

Career Development Candidate Leukemia Spore

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Background

The momentum for target-based cancer therapy has accelerated during the last decade with the clinical success of imatinib mesylate in treating chronic phase CML. Chronic phase patients treated with imatinib have a much lower rate of progression to accelerated phase or blast crisis than patients treated with interferon- α + cytarabine when used as the initial therapy (1). However, in contrast to high remission rates seen in chronic phase patients, imatinib mesylate is less effective in Ph⁺ ALL and CML-BC patients (2). In these patients a Phase I trial of imatinib mesylate illustrated a median disease free survival of < 8 months in patients with myeloid blast crisis and < 2 months in patients with lymphoid blast crisis. Phase II data in lymphoid and myeloid blast crisis have failed to yield improved results (3, 4). Instead, it appears that imatinib treatment in these settings selects for cells that are resistant to this agent. The need for new agents for leukemia is not limited to Ph⁺ ALL and CML-BC. In 2003 there will be an estimated 11,000 new cases and 8,000 deaths from AML in the United States (5). Current induction regimens yield CRs in 65-80% of cases but the majority of patients relapse (6). In these relapsed patients, second remissions do occur but are usually short-lived. Bone marrow transplant does demonstrate a survival advantage (7), but is not feasible for many patients due to advanced age or comorbidity.

The research plan outlined herein focuses on adaphostin, a tyrphostin kinase inhibitor. The term tyrphostin refers to a broad class of inhibitors that mimic the polypeptide substrate rather than ATP. Studies conducted at NCI originally identified the tyrphostin AG957 (the parent compound of adaphostin) as an inhibitor of p210 bcr/abl autophosphorylation in immune complex kinase assays and in intact cells (8). Additional experiments suggested that AG957 also caused crosslinking of p210 to associated signaling molecules in immune complexes (9). Subsequent studies from independent laboratories demonstrated that AG957 exhibited selectivity for CML progenitors including CFU-GEMM, CFU-G, and CFU-GM, as compared to progenitors from normal individuals (10, 11). While these studies established the selectivity of AG957 (and subsequently adaphostin) for Bcr/abl transformed cells, they did not demonstrate dependence on Bcr/abl. To the contrary, studies from a number of laboratories indicated that AG957 and adaphostin kill several bcr/abl negative leukemia cell lines including Jurkat, CEM, HL-60, FDC-P1, and Nalm-6 (11-15), suggesting the possibility of a second cytotoxic mechanism that does not involve Bcr/abl. In particular, both AG957 and adaphostin cause an elevation of reactive oxygen species (ROS) followed by the appearance of DNA single strand breaks and a typical DNA damage response in susceptible cells. The observation that buthionine sulfoximine (BSO), which inhibits glutathione (GSH) synthesis, sensitizes cells to adaphostin and the antioxidant N-acetylcysteine (NAC) protects cells provides further evidence for the importance of ROS in the cytotoxicity of adaphostin (16). Consistent with this Bcr/abl independent mechanism, we and others have observed that adaphostin also kills CLL lymphocytes and AML blasts.

Specific Aims

Further studies are now required to clarify the source of the ROS in adaphostin treated cells and determine the basis for the selectivity of this agent. Towards this end, the following aims are proposed:

Aim 1: Determine the mechanism by which adaphostin causes elevated free radical production and subsequent apoptosis.

Aim 2: To confirm and assess the basis for the selectivity of adaphostin.

Taken together, these studies will provide important new information about the mechanism of action of a potential new antileukemic agent, and also evaluate a novel target pathway for future drug development.

Experimental Design and Methods

Aim 1. Determine the mechanism by which adaphostin causes elevated free radical production and subsequent apoptosis.

Our preliminary data indicates that adaphostin causes intracellular peroxide and superoxide production in leukemic cells regardless of whether they contain bcr/abl protein. Kinetically, ROS generation is followed by DNA damage and induction of apoptosis via a mitochondrial route of caspase activation. Antioxidants prevent apoptosis induction and DNA damage by adaphostin and depletion of glutathione (GSH) by buthionine sulfoximine (BSO) potentiates adaphostin toxicity.

By determining how ROS is produced by adaphostin we can gain an understanding of biochemical pathways altered by this agent in leukemic cells and perhaps target these pathways more effectively. Also, we may gain insight into how oxidants are handled in leukemia cells - which allow a pro-oxidant stimulus to ultimately kill the cell. Experiments posed in this application will focus on ROS generation as byproducts of two major processes: either increased pro-oxidant cellular capacity or decreased antioxidant capacity. Mitochondrial oxidative phosphorylation is a major site of pro-oxidant production. If adaphostin elicits an heightened respiration rate, this could account for the superoxide production noted post treatment. Alternatively, a decrease in antioxidant defenses could also account for the ROS generation seen in adaphostin treated cells. The cellular defenses in place for handling ROS include enzymatic and nonenzymatic processes. Amongst the nonenzymatic antioxidants we will study are the GSH and Trx systems. Of the enzymatic systems we will study the superoxide dismutases (SODs), which convert superoxide ion to hydrogen peroxide. Hydrogen peroxide is then converted to water and oxygen by catalase. This process will be studied in control and adaphostin treated K562, ML-1 and SUP-B15 cells. We have already conducted dose response and time course experiments in these cell lines, therefore appropriate drug concentrations are known.

Experiment 1. Does mitochondrial oxidative phosphorylation change in adaphostin treated cells?

Because respiration yields superoxide generation, we will determine whether adaphostin alters oxygen consumption in cell lines. Oxygen consumption by intact cells will be measured as an indication of the mitochondrial respiration activity. Cells will be resuspended in 1 ml of fresh culture medium pre-equilibrated with 21% oxygen and then placed in the sealed respiration chamber equipped with a thermostat control and a micro-stirring device. Oxygen consumption will be measured polarographically at 37 °C with the Clark-type oxygen electrode disc, using the conditions recommended by the manufacturer. The oxygen content in the suspension medium will be constantly monitored, and the signals will be integrated using the software supplied by the manufacturer. The oxygen contents in the starting medium will be normalized assuming an O₂ concentration of 220 µM in air-saturated medium at 37 °C.

Experiment 2. Does adaphostin deplete GSH levels or alter subcellular compartmentalization of GSH?

We have recently published that adaphostin causes a modest drop in GSH levels in K562 cells using a fluorimetric technique. A HPLC assay that more specifically measures GSH will be employed to determine whether a drop in GSH occurs in other cell types such as Jurkat, ML-1 and SUP-B15. Both mitochondrial and nuclear GSH have been postulated to be important sites of antioxidant action. Therefore, GSH will be measured as described above in isolated mitochondria and nuclei from cell lines.

Experiment 3. Are thioredoxin levels diminished by adaphostin?

We have generated preliminary data indicating that adaphostin causes lowered Trx protein levels in Jurkat cells. We will attempt to extend this finding to K562, ML-1 and SUP-B15 cells by conducting Western blotting with a Trx specific antibody.

Experiment 4. Is SOD expression or enzyme activity altered by adaphostin treatment?

Superoxide dismutase activity will be assayed by preparing homogenates from cell lines in RIPA buffer. Supernatants will be assayed spectrophotometrically for protein and for activity that diminishes the ability of xanthine/xanthine oxidase to oxidize cytochrome c. Protein expression of SOD isoforms will be assessed by Western blotting.

Experiment 5. Is catalase expression or enzyme activity altered by adaphostin treatment?

Enzyme activity assays for catalase will be conducted by generating cell homogenates and measuring their ability to catalyze disappearance of 10 mM hydrogen peroxide as measured by absorbance at 240 nm. Catalase protein expression will also be measured by Western blotting.

Follow-up experiments for Aim 1. An important follow up for the experiments listed above is to determine whether we can confirm the data in cells from patients with CML, ALL and AML. Ideally, ten patients from each disease would be assessed. If we see reproducible changes in activity or expression of the antioxidant proteins listed above, we will test the efficacy of adaphostin in a context where these antioxidants are absent. Either antisense or siRNA may be employed for these experiments.

Aim 2. To confirm and assess the basis for the selectivity of adaphostin.

Our results demonstrate that AG957 and its derivative adaphostin both show selectivity for CML as compared to normal myeloid progenitors (11). Further experiments suggest that generation of ROS plays a major role in the cytotoxicity of adaphostin (16). Interestingly, transformation of cells with p210 Bcr/abl by itself results in elevated ROS (17) and adaphostin causes a further increase in ROS in p210 Bcr/abl transductants as compared to parental FDC-P1 cells. Collectively, these observations raise the possibility that the selectivity of adaphostin for CML progenitors reflects enhanced sensitivity of CML progenitors to oxidative stress. However, enhanced sensitivity to ROS may not be unique to CML. CLL cells have also been reported to be hypersensitive to oxidative stress. Previous studies have shown that a wide range of cancer cells demonstrate elevated peroxide production. Implications for this intracellular peroxide production include enhanced proliferative ability as well as genomic instability. Therefore, determining the source of elevated ROS in transformed cells and exploiting this property is of considerable interest and may be useful in devising therapeutic strategies.

Adaphostin also causes degradation of bcr/abl protein in a ROS independent manner since an antioxidant does not prevent bcr/abl decay. While this data suggests that the drug's effects on bcr/abl are not regulated by ROS, we cannot rule out the possibility that kinase inhibition by adaphostin (whether it be bcr/abl or a downstream kinase) contributes to the mechanism of action. Since adaphostin demonstrates selectivity in AML cells as well, it is plausible that a kinase downstream of bcr/abl is affected by adaphostin. Moreover, immune complex assays with AG957 (the parent compound of adaphostin) indicate that drug treatment alters the normal amounts and physical associations of molecules that are found in complex with bcr/abl such as Shc and Grb2. At the time, these effects were attributed to reflect covalent cross-links induced by the drug.

This aim will try to identify the Bcr/abl activated signal that is responsible for elevated ROS. We will directly assess changes in known downstream targets of bcr/abl. Also, various Bcr/abl activated pathways will be inhibited by pharmacological means or by transient transfection of various pathways. The pathways that will be examined include MAPK, NFkB, and Src kinases.

Experiment 1. Are Shc and Grb2 levels or phosphorylation status altered in cells that do not contain bcr/abl?

Data obtained in K562 cells indicates that the adaptor molecules Shc and Grb2 can bind to bcr/abl and may be targets for adaphostin action. In ML-1 and Jurkat cells, which also undergo apoptosis in response to adaphostin, we will conduct Western blotting for these two proteins in treated and untreated cells. These data will be confirmed in AML patient isolates.

Experiment 2. Is cbl altered in adaphostin treated cells?

Cbl is an adaptor molecule which associates with bcr/abl and with other kinases. Interestingly, cbl is also a ubiquitin ligase which targets its client proteins to the proteasome. We will examine expression of cbl, phospho-cbl and ubiquitinated cbl using Western blotting and immunoprecipitation techniques. Also, the contribution of cbl to adaphostin toxicity will be assessed by testing the efficacy of adaphostin in cbl deficient DT-40 cells.

Experiment 3. Does inhibition of specific kinases prevent ROS production by adaphostin?

In order to determine the relative contribution of other signaling pathways in the cytotoxicity of adaphostin, we will use pharmacological and genetic approaches to block the pathways and then assess intracellular superoxide and peroxide production by adaphostin in K562 and ML-1 cells. Also, dose responses of these compounds will be carried out in the presence of adaphostin to see whether adaphostin's toxicity is diminished, thus implicating these pathways. The following signaling pathways will be examined:

MAPK: Pharmacological inhibition will be attained by using PD98059 and U0126. These structurally distinct inhibitors are commercially available. Genetic inhibition will be accomplished by transient transfection of dominant negative MEK1 and Erk constructs into cell lines and will be assessed by Western blotting with phospho-Erk and phospho-MAPK antibodies.

Src kinases: The structurally distinct src kinase inhibitors BMS354825, PP1, and AG490 will be used. Dominant negative lyn and src constructs will be transiently transfected into cells to determine whether blockade of these pathways alters adaphostin effects. Western blotting with an antibody directed towards the activating phosphorylation site on src kinases will be used to monitor the success of the transient transfections.

NFkB: PS1145 is a small molecule Ikb inhibitor currently in preclinical evaluation. Its effects on adaphostin toxicity will be assessed. Transient transfections with dominant negative Ikb will also be conducted and changes in adaphostin induced ROS generation and DNA fragmentation will be measured. Phospho-Ikb antibodies will be used to track the effectiveness of the transfection strategy.

Follow up experiments for Aim 2: The above experiments will be replicated in AML and CML patient material. For Expt.3 the transient transfections will not be attempted in primary cells due to difficulty associated with this technique in primary material. Instead, the pharmacological inhibition experiments will be conducted.

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