RAPID COMMUNICATION

BB-10010: An Active Variant of Human Macrophage Inflammatory Protein-1α With Improved Pharmaceutical Properties


The stem cell inhibitor, macrophage inflammatory protein-1α (MIP-1α) or LD78, protects multipotent hematopoietic progenitors in murine models from the cytotoxic effects of chemotherapy. Clinical use of human MIP-1α during chemotherapy could therefore lead to faster hematologic recovery and may allow dose intensification. We have also shown that human MIP-1α causes the rapid mobilization of hematopoietic cells, suggesting an additional clinical use in peripheral blood stem cell transplantation. However, the clinical evaluation of human MIP-1α is complicated by its tendency to associate and form high molecular weight polymers. We have produced a variant of rhMIP-1α, BB-10010, carrying a single amino acid substitution of Asp26 > Ala, with a reduced tendency to form large polymers at physiologic pH and ionic strength. This greatly increases its solubility, facilitating its production and clinical formulation. We confirmed the potency of BB-10010 as a human MIP-1α-like agonist in receptor binding, calcium mobilization, inhibition of colony formation, and thymidine suicide assays. The myeloprotective activity of BB-10010 was shown in a murine model of repeated chemotherapy using hydroxyurea. BB-10010 is therefore an ideal variant with which to evaluate the therapeutic potential of recombinant human MIP-1α.

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WE ARE INTERESTED in exploring the clinical application of both the myeloprotective1,6 and progenitor mobilizing3 activities of recombinant human macrophage inflammatory protein-1α (rhMIP-1α). The clinical use of rhMIP-1α is compromised because, at physiologic ionic strength, the 8-kD monomeric molecule self-associates to form heterogeneous, multimeric complexes of mass ranging from 100 to more than 250 kD.8 We and others have determined that, using certain solution conditions, such as high salt concentration, it is possible to dissociate MIP-1α.6,11 However, such conditions might not maintain the dissociated state in vivo and would unacceptably limit the conditions that could be used in manufacture. Biophysical characterization of the polymerization pathway showed that high ionic strength buffers substantially reduced multimerization beyond a tetramer. This indicated that electrostatic interactions were important in the formation and stabilization of higher molecular weight complexes. To dissociate rhMIP-1α to its monomeric subunits, relatively harsh conditions of acidified acetonitrile were required, suggesting that the major stabilizing force for the tetrameric unit is hydrophobic.8

To avoid these production and formulation problems of human MIP-1α, we have used mutagenesis to improve the solution characteristics of the protein while retaining its biologic potency.12 A similar approach has been described by Graham et al.,13 who used a mutagenesis strategy to prevent the aggregation of murine MIP-1α. Based on evidence that the C-terminus of rhMIP-1α was important in multimerization,8 they made three variants with successive neutralization of acidic residues in the C-terminal α helix. Because the C-terminal charge was neutralized, the size of the variant decreased. We chose to adopt a more extensive mutagenesis strategy that did not assume which residues were involved in polymerization. This involved more than 150 variants of the 69 amino acid human protein.12 Our goal was to identify a variant with improved solution properties, wild-type activity, and minimal structural perturbation. We have identified a variant, BB-10010 (Fig 1), carrying a single amino acid substitution of Asp26 > Ala with significant changes in its solution characteristics. The solution properties of rhMIP-1α and BB-10010 were compared using size exclusion chromatography and analytical ultracentrifugation. The biologic activities of the two proteins were compared using receptor binding, calcium mobilization, inhibition of colony formation, and thymidine suicide assays. The myeloprotective activity of BB-10010 in vivo was shown in a murine model of repeated chemotherapy using hydroxyurea. We show here that BB-10010 has improved solution characteristics but retains the biologic activity of rhMIP-1α. We are now evaluating its clinical potential in studies of both stem cell protection and peripheral blood stem cell transplantation.

MATERIALS AND METHODS

Source of MIP-1α. rhMIP-1α and BB-10010 were expressed from synthetic wild-type and Asp26 > Ala variant genes, respectively, according to the methods of Clements et al.3 Both genes encoded a 69 amino acid protein based on the sequence of LD78,14 with an N-terminus of S-L-A-A-D- (Fig 1). Protein was prepared from cell-free supernatants according to the methods described in Patel et al.3 The identity and purity of the rhMIP-1α and BB-10010 were confirmed using electrospray mass spectroscopy, N-terminal sequencing, and analytical reverse-phase high performance liquid chromatography (HPLC). The protein contained less than 2 endotoxin units/mg as determined by the limulus amebocyte lysate (LAL) assay.

Mice. Male B6D2F1 mice weighing 25 to 30 g (Harlan Olac, B6D2F1/Ola/Hsd, Harlan Olac, Bicester, UK) were used and all procedures were performed under Home Office license in accordance with the Animals (Scientific Procedures) Act 1986.

Cell lines and culture conditions. The murine FDCP-mix A4 cell-line15 was cultured in Iscove's modified Dulbecco's medium


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Ser Leu Ala Ala Asp Thr Pro Thr Ala Cys Phe Ser Tyr Thr Ser Arg Gln Ile Pro Gln Asn Phe Ile Ala Ala Tyr Phe Glu Thr Ser Ser Gin Cys Ser Lys Pro Gly Val Ile Phe Leu Thr Lys Arg Ser Arg Gin Val Cys Ala Asp Pro Ser Glu Glu Trp Val Gln Lys Tyr Val Ser Asp Leu Gin Leu Ser Ala

(BIMDM) containing 20% (vol/vol) horse serum, 2% (vol/vol) X63AG8/653 conditioned medium, and 2 mmol/L glutamine. The human monocytic cell line THP-1 (ECACC No. 88081201) was cultured in RPMI 1640 medium containing 2 mmol/L glutamine and 10% (vol/vol) fetal calf serum. The cells were maintained at a density of between 1 and 8 x 10^5 cells/mL, and incubated at 37°C in an atmosphere of 95% air/5% CO₂. L-929 conditioned medium was prepared according to Heyworth and Spooncer. 

Size exclusion fast protein liquid chromatography (IE-FPLC) using a Pharmacia Superdex G-75 HR 10/30 column (Pharmacia, Uppsala, Sweden). Samples at 1 mg/mL in Dulbecco's modified phosphate-buffered saline (PBS) without calcium, magnesium, or bicarbonate (PBS "A") were centrifuged at 10,000 g for 3 minutes and 50 μL of the supernatant was loaded onto a column pre-equilibrated with PBS "A". The sample was then eluted at a flow rate of 1 mL/min and the absorbance of the elute was monitored at 280 nm. The elution profiles of rhMIP-1α and BB-10010 were compared with elution profiles obtained with gel filtration molecular weight markers (BioRad Laboratories, Hercules, CA).

Sedimentation equilibrium analytical ultracentrifugation (AUC). Sedimentation equilibrium AUC was performed using a Beckman XLA analytical ultracentrifuge (Beckman Instruments Inc, Palo Alto, CA) with absorbance optics. Protein samples at 0.1 mg/mL were loaded in a six sector cell with PBS "A" in the reference sector. The samples were spun at 15,000 rpm at 25°C for 18 hours to reach equilibrium and scanned at 230 nm as a function of radius. The scan was repeated after a further 3 hours of centrifugation to confirm that equilibrium had been achieved. Data were analyzed using Microcal Origin software (Micro-C Software, Inc, Northampton, MA) for the Beckman XLA analytical ultracentrifuge. They were fitted to a model assuming a single ideal species (Ideal1) to provide an estimate of the weight average molecular weight. Data sets shown are an average of 20 scans in 0.001 cm increments across the radius of the rotor.

Receptor binding assay. rhMIP-1α was radiolabeled under contract (Amersham International plc, Slough, UK) using the Chloramine T method to a specific activity of 250 μCi/μg. In receptor binding assays, 1 x 10^6 murine FDCP-mix A4 or human THP-1 cells were dispensed into Eppendorf tubes containing different amounts of unlabeled rhMIP-1α or BB-10010 and 3.85 nmol/L [125I]-rhMIP-1α in a total volume of 250 μL of binding medium (RPMI 1640, 20 mmol/L HEPES, and 1 mg/mL bovine serum albumin [BSA]). After incubation at 25°C for 60 minutes, 1 μL of PBS without calcium (PBS "A") was added and mixed, and the cells were harvested by centrifugation. The supernatants were removed and the cells were washed twice with 1 mL of PBS "A". Cell pellets were resuspended in 250 μL of PBS "A" and the radioactivity was determined in a gamma counter. The concentration of unlabeled rhMIP-1α or BB-10010 required to reduce the binding of 3.85 ng/ml [125I]-rhMIP-1α by 50% (IC50) on murine FDCP-mix A4 or human THP-1 cells was estimated in a graph of counts bound against the concentration of cold ligand.

Calcium mobilization assay. Changes in the concentration of intracellular Ca^{2+} were followed using the fluorescent label FURA-2. THP-1 cells were harvested and resuspended to 2 x 10^5 cells/mL in growth medium. FURA-2 AM (Cambridge Bioscience, Cambridge, UK; 1 mg/mL in dimethyl sulfoxide [DMSO]) was added to 1 μmol/L and incubated for 45 minutes at 37°C in an atmosphere of 95% air/5% CO₂. The cells were washed and resuspended in Tyrodes buffer (10 mmol/L HEPES, 129 mmol/L NaCl, 8.9 mmol/L NaHCO₃, 2.8 mmol/L KCl, 0.8 mmol/L KH₂PO₄, 5.6 mmol/L dextrose, 0.8 mmol/L MgCl₂, pH 7.4) to give 2 x 3 x 10^6 THP-1 cells/mL. FURA-2 fluorescence emission intensity was measured at 37°C using a Perkin-Elmer LS-50 fluorimeter with a cell holder and built-in magnetic stirrer. The samples were excited at 340 nm with a 10-nm bandwidth and the emission was continuously recorded at 500 nm with a 5-nm bandwidth. FURA-2-loaded THP-1 cells (2 mL; 2 x 10^6 cells/mL) were transferred to a 4.5 mL UV grade PMMA cuvette (Kartell disposable UV grade PMMA cuvette; Fisons Scientific Equipment, Loughborough, UK); CaCl₂ was added to 1 mmol/L and left to equilibrate for 2 minutes. rhMIP-1α or BB-10010 was added (20 μL, 100X final concentration) and the increase in intracellular calcium was noted. After approximately 60 seconds, digitonin (20 μL, 5 mmol/L digitonin [Sigma, St Louis, MO]) in ethanol (a saturated solution) was added to lyse the cells to obtain maximum fluorescence in 1 mmol/L Ca^{2+} (Fmax). Once the fluorescence level had stabilized, EGTA (20 μL, 1 mol/L EGTA [BDH pH 7.2]) was added to obtain the background fluorescence (Fmin). The increase in intracellular calcium was calculated according to the equation [Ca^{2+}] = [F2 - Fmin]/(Fmax - F2) - [F1 - Fmin]/(Fmax - F1), where F1 is the intensity before agonist addition, F2 is the peak intensity after agonist addition, Fmax is the intensity after digitonin addition, and Fmin is the intensity after EGTA chelation.

Isolation of FACS-1 murine progenitors. Murine hematopoietic progenitor cells were isolated as a FACS-1 population from murine bone marrow by density gradient centrifugation and fluorescence-activated cell sorting with wheat germ agglutinin (WGA) as described by Lord and Marsh. Briefly, low-density femoral marrow cells were obtained by density separation on metrizamide at 1.080 g/mL, labeled with fluorescein isothiocyanate (FITC)-conjugated WGA, and sorted on a Becton Dickenson FACSIV or FACSvantage flow cytometer (Becton Dickinson, Mountain View, CA). The windows were set to exclude small cells, large granular cells, and cells with low WGA-FITC. The cells were sorted at approximately 3,000 cells/s. On completion of the first sort (2 hours), the WGA-FITC-labeled low-density mononuclear cells were resorted at 390 cells/s using the same parameters.

Agar colony-forming unit assay. rhMIP-1α or BB-10010 (15 pg to 500 μg/mL) was added to FACS-1 cells (3,000 to 4,500) in 3 mL of plating medium (IMDM, 20% [vol/vol] Fetal calf serum [FCS], 10% [vol/vol] BSA, 10% [vol/vol] L-929 conditioned medium, and 10% [vol/vol] AFI-19T cell conditioned medium). Molten agar (0.33 mL, 3.3% [wt/vol] agar in sterile water) was added, mixed, and plated out immediately into 3 x 35 mm petri dishes (1 mL dish). The plates were incubated for 10 days at 37°C in an atmosphere
of 5% CO₂, 5% O₂ in a humidified cabinet and the numbers of colonies were assessed microscopically.

**Thymidine suicide assay.** The FACS-1 population of murine hematopoietic progenitors was used. The cells were washed with IMDM (GIBCO BRL, Life Technologies Inc, Gaithersburg, MD) and resuspended in Iscoves’ incubation medium (IIM; IMDM containing 20% [vol/vol] FCS, 10% [wt/vol] BSA, 10% [vol/vol] L-929 cell conditioned medium, 10% [vol/vol] AF1-19T conditioned medium, 2 mmol/L glutamine) at 3 × 10⁵ cells/mL. The resuspended cells were dispensed as 1-mL aliquots into 15-mL polypropylene tubes and incubated overnight at 37°C in an atmosphere of 95% air/5% CO₂. The 1-mL aliquots of cells were split into 0.5-mL duplicates. rhMIP-1α and BB-10010 at the required concentration was added to one of the duplicates and PBS (control) was added to the other duplicate and incubation continued. After 2 hours, ³H-thymidine (10 µCi/mL) was added to all appropriate tubes and incubation continued for 30 minutes. Thymidine incorporation was terminated by the addition of excess unlabeled thymidine (4 mL, 100 µg/mL thymidine in IMDM). The cells were harvested and resuspended in 3 mL of plating medium and plated into 3 × 35 mm petri dishes as described above. The plates were incubated at 37°C in an atmosphere of 5% CO₂, 5% O₂ in a humidified cabinet. After 7 to 12 days of incubation, the number of colonies was assessed microscopically.

Protection of hematopoietic progenitor cells from the cytotoxic effects of hydroxyurea in vivo by BB-10010. A murine model of repeated chemotherapy using hydroxyurea was used to show the in vivo myeloprotective activity of BB-10010. Nine groups of five mice were injected intraperitoneally (IP) at 0 and 7 hours with 1 g/kg hydroxyurea and with BB-10010 or vehicle control (PBS “A”) at 3 and 6 hours (day 0). Three of the nine groups received BB-10010 at 100 µg/kg; three groups received BB-10010 at 500 µg/kg, and three groups received PBS control, all injected subcutaneously. An additional three groups received PBS injected IP at 0 and 7 hours. PBS “A” was injected subcutaneously (SC) at 3 and 6 hours to act as vehicle controls for both hydroxurea and BB-10010 administration. One group of mice receiving each treatment was killed on day 1, day +2, and day +3. A pooled femoral bone marrow suspension was prepared using a single femur from each mouse in each group on days +1, +2, and +3 for colony-forming unit-spleen (CFU-S) assay. On days +1 and +2, unpooled bone marrow cell suspensions were prepared from mice in each group to allow an assessment of the CFU-femur in individual mice using methylcellulose CFU assays.

**CFU-S assay.** CFU-S were assayed in irradiated (15.25 Gy Co ß-rays; dose rate, 0.95 Gy/h) mice as previously described. The irradiated recipient mice (10/group) were injected intravenously with 0.2 mL of a femoral bone marrow suspension and killed 8 days later for spleen colony counting.

**Methylcellulose CFU assays.** Femoral bone marrow suspensions were plated in methylcellulose semisolid medium (Readymix HCC3430; Stem Cell Technologies Inc, Vancouver, British Columbia, Canada) containing a complex mixture of growth stimulatory cytokines, supplemented with additional stem cell factor (SCF; 20 ng/mL), and incubated at 37°C and 5% CO₂ and 5% O₂. The number of colonies was assessed microscopically after 7 days of growth. The colonies were counted regardless of size and cell type, but were generally CFU-granulocyte-macrophage (CFU-GM) colonies of greater than 40 cells. The number of colonies per nucleated white blood cell plated was calculated. The results were transformed into methylcellulose CFU per femur for each animal. Comparisons between treatments were made using the unpaired Student’s t-test.

**RESULTS**

**Comparison of the self-association properties of rhMIP-1α and BB-10010.** The solution properties of rhMIP-1α and BB-10010 were initially compared using SE-FPLC (Fig 2). The elution profiles are strikingly different. The rhMIP-1α peak is asymmetric, with the bulk of the protein excluded from the column and the remainder trailing to positions equivalent to lower molecular masses. This reflects a heterogeneous mixture of soluble multimeric complexes ranging in mass from approximately 20 to greater than 100 kD. In contrast, BB-10010 elutes as a symmetrical peak, within the analytical range of the column. By comparison with molecular weight markers, the BB-10010 peak was estimated to be between 17 and 44 kD. A more rigorous comparison of the molecular masses was obtained by sedimentation equilibrium AUC at 0.1 mg/mL in PBS “A” (Fig 3). The samples reached equilibrium after centrifugation at 15,000 rpm for 18 hours. The absorbance at 230 nm was plotted against the position in the cell (radius) and a line of best fit was calculated. The calculation assumes that the protein does not self-
associate or interact with the PBS "A" or the cell (a single component ideal solution) and forms a model with which to test these assumptions. The differences between the data and the best fit model, the residuals, are also plotted against cell radius. A good correlation between the data and the model is indicated when the residuals are randomly distributed across the radius. The weight average molecular weight of rhMIP-1α was 100 kDa. rhMIP-1α is a poor fit to the model, as shown by the nonrandom residuals. The higher weight average molecular weight of rhMIP-1α and the distribution of the residuals indicate an aggregating system. In comparison, the data for BB-10010 are a good fit to the model because the residuals are randomly distributed across the radius. Thus, BB-10010 behaves as if it does not self-associate. The average molecular weight of BB-10010 was 19 kDa.

Comparison of the receptor binding and signal transduction activities of rhMIP-1α and BB-10010. The effect of the Asp26 > Ala substitution on the biologic activity of BB-10010 was assessed using receptor binding and signal transduction assays. The receptor binding activities of rhMIP-1α and BB-10010 were compared on murine FDCP-mix A4 cells (Fig 4). This assay measured the concentration of unlabeled rhMIP-1α or BB-10010 required to reduce the binding of 3.85 ng/mL [125I]-rhMIP-1α on murine FDCP-mix A4 cells by 50% (IC50). IC50 values of 3.25 ± 0.03 ng/mL for rhMIP-1α (mean ± SEM; n = 84) and 7.71 ± 0.55 ng/mL (mean ± SEM; n = 10) for BB-10010 were estimated. The increase in IC50 value for BB-10010 was statistically significant (P < .0001, using the Student’s t-test).

The abilities of rhMIP-1α and BB-10010 to transduce a signal at MIP-1α receptors on THP-1 cells were compared by estimating the increase in intracellular free calcium ions as a function of agonist concentration (Fig 5). The responses induced by rhMIP-1α and BB-10010 were similar in the linear portion of the assay and were between 10 and 40 ng/mL of either protein. BB-10010 may induce a greater response than rhMIP-1α at the extremes of the dose response presented, but the sensitivity of the assay is compromised at these agonist concentrations.

Agar CFU assay. The inhibitory properties of rhMIP-1α and BB-10010 on stem cell growth were compared using their ability to inhibit the formation of FACS-1 colonies in the agar CFU assay as a surrogate parameter. Significant inhibition of colony formation was obtained with doses of 15 ng/mL or greater of both proteins (P < .05), at which an approximately 25% reduction in colony number was observed (Fig 6).

Thymidine suicide assay. To obtain an in vitro assessment of the myeloprotective activity of BB-10010, we used the 3H-thymidine suicide assay to compare the abilities of rhMIP-1α and BB-10010 to reduce the percentage of FACS-1 progenitors in cycle (Fig 7). Half-maximal protection was observed between 15 pg/mL and 1.5 ng/mL for both proteins; thus, both had similar potencies as assessed by their ability to inhibit the cycling of purified murine progenitors.
Protection of multipotent hematopoietic progenitor cells from the cytotoxic effects of hydroxyurea in vivo. We used a murine model of repeated chemotherapy with hydroxyurea to show the in vivo myeloprotective activity of BB-10010 on multipotent (CFU-S) hematopoietic progenitors (Fig 8). This assay has previously been used to show a protective effect with rmMIP-1α. BB-10010 treatment gave increased CFU-S survival seen on day +1. On day +2, the BB-10010 protected groups recovered to control levels, whereas the unprotected group was still depleted of CFU-S. By day +3, the unprotected group recovered to control levels and the BB-10010 protected groups displayed a slight overshoot. This experiment showed that BB-10010 retains the multipotent hematopoietic progenitor protection activity associated with rMIP-1α.

Protection of lineage-restricted hematopoietic progenitor cells from the cytotoxic effects of hydroxyurea in vivo. In addition to the myeloprotective effects of BB-10010 on multipotent hematopoietic progenitors (CFU-S), we also assessed the effect of BB-10010 on the survival and repopulation of more mature, lineage-restricted progenitors, using the methylcellulose CFU assay (Fig 9). On day +1, hydroxyurea caused a significant reduction in the mean CFU per femur compared with control. Protection by BB-10010 on day +1 posttreatment was not significant. However, by day +2, significant differences between treatment groups were apparent. In contrast to the group receiving hydroxyurea alone, the groups receiving 100 μg/kg and 500 μg/kg doses of BB-10010 had CFU per femur counts that were statistically indistinguishable from the control group. This shows that BB-10010 treatment leads to an increased rate of recovery in more committed progenitors.

DISCUSSION

Since the discovery of the stem cell inhibitor, later identified as mMIP-1α, the use of such a regulator of stem cell division as a protective agent has been an attractive possibil-
Fig 6. Growth inhibitory properties of rhMIP-1α and BB-10010. The abilities of rhMIP-1α and BB-10010 to inhibit the growth of FACS-1 colonies were compared in the agar CFU assay. The stem cell inhibitors were added directly to the plates at various concentrations. The results expressed as means from between 2 and 8 experiments at each agonist concentration and are presented with SEM. The significance of the inhibition of colony formation was determined with the Student’s t-test, comparing the percentage of colony formation at the lowest stem cell inhibitor concentration (15 pg/mL) with that at the higher concentrations. Points showing significant inhibition (P < .05) of colony formation with both proteins are marked with an asterisk.

Fig 7. Reversible inhibition of cell cycle by rhMIP-1α and BB-10010 measured by thymidine suicide. The potencies of rhMIP-1α and BB-10010 were compared using the thymidine suicide assay. Murine FACS-1 bone marrow progenitors were subjected to high specific activity [3H]-thymidine in the presence of various concentrations of stem cell inhibitor. The number of progenitor cells surviving [3H]-thymidine treatment was determined using the agar CFU assay. Results are expressed as the percentage of survival compared with controls without [3H]-thymidine treatment. Data from between 3 and 7 experiments at each agonist concentration have been averaged. The error bars represent SEM. The significance of the protection of colony formation was determined with the unpaired Student’s t-test, comparing the percentage of colony survival without rhMIP-1α or BB-10010 with survival in the presence of increasing concentrations of agent. Points showing significant protection (P < .05) compared with the controls are marked with an asterisk.

The identification of its human homologue and the ability to prepare large quantities of pure protein for preclinical studies brought the clinical evaluation of stem cell inhibitors a step closer. Importantly, these advances allowed a clear demonstration that stem cell inhibition could lead to protection in vivo. Thus, rmMIP-1α has been shown to protect multipotent hematopoietic progenitor cells (CFU-S) from the cytotoxic effects of hydroxyurea in vivo in a murine model of repeated cycles of chemotherapy. Using rhMIP-1α, stem cell protection was shown to reduce the duration of neutropenia associated with cytosine arabinoside administration in a murine model. More recently, the observation that rMIP-1α can mobilize hematopoietic stem cells means that it might have a role in peripheral blood stem cell mobilization. These preclinical observations were sufficiently compelling for us to test the clinical potential of MIP-1α. However, the clinical development of MIP-1α was complicated by its tendency for self-association. This property led to difficulties in controlling production processes, in pharmaceutical formulation, and in the stability of the final product.

We have used mutagenesis to improve the solution properties of rhMIP-1α and hence facilitate its clinical development. During our mutagenesis program, which will be reported in detail elsewhere, we found that a number of amino acid substitutions that altered solution properties also de-
is possible to manipulate the self-association pathway of rhMIP-1α by mutagenesis. We have also identified regions other than the C-terminus of MIP-1α that can be manipulated to give active nonaggregating variants. BB-10010 was chosen for clinical evaluation because of its molecular weight, its activity, and the fact that it has a single amino acid substitution, thus reducing the potential for immunogenicity.

Comparison of the SE-FPLC and AUC data for both rhMIP-1α and BB-10010 show that the Asp26 → Ala substitution in BB-10010 greatly affected the solution properties, producing a more homogeneous solution with an average molecular weight of 19 kDa in PBS "A" at 0.1 mg/mL. It is impossible to determine the precise association state of BB-10010 from these data, although it falls in the range dimer/trimer/tetramer in these conditions. In comparison, the rhMIP-1α is clearly heterogeneous, with an average size of at least 100 kDa. This difference is striking at higher protein concentrations and is emphasized in the formulations shown

increased biologic potency. We therefore undertook to assess the activity and detailed solution characteristics of a larger number of variants to identify a suitable candidate for clinical development. In our library of rhMIP-1α mutants, we identified several single amino acid substitutions that yielded proteins that are substantially dissociated in PBS. These include variants that are predominantly monomeric, dimeric, or tetrameric when analyzed using AUC. These results show that it
An Improved Variant of rhMIP-1α

Fig 10. Improved formulation properties of BB-10010. BB-10010 is more soluble than rhMIP-1α. The left ampoule contains 10 mg/mL rhMIP-1α in PBS "A" and the right ampoule contains 10 mg/mL BB-10010 in PBS "A." The opaque rhMIP-1α precipitate contrasts with the clear solution of BB-10010. The bright meniscus is seen in both protein preparations.

As a result of the polymerization observed on the molecular scale by the SE-FPLC and AUC, the rhMIP-1α solution contains a large amount of aggregated material, with molecular species so large that they become insoluble. Under these conditions, BB-10010 is still soluble.

The biologic activity of MIP-1α is initiated by the binding of the chemokine to receptors and subsequent signal transduction. It was therefore important to show that BB-10010 had similar receptor binding and signal transduction activities to rhMIP-1α. BB-10010 retained receptor binding activity, but with an IC₅₀ approximately twice that of rhMIP-1α on FDCP-mix A4 cells. We confirmed that BB-10010 receptor binding was able to trigger signal transduction using a calcium mobilization assay. The human monocytic cell line THP-1 was used in this assay because the FDCP-mix A4 cells were not suitable. Both cell lines possess MIP-1α receptors with similar affinities for rhMIP-1α and BB-10010 (data not shown). The rhMIP-1α and BB-10010 dose responses were similar over the linear portion of the calcium mobilization assay. This suggested that the twofold increase observed in the receptor binding IC₅₀ of BB-10010 was pharmacologically insignificant. In addition to retaining receptor binding and signal transduction activities, BB-10010 retains the stem cell inhibitory properties of rhMIP-1α. Thus, the in vitro growth-inhibitory activities of rhMIP-1α and BB-10010 in the agar CFU assay, and their ability to protect progenitors from thymidine suicide was similar.

BB-10010 retains the ability to protect murine multipotent (CFU-S) and lineage-restricted hematopoietic progenitor cells from the cytotoxic effects of hydroxyurea in vivo. Approximately twice as many multipotent hematopoietic progenitor cells, capable of forming spleen colonies, survived hydroxyurea in groups that were protected by BB-10010. Furthermore, they recovered to control levels 1 day faster. BB-10010 also effected a more rapid recovery of lineage-restricted hematopoietic progenitors. These results confirmed the in vivo activity of BB-10010 and showed its suitability for clinical evaluation.

Some nonaggregating preparations and variants of rhMIP-1α, like BB-10010, retain biologic activities and have led to the suggestion that the monomer is the active form of the molecule at the receptor. It has been reported that monomeric interleukin-8, MCP-1, and I-309 are active as monomers at physiologically relevant concentrations. It is tempting to assume that the MIP-1α monomer is the active form because it suggests a commonality of action throughout the chemokines. Our structure-activity data do not address this issue and cannot be used to conclude that the monomer is the active species. The aggregation problems of rMIP-1α caused Paolini et al difficulties in AUC analysis of their preparations. Because of the improved solution properties of BB-10010, we have not experienced these problems.

BB-10010 is a genetically engineered variant of rhMIP-1α, with a single amino acid substitution of Asp26 > Ala that dramatically improves its pharmaceutical properties. It is active in a range of in vitro assays of biologic function and retains the ability to protect hematopoietic progenitor cells from the myelosuppressive effects of hydroxyurea in vivo. The development of this agent now enables us to test the hypothesis that stem cell protection will enhance hematopoietic recoveries in cancer patients undergoing intensive chemotherapy. BB-10010 is currently in clinical trials to evaluate its potential as an adjunct to chemotherapy regimens and as a mobilizer of hematopoietic stem and progenitor cells.

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