appeared to be unchanged, the phosphorylation state of BAD was altered, as demonstrated in the immunoblots with phospho-BAD antibody (Fig. 4). Dephosphorylation of BAD was demonstrated as early as 2 hr after exposure to AG957. Similarly, dephosphorylation of Akt kinase was demonstrated 60 min after exposure to AG957, with no change in Akt protein on the immunoblots.

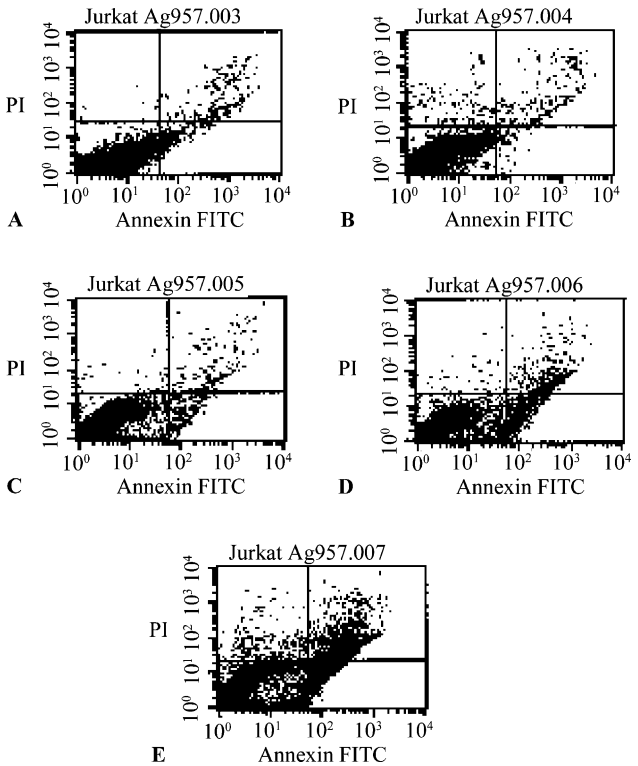

Fig. 2. Annexin-V staining and flow cytometry. (A) Untreated cells. (B–E) Cells at 2 hr (B), 4 hr (C), 6 hr (D), and 8 hr (E) post-exposure to 50 μM AG957.

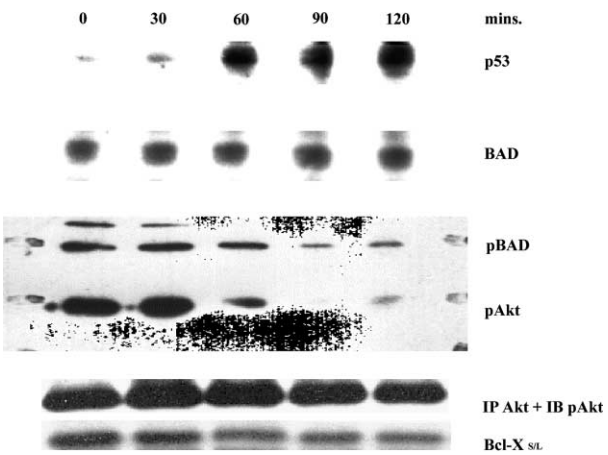

Fig. 4. Western blot analysis of rapid dephosphorylation of BAD and Akt in Jurkat cells at 0–120 min after exposure to 50 μM AG957. Results are an autoradiograph of blots developed *via* chemiluminescence.

4. Discussion

Our data demonstrated that AG957-induced apoptosis is an early event in leukemia cells and is most likely mediated by dephosphorylation of Akt and subsequently BAD, thereby destabilizing the stoichiometric relationship between the Bcl-2 family members and their regulatory proteins. BAD function is regulated by the Akt/protein kinase B pathway, which is downstream of phosphatidylinositol-3 (PI-3) kinase, a ubiquitous component of signal transduction for survival factors [5]. To date, direct effects of a chemical mediator of apoptosis on Akt and BAD phosphorylation have been demonstrated using the PI-3 kinase inhibitors wortmannin and LY294002, as well as a platelet-derived growth factor receptor inhibitor, AG1295 [6].

The various mechanisms by which AG957 may induce apoptosis demonstrate that AG957 is not a specific inhibitor of bcr–abl. However, recent reports demonstrate that bcr–abl-expressing progenitor cells from CML patients are more sensitive to AG957-induced growth inhibition than are normal counterparts [7,8]. Svingen *et al.* [8] demonstrated selectivity of AG957 for CML progenitors compared to normal cells with an IC_{50} value of 7.3 vs. 20 μM. In this study, AG957-induced apoptosis was shown to be mediated through the cytochrome *c*/Apaf-1/caspase-9 pathway rather than the FAS/FADD-mediated death receptor pathway.

In this study, we demonstrate that the upstream target of AG957 may be the Akt kinase. Prior studies by Skorski *et al.* [9] have described activation of the PI-3 kinase/Akt pathway as a critical element of bcr–abl-mediated transformation. In their studies, kinase-deficient Akt mutants with dominant negative activity inhibited bcr–abl transformation of murine bone marrow cells and suppressed leukemia development in SCID mice. Aside from modulating BAD phosphorylation, Akt has also been demonstrated to inhibit the progression of apoptosis by restricting the release of cytochrome *c* from the mitochondria by an unknown mechanism distinct from BAD phosphorylation, so our results are consistent with the findings of Svingen *et al.*, who demonstrated the release of cytochrome *c* after exposure of CML cells to AG957 [8,10].

Recently, Akt has been shown to promote hematopoietic cell survival *via* activation of mitochondrial Raf-1 in a ras-independent, but protein kinase C-dependent manner [11]. Salomoni *et al.* [12] also demonstrated that transfection of constitutively active Raf-1 restored the leukemogenic potential of transformation-deficient bcr–abl mutants. Interestingly, this pathway was inhibited by AG957 in studies by Jamieson *et al.* [13] to describe the necessity for PKCι in bcr–abl leukemogenesis. Taken together, these findings suggest that activation of the PI-3 kinase/Akt pathway could be the limiting event in bcr–abl transformation.

In summary, we demonstrated that the tyrphostin AG957 is capable of inducing apoptosis in leukemia cells by regulating the PI-3 kinase/Akt-mediated dephosphorylation of BAD. Evaluation of the effects of other tyrphostins with structural features similar to those of AG957 on Akt kinase and BAD phosphorylation may lead to identification of a novel targeting strategy for leukemias.

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