Tumor Growth Inhibition Induced in a Murine Model of Human Burkitt's Lymphoma by a Proteasome Inhibitor

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ABSTRACT

Cell growth and viability are dependent on the function of the multi-catalytic protease complex (proteasome), a multisubunit particle that affects progression through the mitotic cycle by degradation of cyclins. Exposure of rodent fibroblasts and human lymphoblasts in culture to benzyloxycarbonyl-leucyl-leucyl-phenylalaninal (Z-LLF-CHO), a cell-permeable peptidyl aldehyde inhibitor of the chymotrypsin-like activity of the proteasome, resulted in the induction of apoptosis in a rapid, dose-dependent fashion. Fibroblasts transformed with ras and myc, lymphoblasts transformed by c-myc alone, and a Burkitt's lymphoma (BL) cell line that overexpresses c-Myc were up to 40-fold more susceptible to apoptosis than were either primary rodent fibroblasts or immortalized nontransformed human lymphoblasts, respectively. To determine whether such preferential apoptosis could impact upon tumor growth in vivo, toxicological studies were performed in mice with severe combined immunodeficiency and showed that mice tolerated single intraperitoneal doses of Z-LLF-CHO without unacceptable toxicity. Severe combined immunodeficient mice bearing c-Myc tumors in the flank were treated intraperitoneally with Z-LLF-CHO or a comparable dose of the peptidyl alcohol (Z-LLF-OH), which does not induce proteasome inhibition or apoptosis. Single doses of Z-LLF-CHO induced statistically significant (P < 0.0001) early tumor regression and a significant (P < 0.0001) delay in tumor progression. Analysis of tumor specimens revealed increased apoptosis in BL tumors from mice treated with Z-LLF-CHO. These results, showing a 42% tumor growth delay, indicate that proteasome inhibitors have the potential of curbing the growth of a c-myc-related tumor.

INTRODUCTION

Degradation of many cytoplasmic and nuclear proteins depends on the function of the proteasome, which is emerging as the major factor in extralysosomal cytoplasmic and nuclear proteolysis (1, 2). Proteasomal activity is necessary for cell growth by regulating the entry and exit to and from the mitotic cycle through timely degradation of cyclins and cyclin-dependent kinases (3). The proteasome is also associated with many other important functions, including degradation of transcription factors and other short-lived regulatory proteins, antigen processing, and both Ub-dependent and Ub-independent proteolysis (1, 2). Studies have shown that the proteasome is absolutely necessary for cell survival and proliferation. Indeed, disruption of any one of 13 of the 14 genes encoding its subunits has been shown to be lethal to cells (4). Substrate and inhibitor studies of the proteasome from bovine tissues have identified five distinct catalytic components (5-7), designated as ChT-L, BrAAP, trypsin-like, small neutral amino acid-prefering, and peptidylglutamyl peptide-hydrolyzing, based on the nature of the amino acid residue providing the carbonyl group to the bond undergoing cleavage (5-7). Two of these, the ChT-L and the BrAAP activities, constitute major factors in the protein-degrading activity of the proteasome (8, 9).

A series of substrate-related peptidyl-aldehyde inhibitors have been synthesized with marked selectivity toward the proteasome and its various catalytic components (8, 9). Exposure of cells in culture to inhibitors of the ChT-L and BrAAP activities causes accumulation of Ub-protein conjugates, indicating interference with the Ub-dependent pathway of intracellular proteolysis (9, 10). Cells grown in the presence of peptidyl-aldehydes or lactacystin, which inhibit all of the activities of the proteasome or the ChT-L activity alone, are induced to undergo apoptosis (11-16). In fibroblastic cell lines, this activation of programmed cell death has been attributed to the accumulation of the p53 tumor suppressor protein, because dominant-negative p53 mutants interfere significantly with this process (13). In p53-null HL60 cells, the p27Kip1 cyclin-dependent kinase inhibitor is believed to be responsible for this apoptosis, because p27 accumulates in a time course that parallels the induced cellular death (17), and its overexpression has been demonstrated to result in apoptosis (18, 19).

c-myc is part of a family of related genes the protein products of which function as nuclear transcription factors and have roles in a variety of cellular processes (20). Expression of c-myc is associated with proliferation and progression through the cell cycle (21). Such expression can inhibit differentiation in some systems (22, 23). c-myc, in combination with an activated ras gene, transforms primary rat embryo cells (24), and c-myc alone can transform already established immortal cell lines (25, 26). Most recently, expression of c-myc has been noted to induce apoptosis, or programmed cell death, after withdrawal of mitogenic stimuli (27-29). The c-myc gene has clinical importance as well, because mutations of c-myc have been discovered in many tumors, including breast carcinomas (30, 31), colorectal carcinomas (32, 33), gynecological carcinomas (34, 35), and BLs and B-cell acute lymphoblastic leukemias (36-38). Thus, stringent control of the activity of c-myc is essential for the viability of normal cells.

In the course of examining the involvement of the Ub-proteasome pathway in degradation of the human c-myc oncogene product, we noted that inhibition of proteasome activity by a cell-permeable peptidyl aldehyde causes accumulation of the c-Myc protein and, as reported here, induces apoptosis preferentially in c-myc-transformed fibroblasts and lymphoblasts. To determine whether this effect could be exploited to slow tumor growth in vivo, mice with SCID were studied and found to tolerate single s.c. injections of Z-LLF-CHO, an inhibitor with marked selectivity toward the ChT-L activity of the proteasome. SCID mice bearing c-myc-transformed tumors that re-
ceived this inhibitor showed a tumor growth delay compared with control animals, and their tumors underwent increased apoptosis in vivo. These results indicate that proteasome inhibitors merit further study as agents that could slow tumor growth.

MATERIALS AND METHODS

Cell Lines and Cell Culture. REF cells are primary fibroblasts harvested previously from 13-day-old pregnant Fisher rats (15, 16). REF-ras/myc cells are REF cells transformed with the EJ ras and c-myc oncogenes (15, 16). Fibroblast lines were grown in DMEM containing 9% fetal bovine serum, penicillin G, and streptomycin (all reagents from Life Technologies, Inc., Grand Island, New York). CB33 cells are a line established from human umbilical cord blood lymphocytes that were immortalized by cocultivation with culture fluid of the B95-8 mammoset lymphoma line containing EBV (39). CB33/myc cells are derived from the parental CB33 line and overexpress exogenous human c-Myc protein. They were generated by transfection of a vector containing human c-myc under the control of the SV40 enhancer/promoter region into CB33 cells (Ref. 39; both were generous gifts of R. Dalla-Favera, Columbia University College of Physicians and Surgeons, New York, NY). The Ramos cell line is an EBV-negative immortal transformed line derived originally from a BL tumor specimen (Ref. 40; American Type Culture Collection, Rockville, MD). Lymphocyte-derived cell lines were grown in suspension culture in RPMI 1640 containing 9% fetal bovine serum, penicillin G sodium at 100 units/ml, and streptomycin sulfate at 100 ¡g/ml, respectively (Life Technologies, Inc.). All cells were propagated in incubators providing a humidified atmosphere, 5% CO2, and were passed at least three times per week. For inhibitor-related assays, REF and REF-ras/myc fibroblasts were trypsinized (Life Technologies, Inc.), counted using a hemocytometer (Hauser Scientific; Horsham, PA), seeded onto Falcon 3025 tissue culture plates (Becton Dickinson Co., Cockeysville, MD) at a density of 2 × 105 cells per plate, fed with fresh DMEM, and allowed to recover for 12–15 h. CB33, CB33/myc, and Ramos cells were also counted using a hemocytometer (Hauser Scientific), and 1.5 × 105 cells were seeded onto Corning 25020 tissue culture plates (Corning Glass Works, Corning, NY). They were fed with fresh RPMI and allowed to recover for 12–15 h. For SCID mice-related assays, Ramos cells were grown in the absence of antibiotics and then certified as Mycoplasma free by the Johns Hopkins Tissue Culture Facility. They were then collected, washed twice with PBS (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, pH 7.4), and counted prior to injection.

SCID Mice. Male Fox Chase CB-17 SCID mice were obtained at 5–7 weeks of age (Taconic, Germantown, NY) and housed under aseptic, virus-free conditions in the Johns Hopkins Animal Care Facility. They were provided with autoclaved food and water containing trimethoprim sulfamethoxazole (Alpharma USPD, Inc., Baltimore, MD), which they partook of ad libitum.

Proteasome Inhibitors. Z-LLF-CHO was synthesized by oxidation of the corresponding peptidyl-alcohol Z-LLF-OH using the method of Pfister and Moffatt (41) essentially as described previously (8) and purified by crystallization from ethanol-water. Z-LLF-CHO was found to be a slow binding potent inhibitor of the Cch-L activity of the bovine pituitary MPC with Ks of 0.46 ¡mM (8). For cell experiments, stock solutions of protease inhibitors were prepared at concentrations of 10 mM in 100% ethanol (The Warner-Graham Co., Cockeysville, MD) and stored at 4°C. These were added to cells in tissue culture to the final concentrations indicated in the text, and final volumes were equalized using 100% ethanol. For animal experiments, stock solutions of Z-LLF-CHO and Z-LLF-OH were prepared at concentrations of 45 mg/ml in 100% DMSO (J. T. Baker, Inc.,Phillipsburg, NJ). These were filter sterilized prior to injection using 0.2 ¡m Millex-FG filters (Millipore Products Division, Bedford, MA) and stored at 4°C prior to use.

Apoptosis Assays. To specifically identify cells undergoing apoptosis in tissue culture specimens, an in situ TUNEL assay adapted from Gorczyca et al. (42) was performed. Briefly, after incubation of cells with proteasomal inhibitors, the cultures were collected and fixed in PBS containing 1% formaldehyde, permeabilized with 70% methanol, and stored at –20°C (these reagents were from J. T. Baker, Inc.). To perform the TUNEL assay, cells were washed with PBS and then 1× TDT buffer, consisting of 0.2 M sodium cacodylate, 0.25 mg/ml BSA, 2.5 mM CoCl2, and 25 mM Tris (pH 6.6; these reagents from Sigma Chemical Co., St. Louis, MO). Samples were resuspended in 1× TDT buffer containing 5.0 units of TDT enzyme and 0.50 nmol of biotinylated dUTP (both from Boehringer Mannheim Corporation, Indianapolis, IN). These reactions were incubated at 37°C for 30 min, and 1.0 ml of staining solution was added, consisting of 4× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate; Sigma Chemical Co.), 0.1% Triton X-100 (Sigma Chemical Co.), 0.2% nonfat dry milk (Super G, Inc., Landover, MD), and 50 ¡g/ml of heat-inactivated RNase A (Boehringer Mannheim Corp.). These were incubated at room temperature for 30 min before analysis by FACS. This analysis was performed on a Coulter Epics V flow cytometer (Coulter Corp., Hialeah, FL), with red propidium iodide fluorescence being acquired on a linear scale and green FITC staining being acquired on a log scale. The data from 1 × 106 cells were collected, stored, and analyzed using Multiparameter Data Acquisition and Display System-86 version 2.0 software (Coulter Corp.).

To generate the graphs shown in the figures, percentages of cells undergoing apoptosis derived from analyses by Multiparameter Data Acquisition and Display System-86 software were tabulated in KaleidaGraph version 3.0.1 (Synergy Software, Reading, PA). All cell lines, even without the addition of a proteasome inhibitor, were noted to have small, measurable baseline levels of apoptosis staining. The data were adjusted so that the percentage of apoptosis without added proteasome inhibitor was set at zero (control), and the remaining data points for each experiment were adjusted accordingly. Mean percentages and SEs were then calculated and plotted in KaleidaGraph (Synergy Software).

To specifically identify cells undergoing apoptosis in tumor specimens, an in situ TUNEL assay was performed to label apoptotic DNA with digoxigenin using the Oncor ApoTag kit according to the manufacturer's specifications (Oncor, Inc., Gaithersburg, MD). Digoxigenin-labeled DNA was then detected in an enzyme-linked immunoassay using anti-digoxigenin antibody conjugated to alkaline phosphatase with the DIG DNA Labeling and Detection kit (Boehringer Mannheim Corp.) according to the manufacturer's specifications. Counterstaining was performed using a standard eosin staining protocol.

Toxicology. These experiments were performed under a protocol approved by the Johns Hopkins Animal Care and Use Committee. SCID mice were weighed using an Ohaus SC6010 scale (Ohaus Corp., Florham Park, NJ). They were then injected s.c. in the interscapular region using 25-gauge, 1 ml syringes (Becton Dickinson Co.) with 100 ¡l of either DMSO alone or 100 ¡l of a solution of Z-LLF-CHO in DMSO diluted from the stock solution with filter-sterilized DMSO to deliver the necessary inhibitor doses. Mice were then observed daily and weighed three times a week to monitor for any unacceptable toxicity, as described in the text.

In Vivo Tumor Experiments. These experiments were performed under a protocol approved by the Johns Hopkins Animal Care and Use Committee. SCID mice were injected s.c. in the right flank with 100 ¡l of filter-sterilized PBS containing 100 ¡g Mycoplasma-free Ramos BL cells. They were then monitored for the development of s.c. nodules. Once these tumors developed, their bidirectional dimensions in millimeters were measured using Vernier type calipers (Bel-Art Products, Pequannock, NJ), and tumor weights in milligrams were calculated using the formula for a prolate ellipsoid, \( V = \frac{4}{3} \pi a b c \), where \( a \) is the longer of the two dimensions (43, 44). When tumors reached a size of 150–250 mg, animals were sequentially assigned to receive 100 ¡l s.c. of interscalpular injections of DMSO containing either 150 mg/kg of Z-LLF-CHO or Z-LLF-OH. Injections were performed on day 0 only, and no subsequent injections were administered. These mice were then followed by serial daily tumor mass measurements that were performed in a fashion blinded to the treatment assignment. To minimize distress, those animals whose tumors exceeded 1500 mg in size were euthanized according to guidelines established by the American Veterinary Medical Association’s Panel on Euthanasia (43, 44). Comparisons that were made between the two groups included changes in the tumor mass, days to reach the 1500-mg end point, and growth delay of tumors in treated animals (equations of 7 – CYC × 100, where T and C are median times for tumors to reach a weight of 1500 mg for the treated and control groups; Refs. 43 and 44). Statistical comparisons were made using the JMP IN 3 Software package for the Macintosh (SAS Institute, Inc. and Wadsworth Publishing Co., Belmont, CA).
For direct analysis of tumor tissue, SCID mice were injected with $10^7$ Mycoplasma-free Ramos BL cells and monitored as described above. Once tumors reached a size of 150–200 mg, mice were injected with either saline, DMSO, Z-LLF-OH in DMSO, or Z-LLF-CHO in DMSO, and then sacrificed 24 h later according to guidelines established by the American Veterinary Medical Association’s Panel on Euthanasia (43, 44). Tumors were immediately removed by dissection, frozen in Tissue-Tek OCT 4583 Compound (Sakura Finetek U.S.A., Torrance, CA) by immersion in 2-methylbutane (Sigma Chemical Co.) in a dry ice bath, and stored frozen until use. Sections of these specimens were prepared using a Reichert-Jung model 2800 Frigocut E microtome (Heizscheibe, Germany) and allowed to desiccate on glass slides at 4°C overnight. Apoptosis was detected using an in situ TUNEL assay as described above, slides were visualized by light microscopy, and representative fields were photographed by the Pathology Photographic Service at Johns Hopkins. Composite figures were prepared by the Pathology Photographic Service using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

RESULTS

Preferential Apoptosis of Transformed Cells Due to Inhibition of the Cht-L Activity of the Proteasome. In the course of investigating the pathways responsible for degradation of the c-myc oncogene product, primary REF cells transformed with the oncogenes EJras and c-myc (REF-ras/myc; Refs. 15 and 16) were exposed to Z-LLF-CHO, a cell-permeable inhibitor with marked specificity for the Cht-L activity of the proteasome found in pituitary tissue (7, 8). These cells underwent dramatic morphological changes at the light microscopic level, during which their plasma membranes became less distinct, the fibroblasts lost their characteristic spindly protrusions, becoming round instead, and finally, they would detach from the culture plate and float freely (data not shown). The parental REF cells underwent similar morphological changes but did so at a lower frequency (data not shown). To determine whether these changes were due to apoptosis and to quantitatively compare the extent of these changes, an in situ TUNEL assay was performed, and samples were analyzed by FACS scanning (42). Comparison of REF cells with REF-ras/myc cells revealed that both developed apoptotic changes in proportions that depended on the dose of inhibitor used (Fig. 1). At all of the concentrations tested, transformed REF-ras/myc cells were more sensitive than primary REF cells to the induction of apoptosis. This difference was most notable at 5 μM, where 34.7% (± 3.4%) of REF-ras/myc cells were apoptotic compared with 12.7% (± 4.0%) of REF cells, indicating that the transformed cells were 2.7-fold more sensitive to Z-LLF-CHO.

To determine whether this finding could be confirmed in other cell lines and to attempt to determine whether the activation of c-myc alone increased this sensitivity to apoptosis, the EBV-immortalized CB33 human lymphoblastoid line was compared in culture with CB33-myc cells. These two cell lines were exposed to Z-LLF-CHO at varying concentrations, and the extent of induced apoptosis was evaluated by a TUNEL assay (42). In addition, the EBV-negative lymphoblastoid cell line Ramos, derived originally from a patient’s BL tissue (40), was also evaluated in this fashion. A comparison of the three cell lines studied revealed that they all developed apoptotic changes in a dose-dependent fashion (Fig. 2). The sensitivities of the transformed cell lines CB33-myc and Ramos were comparable to each other, and both were significantly more sensitive to the induction of apoptosis than was the CB33 cell line, which is immortalized but not transformed. This difference was most notable at an inhibitor concentration of 1 μM, where 34.9% (± 3.8%) of CB33-myc and 40.5% (± 2.3%) of Ramos cells were apoptotic, compared with 9.9% (± 1.5%) of CB33 cells, indicating that the transformed cells were 38.8- to 45.0-fold more sensitive to Z-LLF-CHO, respectively. The wide difference in the extent of apoptosis tended to decrease with increasing inhibitor concentrations. Even at 50 μM, however, where the difference was least notable, 66.4% (± 4.6%) of CB33-myc and 77.5% (± 1.1%) of Ramos cells were apoptotic, compared with 27.0% (± 2.7%) of CB33 cells, representing a 2.5- to 2.9-fold increased sensitivity to Z-LLF-CHO, respectively.

Toxicological Studies in Mice Show That Single Doses of a Proteasome Inhibitor Are Well Tolerated. The 45-fold increased sensitivity of Ramos cells to the induction of apoptosis by Z-LLF-CHO compared with nontransformed CB33 cells raised the possibility that a proteasome inhibitor might be effective in curbing the growth of this c-myc-related tumor in vivo with limited toxicity. To evaluate the toxicity of the inhibitor in an animal model system, experiments were initiated in SCID mice. Stock solutions of Z-LLF-CHO were prepared in DMSO to allow a broad range of doses to be tested in a small volume of injectate. SCID mice were then injected interscapularly with single 100 μl doses of DMSO alone, or Z-LLF-CHO in DMSO, and toxicity was assessed for 2 weeks by evaluating the mice for weight loss, distress, or death (43, 44). These experiments were performed under a protocol approved by the Johns Hopkins Animal Care and Use Committee. Injections of DMSO alone were well tolerated with no appreciable weight loss (Fig. 3). Toxicological studies with Z-LLF-CHO were initiated at a dose of 1 mg/kg which, if the inhibitor were to distribute evenly throughout the total body water, would have been expected to result in a final concentration of ~2.5 μM. This was within the concentration range that had shown the ability to preferentially induce apoptosis in c-myc-transformed cells in culture (Fig. 2). Furthermore, given the hydrophobic nature of the compound, a substantial portion of the inhibitor could have been expected to accumulate in tissue lipids, resulting in an even lower final concentration. Consistent with this possibility, doses of Z-LLF-CHO of 1 and 10 mg/kg were well tolerated and did not impact appreciably on the weight of the mice (Fig. 3). At 100 mg/kg, the first signs of systemic effects were noted, with an average weight loss of 4.4% and a range from 2.7 to 6.8% (Fig. 3). The next and final dose escalation was to 150 mg/kg, because the solubility of Z-LLF-CHO did not allow higher doses to be administered in a well-tolerated single injection of DMSO (45–47). At this dose level, the average

Fig. 1. Apoptosis of REF and REF-ras/myc fibroblasts induced by proteasome inhibition. REF cells and REF cells transformed with the oncogenes ras and myc were exposed to Z-LLF-CHO at the indicated concentrations for 8 h. Monolayer cultures were then collected, subjected to the TUNEL apoptosis assay, and analyzed by PACScan. The mean percentage of cells undergoing apoptosis is shown at each concentration, along with the SE (bars) from eight experiments.
weight loss was 6.7%, with a range from 4.4% to 8.1% (Fig. 3). None of the animals evaluated in this fashion suffered any obvious distress, and all survived the toxicological studies. Thus, single doses of the inhibitor were well tolerated, without the appearance of any unacceptable complications.

Tumor Growth Delay Can Be Induced in Tumor-bearing Mice Injected with a Single Dose of a Proteasome Inhibitor. To determine whether a single inhibitor dose had any potential to limit the growth of a c-myc-related tumor, experiments were performed using the Ramos cell line, which had shown high sensitivity to apoptotic induction by proteasome inhibition (Fig. 2). SCID mice were therefore injected s.c. with 10^7 Mycoplasma-free Ramos cells in the right flank (48, 49), the sizes of any evident tumor masses were measured using calipers, and calculations of the tumor weight were made using the formula for a prolate ellipsoid (43, 44). These experiments were performed under a protocol approved by the Johns Hopkins Animal Care and Use Committee. Once animals developed a tumor mass of 150-200 mg, a process that generally took 2-4 weeks, they were treated with Z-LLF-CHO at 150 mg/kg in DMSO. Twenty-four h later, each animal was treated either by s.c. interscapular injection of 100 µl of saline, DMSO, Z-LLF-OH at 150 mg/kg in DMSO, or Z-LLF-CHO at 150 mg/kg in DMSO. Twenty-four h later, each animal was euthanized according to guidelines established by the American Veterinary Medical Association’s Panel on Euthanasia (43, 44). The s.c. tumor nodules were then dissected free and frozen for routine histology. These revealed a large, monotonous population of small cells with round or oval nuclei containing several nucleoli, characteristic of the appearance of BL cells, thereby confirming the presence of these tumors (data not shown). Adjacent sections were evaluated for the presence of apoptosis using an in situ TUNEL assay (Fig. 5). Sections of tumor specimens from animals treated with Z-LLF-OH as controls showed some background level of apoptosis (Fig. 5, A and B), which was comparable with that seen in sections

the alcohol analogue of the inhibitory peptidyl-aldehyde, that neither inhibits proteasome activity nor induces apoptosis in cells grown in culture. Furthermore, mice treated in the small pilot trial with DMSO alone reached the 1500-mg tumor size end point in 7.3 days, whereas Z-LLF-OH-treated mice in the current trial reached the same end point in 6.5 days. Thus, the peptidyl-alcohol does not induce any tumor growth delay and serves as an adequate control for the active peptidyl-aldehyde proteasome inhibitor. After the initial injections of either Z-LLF-CHO or Z-LLF-OH on day 0, no subsequent treatment was administered to either of the two groups of animals. Mice treated with Z-LLF-CHO underwent early tumor regression, with an average tumor weight decrease of 13.3% from day 0 to day 1, compared with an increase of 89.1% for the Z-LLF-OH group (P < 0.0001; Fig. 4). After this initial decrease, Z-LLF-CHO-treated tumors began to expand and reached the 1500-mg end point within 9.2 days (±0.3), whereas Z-LLF-OH-treated tumors did so in 6.5 days (±0.4; P < 0.0001). These results demonstrate a 42% tumor growth delay and indicate that a single dose of a proteasome inhibitor can curb the growth of a c-myc-related tumor in this system.

BL Tumors Undergo Apoptosis in Vivo after Proteasome Inhibitor Administration. To determine whether the Ramos BL cells in SCID mice tumors underwent increased apoptosis after proteasome inhibition as they did in cell culture, a third group of tumor-bearing animals was examined. Once tumors of 150–200 mg developed, one animal each was treated either by s.c. interscapular injection of 100 µl of saline, DMSO, Z-LLF-OH at 150 mg/kg in DMSO, or Z-LLF-CHO at 150 mg/kg in DMSO. After this initial decrease, Z-LLF-CHO-treated tumors began to expand and reached the 1500-mg end point within 9.2 days (±0.3), whereas Z-LLF-OH-treated tumors did so in 6.5 days (±0.4; P < 0.0001). These results demonstrate a 42% tumor growth delay and indicate that a single dose of a proteasome inhibitor can curb the growth of a c-myc-related tumor in this system.
Fig. 4. Statistical evaluation of tumor growth in mice receiving a proteasome inhibitor. A group of 20 SCID mice bearing s.c. lymphomas of 150–250 mg in calculated weight were injected once on day 0 with either Z-LLF-CHO in DMSO to deliver a dose of 150 mg/kg in the interscapular region or Z-LLF-OH in DMSO at a comparable dose. No injections were performed after this first administration of agent on day 0. These mice were then examined daily for changes in tumor size. When tumors reached 1500 mg, animals were euthanized to avoid undue distress according to guidelines from the American Veterinary Medical Association’s Panel on Euthanasia (43, 44). The mean tumor weight for all mice surviving at each day of treatment is indicated in the graph, and SEs (ns) are indicated as well. The apparent decrease in tumor size of the Z-LLF-OH-treated group from day 7 to day 8 is artifactual in that it does not indicate actual tumor shrinkage. It is due to the euthanasia of animals with large tumors on day 7, resulting in only a single animal surviving to day 8 and 9, which had a smaller tumor on day 8.

from animals that had received either saline or DMSO alone (data not shown). Those sections of tumors from animals that had received Z-LLF-CHO, however, showed an increased level of apoptosis compared with the controls (Fig. 5, C and D) 24 h after administration of the inhibitor. Thus, administration of the proteasome inhibitor Z-LLF-CHO resulted in apoptosis of c-myc-transformed cells in vivo as it did in cell culture. It is therefore likely that apoptosis induced by the inhibitor is responsible for the tumor shrinkage and growth delay observed in this model of human BL.

**DISCUSSION**

**Enhanced Apoptosis Due to Proteasome Inhibition.** The multicatalytic proteinase complex, or proteasome, is an integral part of the cellular machinery responsible for turnover of intracellular proteins. Given its role in the degradation of cyclins and cyclin-dependent kinases, as well as its role in the degradation of many short-lived transcription factors and oncogene products (1, 2), one would suspect that interference with its activity would have deleterious consequences for cells. Consistent with this prediction, several investigators have noted that inhibition of the proteasome with synthetic, cell-permeable peptidyl aldehydes or naturally occurring inhibitors such as lactacystin induces a variety of cell lines in culture to undergo apoptosis (11–16). Our studies with the REF, REF-ras/myc, CB33, CB33-myc, and Ramos cell lines indicate that rodent fibroblasts and human lymphoblastoid cells are similarly sensitive to proteasome inhibition and react by activating the programmed cell death pathway. A unique finding is that the transformed REF-ras/myc, CB33-myc, and Ramos cell lines indicate that rodent fibroblasts and human lymphoblastoid cells are similarly sensitive to proteasome inhibition and react by activating the programmed cell death pathway. A unique finding is that the transformed REF-ras/myc, CB33-myc, and Ramos cells are much more sensitive to this apoptotic induction than are primary REF cells or EBV-immortalized but not transformed CB33 cells, respectively, which is seen with short exposures to this proteasome inhibitor. Because proteasome function is essential to cell viability, prolonged exposure to sufficient concentrations of such an inhibitor ultimately results in apoptosis of the entire cellular population. Ramos BL cells, which are myc-transformed and immortalized but EBV negative, have a sensitivity comparable with that seen in the

Fig. 5. Induction of apoptosis in vivo by a single injection of a proteasome inhibitor in SCID mice. To determine whether administration of a proteasome inhibitor had induced apoptosis of lymphoma cells in vivo, SCID mice bearing 150–200 mg Ramos tumors were treated with either Z-LLF-OH, the inactive alcohol analogue of the active inhibitor, or Z-LLF-CHO itself, and sacrificed 24 h later. The tumor specimens were dissected free, frozen, and subsequently sectioned; apoptosis was detected using an in situ TUNEL-based method with eosin as a counterstain. Areas of the tumor that are undergoing apoptosis appear blue with this alkaline phosphatase-based technique. All fields shown are magnified 250-fold.
CB33-myc cells (Fig. 2), indicating that this phenomenon is not likely to be due to an EBV-related activity. In the REFIREF-ras/myc system, increased apoptotic sensitivity could be due to the action of ras, myc, some combination of these oncogenes, or due to the transformation process itself. In the CB33/CB33-myc system, results showing that CB33-myc cells are also more sensitive imply that the presence of ras is not required. Finally, we have also compared the transformed human small cell lung carcinoma cell line NCI H209 with NCI H209-myc cells and found that the myc-overexpressing cells again undergo enhanced apoptosis in the presence of a proteasome inhibitor. Thus, this enhanced apoptosis seems to be myc related and not due to the transformation event.

Cells exposed to proteasome inhibitors have been noted to arrest at various points in the cell cycle, but those that seem to undergo apoptosis most readily appear to be traversing the G1-S boundary (11–16). As a result, proteins that impact on this transition point, such as p53 and p27Kip1, have been suggested to be of importance in the mechanism of induction of apoptosis by proteasome inhibitors. In Rat 1 cells, dominant-negative p53 mutations seem to interfere with this apoptotic process (13), and we have noted that, although p53 is not absolutely necessary for inhibitor-mediated apoptosis, it is required for the enhanced apoptosis in myc-transformed fibroblasts. Ramos cells carry a mutated p53 allele (50), however, and therefore the enhanced apoptosis seen in these cells compared with the CB33 line must be due to the involvement of a different pathway. In p53-null HL60 cells, the p27Kip1 cyclin-dependent kinase inhibitor is believed to be responsible for this apoptosis because its accumulation parallels cellular death (17), and recent studies have shown that overexpression of p27 results in cell cycle arrest and apoptosis (18, 19). Proteasome inhibition results in accumulation of c-Myc protein5; therefore, in c-Myc-overexpressing cells, even higher levels of Myc protein would be expected to accumulate than in cells with baseline Myc levels. Because such cells would be driven to cycle more rapidly and proteasome inhibition also results in accumulation of p27, these cells would be faced with conflicting growth-promoting and growth-inhibiting signals. This conflict, which has been suggested to trigger apoptosis (11–16), could lead to higher levels of apoptosis in c-Myc-overexpressing cells than in comparable cells with normal c-Myc levels, as observed in this study. Direct experimental verification of this hypothesis will be necessary, however, before it or some other mechanism is accepted as accounting for the enhanced apoptosis seen in c-myc-transformed lymphoblastoid cells.

**Tumor Growth Delay Due to Proteasome Inhibition.** The more than 40-fold enhanced apoptosis induced in Ramos BL cells by proteasome inhibition suggested that in vivo growth of a lymphoma could be impacted upon as well. A well-tolerated dose of 150 mg/kg that did not cause undue weight loss or obvious distress was therefore administered s.c. in the interscapular region to SCID mice bearing BL tumors. Small hydrophobic peptides such as Z-LLF-CHO dissolved in DMSO would be expected to distribute rapidly from the s.c. space into the bodies of SCID mice (42–44) and reach tumors growing in the flank. Indeed, such single injections caused a significant decrease in tumor mass when measured on day 1 after administration and a 42% growth delay of the tumor. However, because of the high lipid solubility of the inhibitor, plasma levels after a single injection would be expected to result in a short-lived peak concentration, followed by a rapid fall to very low levels because of extensive distribution to tissues. Thus, an increased effect of the inhibitor on tumor growth might be produced by multiple injections or prolonged infusions at a rate that would result in concentrations optimal for inducing apoptosis while minimizing toxicity to normal tissues. Mice will not tolerate higher doses of DMSO than those used in this study, however; therefore, less toxic means of solubilizing this hydrophobic compound are being actively investigated. The availability of such techniques should allow delivery of increased doses, and in combination with improved delivery schedules, will allow better delineation of the potential efficacy of this compound, as well as a more detailed evaluation of its toxicity.

Experiments indicate that the ChT-L activity is expressed by the X subunit of the proteasome, and that the NH2-terminal threonine residue of this subunit is involved in the catalytic process (51). The inhibition of the ChT-L activity by Z-LLF-CHO is apparently based on the formation of a reversible hemiacetal adduct with the hydroxyl group of the NH2-terminal threonine. Aldehydes, however, are reactive compounds susceptible to enzymatic oxidation reactions that would be expected to shorten their half-lives in vivo. These factors would tend to limit the duration and antitumor efficacy of such inhibitors. It is therefore not surprising that tumor regression after a single injection was of short duration and also supports the possibility that longer inhibitor infusions would be needed to maintain concentrations in the plasma that would be optimal for greater tumor regression.

**In Vivo Apoptosis.** To verify that tumor regression and growth delay in vivo were due to the induction of apoptosis rather than some other mechanism, sections of the tumors were prepared and analyzed histochemically. H&E staining verified that the s.c. nodules contained cells characteristic of a BL. A specific in situ TUNEL-based assay demonstrated that administration of a proteasome inhibitor indeed induced apoptosis in these lymphoma cells in vivo as it had in tissue culture. That a single s.c. dose of a proteasome inhibitor can result in significant tumor regression, in vivo tumor cell apoptosis, and a 42% tumor growth delay indicates that the proteasome deserves further study as a potential target for antineoplastic therapy.

**ACKNOWLEDGMENTS.** We thank Dr. Ricardo Dalla-Favera (Columbia University College of Physicians and Surgeons, New York, NY) for his gift of the CB33 and CB33-myc cell lines. We also thank H. Shim and S. Campbell for technical advice, C. Ytkin for support, and H. Shim for critical review of the manuscript.

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