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Adaphostin and bortezomib induce oxidative injury and apoptosis in imatinib mesylate-resistant hematopoietic cells expressing mutant forms of Bcr/Abl

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Abstract

Effects of the tyrphostin adaphostin and bortezomib were examined in Bcr/Abl$^+$ leukemia cell resistant to imatinib mesylate secondary to Bcr/Abl point mutations. Adaphostin was equally effective in inducing mitochondrial damage, caspase activation, JNK activation, and Raf-1, phospho-Stat3 and -Stat5 inactivation in mutant and wild-type cells, but differentially down-regulated phospho-Bcr/Abl. Adaphostin and bortezomib synergistically induced apoptosis in wild-type and mutant cells, including T315I mutants. Notably, adaphostin ± bortezomib potently induced ROS and lethality in mutant cells, effects attenuated by the antioxidant NAC. These findings indicate that adaphostin ± bortezomib circumvent imatinib resistance due to Bcr/Abl point mutations most likely through ROS generation.

Keywords: Adaphostin; Bortezomib; ROS; Phospho-Bcr/Abl; Imatinib resistance

1. Introduction

Chronic myelogenous leukemia (CML) is a hematopoietic disorder characterized by the (9:22) translocation which encodes the mutant chimeric protein Bcr/Abl, a constitutively active tyrosine kinase responsible for leukemogenic transformation [1]. Bcr/Abl signals downstream to multiple survival signaling pathways, including Akt, NF-κB, Stat5, Bcl-x_L, and ERK (extracellular signal regulated kinase), among others [2–3], which collectively confer on Bcr/Abl$^+$ cells a survival advantage compared to their normal counterparts [4]. The treatment of CML and related disorders has been revolutionized by the development of imatinib mesylate (STI571; Gleevec), which binds to and traps Bcr/Abl in an inactive conformation [5], resulting in cell death [6]. Imatinib mesylate has proven highly active in patients with chronic phase CML, although it is less effective in patients with accelerated and blast phase disease [7–8]. A major barrier to cure of patients with Bcr/Abl$^+$ hematopoietic malignancies is the development or pre-existence of imatinib mesylate resistance due to multiple factors, including Bcr/Abl amplification, increased Bcr/Abl expression, Pgp-related resistance, or plasma proteins binding [9]. Probably the most common basis for resistance, however, is the development of mutations in various regions of the Bcr/Abl protein, including the kinase domain, the ATP binding domain, the P-loop, or in regions outside of the kinase domain [10–11]. These mutations interfere with binding of imatinib mesylate to Bcr/Abl, and render it ineffective in blocking Bcr/Abl survival signaling. Recently, newer generation Bcr/Abl kinase inhibitors have been developed, including AMN107 and BMS 354825 [12], which are active

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against some Bcr/Abl mutations conferring resistance to imatinib mesylate. However, certain mutant proteins (e.g., those expressing the T315I mutation) are not inhibited by these agents [12], and cells bearing them survive drug exposure. Consequently, a need to develop new strategies targeting mutant Bcr/Abl proteins exists.

The tyrphostin family of tyrosine kinase inhibitors comprise a group of small molecules that interfere with peptide binding rather than the kinase ATP binding site [13]. The tyrphostin AG957 was originally developed as an alternative to imatinib mesylate as an inhibitor of the Bcr/Abl kinase [14]. Adaphostin (NSC 680410) is an adamantyl ester of AG957 that is more potent on a molar basis than AG957 in vitro and in vivo, and is currently undergoing preclinical development. Previous studies demonstrated that adaphostin induces apoptosis more rapidly than imatinib mesylate in Bcr/Abl$^+$ cells in association with Bcr/Abl down-regulation as well as Stat5 inactivation [15]. Furthermore, results of a very recent study suggests that it triggers cell death in certain imatinib mesylate-resistant cells expressing point mutations [16]. Adaphostin is also relatively less toxic toward normal hematopoietic progenitors [15]. However, the actions of adaphostin are not restricted to CML cells, as it also induces apoptosis in Bcr/Abl$^-$ human leukemia lines (e.g., Jurkat, U937), as well as glioblastoma cells [17,18]. Recently, reports from several laboratories including our own have shown that adaphostin initiates apoptosis in human leukemia cells in association with generation of reactive oxygen species (ROS) [16,18,19]. Together, these findings suggest a possible therapeutic role for adaphostin in CML and potentially other leukemias. Currently, however, no information is available concerning the effects of adaphostin-mediated ROS generation on downstream targets of Bcr/Abl, including Raf-1, Stat 3, Stat 5, or Lyn, particularly in imatinib mesylate-resistant cells.

Recently, our group reported highly synergistic interactions between adaphostin and the proteasome inhibitor bortezomib (previously known as PS-341; Velcade) in human leukemia (but not normal hematopoietic) cells, a phenomenon associated with a marked increase in oxidative injury (ROS generation) [20]. Proteasome inhibitors such as bortezomib inhibit the chymotryptic activity of the 26S proteasome, and in so doing, modulate the disposition of diverse proteins involved in signal transduction, cell cycle regulation, and apoptosis [21]. They also exert selective lethality toward transformed cells [22], and kill human leukemia cells via an ROS-dependent mechanism [23]. Given the synergistic lethality of adaphostin and bortezomib toward Bcr/Abl$^-$ leukemia cells, the question arose whether this strategy would be effective against Bcr/Abl$^+$ hematopoietic cells, particularly those bearing mutations conferring high degrees of imatinib mesylate resistance. To this end, BaF/3 cells expressing three clinically relevant Bcr/Abl mutations (E255K, M351T, and T315I) [8–10] were employed to assess the response of such cells to adaphostin and particularly the adaphostin/bortezomib regimen. Our results indicate that a strategy designed to enhance oxidative injury by combining adaphostin and bortezomib is highly effective in triggering cell death in highly imatinib mesylate-resistant Bcr/Abl$^+$ cells bearing point mutations in the Bcr/Abl kinase.

2. Materials and methods

2.1. Cells

BaF/3 cells expressing wild-type or mutant Bcr/Abl were kindly provided by Dr. Brian Druker (Oregan Health and Sciences University Cancer Center, Portland, OR) and have been described in detail previously [24]. Cells were cultured in RPMI 1640 supplemented with sodium pyruvate, MEM essential vitamins, L-glutamate, penicillin, streptomycin, and 10% heat-inactivated FCS (Hyclone, Logan, UT). They were maintained in a 37 °C, 5% CO_2, fully humidified incubator, passed twice weekly, and prepared for experiment when in log-phase growth (cell density $\leq 4 \times 10^5$ cells/ml).

2.2. Reagents

Adaphostin was provided by the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. Bortezomib (Velcade) was provided by Millennium Pharmaceuticals, Cambridge, MA. All chemicals were formulated in sterile DMSO before use. Annexin V/PI was supplied by BD PharMingen, San Diego, CA, and was formulated as per the manufacturer's instructions. NAC was purchased from Sigma (St. Louis, MO). 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) was obtained from Molecular Probes Eugene, OR. All other cell culture products, including sodium pyruvate, MEM essential vitamins, and L-glutamate, were obtained from Invitrogen, Carlsbad, CA.

2.3. Experimental format

Logarithmically growing cells were placed in sterile plastic T-flasks (Corning, NY) to which the designated drugs were added. Then flasks were placed in the incubator for the indicated intervals, after which cells were transferred to sterile centrifuge tubes, pelleted by centrifugation at $400 \times g$ for 10 min at room temperature, and prepared for analysis as described below.

2.4. Assessment of apoptosis

After drug exposure, cells were stained with Annexin V/PI as described previously [19]. Briefly, cells are washed with 1× PBS and stained with Annexin V/PI (BD PharMingen) for 30 min at room temperature. Cells were then processed and analyzed using a Becton-Dickinson FACScan cytofluorometer (Mansfield, MA) with the use of Cell Quest software. Cells