Use and Mechanism of Action of AS101 in Protecting Bone Marrow Colony Forming Units-Granulocyte-Macrophage following Purging with ASTA-Z 7557

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ABSTRACT

Ammonium trichloro(dioxyethylene-\(O,\O' \))tellurate (AS101) has been shown previously to provide radioprotective effects when given to mice 24 h prior to irradiation and to protect mice from lethal and sublethal doses of cyclophosphamide (CTX). In this study we examined the ability of AS101 to protect mice bone marrow colony forming units-granulocyte-macrophage treated in vitro with various doses of ASTA-Z 7557, a potent derivative of cyclophosphamide. We demonstrate that prior incubation with AS101 protects colony forming units-granulocyte-macrophage from toxic effects of ASTA-Z. This protection can also be conferred by injection of mice with AS101 prior to incubation of their bone marrow in vitro with ASTA-Z. Prior incubation with AS101 was shown not to protect K562 leukemic cells or HL-60 cells from the toxic effects of ASTA-Z. We show that AS101 protection from the toxic effects of ASTA-Z in vitro and CTX in vivo can be partly ascribed to increased aldehyde dehydrogenase (ALDH) activity induced by AS101. This was shown directly by measuring cellular ALDH activity and indirectly by measuring the toxicity of ASTA-Z and CTX in the presence of cyanomamide, an inhibitor of ALDH. AS101 is also demonstrated in this study to protect spleen cells from the toxic effects of 5-fluorouracil, probably through a different mechanism. These properties of AS101 make it a useful candidate for increasing the qualitative potential of bone marrow used for autologous transplantation after purging with ASTA-Z. In addition, the results suggest an increase in ALDH activity by AS101 as one of the mechanisms of protection from the toxic effects of ASTA-Z and CTX. However, the chemoprotectiveness of AS101 was found not to be restricted to cyclophosphamide, since as shown in this study, AS101 helped by other mechanisms to reconstitute the number of spleen cells after 5-fluorouracil treatment.

INTRODUCTION

AS101\(^1\) has been developed and previously shown by us to stimulate the production of IL-1, CSF, tumor necrosis factor, and other cytokines in vitro (1-5). Current phase I clinical trials on cancer and acquired immunodeficiency syndrome patients show an enhancement in the secretion of tumor necrosis factor, \(\gamma\)-interferon, and IL-2 in vivo (6, 7). AS101 was recently shown by us to have radioprotective properties when injected prior to sublethal and lethal doses of irradiation (4). In addition, AS101 was found to protect mice from hemopoietic damage caused by sublethal doses of cyclophosphamide and to increase the rate of survival of mice treated with lethal doses of CTX (8). AS101 could significantly increase the number of BM and spleen cells, the absolute number of CFU-GM, and the secretion of CSF by either BM or spleen cells in mice treated with CTX. We have recently suggested that radioprotection conferred by AS101 results in part from enhanced stimulation of CFU-S toward proliferation and self-renewal.\(^4\) However, the mechanism of protection from CTX damage is unclear and remains to be established. Also, these data suggest the possible therapeutic potential of AS101 in the treatment of chemotherapy or radiation-induced myelosuppression.

BM used for autologous BM transplantation is usually treated in vitro with pharmacological agents in order to purge residual tumor cells possibly present in the mixture of BM cells. The most commonly used cytotoxic agents are cyclophosphamide derivatives, 4-HC, and ASTA-Z, the prototype of a new class of stabilized congeners of CTX (9-11). In order to be effective, the chemical purge of the bone marrow must fulfill two requirements; the treatment should eliminate the occult neoplastic cells; and it should not affect the hematopoietic cells that are necessary to ensure the recovery of the patient. Data from animal models (12) and from clinical studies (9) suggest a relative sparing of the most primitive pluripotential precursors. In rodents, it was clearly demonstrated that the pluripotent stem cells (CFU-S) are more resistant to 4-HC and ASTA-Z than both leukemic cells (13) and committed progenitor GM-CFC and blast forming units-erythrocyte (14). This has been ascribed to the aldehyde dehydrogenase activity contained in CFU-S (15). This enzyme catalyzes the reaction in which carboxyphosphamide, a metabolite with little cytotoxic activity, is produced (16). In this study we tested whether prior incubation of BM cells in vitro with AS101 or injection of AS101 into normal mice can provide protection to progenitor cells (CFU-GM) treated in vitro with various doses of ASTA-Z. We show that this protection can be partly ascribed to increased aldehyde dehydrogenase activity induced by AS101.

MATERIALS AND METHODS

Mice. Male BALB/c mice, 2 months of age, were purchased from The Jackson Laboratory, Bar Harbor, ME, and housed 10 mice/cage.

Treatment with AS101. AS101 was supplied by Wyeth-Ayerst Research (Radnor, PA) in a solution of phosphate buffered saline, pH 7.4, and maintained at 4°C. Before use, AS101 was diluted in PBS and the appropriate concentrations were either injected into normal mice in 0.2 ml volume, or introduced to BM cultures in 50 \(\mu\)l volume for in vitro experiments.

Recovery of Bone Marrow and Spleen Cells. Femurs and spleens were removed and placed in PBS. Single cell suspensions of BM were prepared by washing each cavity of the femur with 5 ml PBS with a sterile syringe and a 26-gauge needle. Spleen cells were passed through stainless steel mesh nets, treated with hypotonic solution to lyse erythr...
cytocytes, and washed 3 times. Cell counts were obtained using a hemocytometer. Viability, as assessed by trypan blue exclusion method, was always found to be >95%.

Incubation with ASTA-Z. ASTA-Z 7557 (ASTA-Werke, Bielefeld, Germany) powder was dissolved in PBS at a concentration of 10 mg/ml. Before use, the appropriate dilutions were made in PBS. Normal mouse BM cells were treated with hypotonic solution to lyse erythrocytes and were thereafter placed in culture tubes in PBS supplemented with 4% FCS at 6 x 10^6/ml. The cultures were incubated at 37°C for 24 h with or without AS101 at 0.1 and 0.5 µg/ml. After incubation, various doses of ASTA-Z were added to the cultures and the incubation was continued for an additional 30 min in a 37°C incubator. The cells were then washed twice with chilled PBS to remove the ASTA-Z and cultured in semisolid medium for the growth of hematopoietic CFC.

For in vivo experiments, AS101 at 10 µg/0.2 ml was injected into mice 24 h before recovery of BM cells. BM cells were treated in vitro with various concentrations of ASTA-Z for 30 min as described above, washed, and cultured in semisolid agar.

Quantitation of CFU-GM. BM cells were seeded in agar cultures as described previously (17). Briefly, rat spleen cells conditioned medium, as a source of colony stimulating activity, was incorporated in 2 ml of hard agar medium (0.5%) in a 35-mm Petri dish. BM cells [10^3 in 1 ml of soft agar medium (0.3%) were cloned above the hard agar layer. After 7 days of incubation at 37°C in a humidified atmosphere of 8.5% CO2 in air, the number of colonies that had grown in the soft agar was scored.

Cultures of Leukemic Cells. HL-60 and K562 leukemic cell lines were maintained in suspension culture in RPMI 1640 supplemented with 10% FCS. HL-60 and K562 cells (10^6/ml) were incubated with or without the conditioned medium obtained from BM cells previously incubated with AS101 for 24 h at 37°C in a humidified atmosphere of 5% CO2 in air. At the end of this period, various amounts of ASTA-Z were added and the incubation was continued for an additional 30 min. The cells were then washed in chilled medium to remove the ASTA-Z and cultured in agar at 10^3 cells/plate. Colonies were scored at day 7 of culture. In mixing experiments, 6 x 10^4 BM cells/ml were cultured with 6 x 10^4 leukemic cells/ml in the presence of AS101 for 24 h. They were then incubated with ASTA-Z as described above and seeded in agar in the presence of rat spleen cell conditioned medium in order to allow both CFU-GM and leukemic cells to develop.

Incubation with Cyanamide. BM cells (6 x 10^6/ml) treated with hypotonic solution were incubated with or without AS101 for 24 h at 37°C. Cyanamide (Sigma), freshly dissolved in PBS, was added to the cultures at 40 µg/ml. After 45 min incubation at 37°C, ASTA-Z at various concentrations was added and the incubation was continued for an additional 30 min. The cells were washed and cultured in agar at 1 x 10^5 cells/plate.

Treatment with Cyanamide in Vitro. Mice were given injections of 120 mg/kg cyanamide in a volume of 0.2 ml, 24 h after injection of 10 µg/0.2 ml AS101 or 0.2 ml PBS. After 45 min, the animals were given injections of different concentrations of cyclophosphamide (Sigma). Following a further 48-h period, mice were sacrificed, their spleens were removed, and single cell suspensions were recovered. Spleen cells were counted and cultured for proliferation assays in the presence of Con A.

Proliferation of Spleen Cells. Spleen cells were brought to a concentration of 1 x 10^6 cells/ml in RPMI 1640 plus 10% FCS and 2.5 µg/ml Con A (Sigma). The cells were seeded in triplicate 0.2-ml cultures in 96-well tissue culture trays. Cultures were incubated for 72 h and pulsed with 1 µCi of [3H]thymidine/well during the last 14 h. [3H]thymidine uptake was determined in a liquid scintillation counter.

Cell Preparation for Aldehyde Dehydrogenase Activity Determination. BM or spleen cells were recovered from AS101 (10 µg) or PBS injected mice. The cells were washed with cold 1.15% KCl and then treated with hypotonic solution to lyse erythrocytes. The suspension was centrifuged and the supernatant was aspirated. The pellet was washed three times with 1.15% potassium chloride solution. The cell suspension in KCl was homogenized and the homogenate was centrifuged at 9000 x g for 20 min. The supernatant fraction was removed and tested immediately for enzyme activity.

Spectrophotometric Assay for NAD Linked Aldehyde Dehydrogenase Activity. The complete reaction mixture contained NAD (12 µmol), butyraldehyde (12 µmol), pyrazole (600 µmol), the cellular fraction obtained from 4 x 10^7 BM cells or 8 x 10^5 spleen cells in sodium pyrophosphate buffer (96 µmol) adjusted to pH 8.4, 10 units of a control commercial NAD linked aldehyde dehydrogenase (Sigma), in a final volume of 1 ml. The reaction is started by adding butyraldehyde and was followed by monitoring NADH formation at 340 nm in a spectrophotometer at a constant temperature of 30°C. The buffer and pyrazole solutions were warmed to 30°C before addition to the cuvettes. Cellular fractions and butyraldehyde were kept on ice prior to their introduction to the incubation media. Butyraldehyde was omitted from the blank mixture.

Treatment with 5-FUra. Mice were given i.v. injections with 150 mg/kg 5-FUra 24 h after being treated with AS101 at 10 µg/mouse. Mice were sacrificed 24–72 h after 5-FUra treatment.

Statistical Analysis. Comparisons of means among the various groups were by t test. In in vivo studies in which AS101, at two dose levels, or PBS were injected into mice, inhibition by ASTA-Z relative to RPMI was compared in the 3 groups of mice in the following way. A two-way analysis of variance model was fitted, the effects being the group (PBS or AS101) and the treatment (RPMI or ASTA-Z). The inhibition hypothesis is defined by the interaction of group/treatment, which is highly significant.

RESULTS

Effect of Preincubation with AS101 on Recovery of GM-CFC after Being Cultured with ASTA-Z. The doses of 0.1 and 0.5 µg/ml of AS101 were chosen for this study because they have been previously shown in various studies to be the effective doses in vitro. Based on our experience, preincubation of BM cells with AS101 for 24 h gave optimal results. Table 1 shows that in the absence of prior incubation with AS101, a dose-response curve of ASTA-Z concentrations can be observed. At 100 µg/ml ASTA-Z, only occasional colonies could be seen. In contrast, preincubation of bone marrow cells with AS101 for 24 h before exposure to ASTA-Z results in an increased number of CFU-GM. At all concentrations of ASTA-Z, a pronounced effect was seen when AS101 at both 0.1 and 0.5 µg/ml was incubated in cultures. Significantly higher numbers of CFU-GM were observed at AS101 cultures with 25 µg/ml ASTA-Z (P < 0.01), 50 µg/ml ASTA-Z (P < 0.02), and 100 µg/ml ASTA-Z (P < 0.003).

Effect of AS101 Injection Prior to Incubation with ASTA-Z in Vitro on GM-CFC Recovery. The 10 and 30 µg AS101 injected into mice were chosen on the basis of our experience.

Table 1 Effect of preincubation with AS101 on recovery of GM-CFC after culture with ASTA-Z

<table>
<thead>
<tr>
<th>BM cultures (µg/ml)</th>
<th>No. of CFU-GM/10^6 BM cells</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>182.6 ± 5.8</td>
</tr>
<tr>
<td>AS101 0.1</td>
<td>181.0 ± 6.5</td>
</tr>
<tr>
<td>AS101 0.5</td>
<td>188.3 ± 12.9</td>
</tr>
<tr>
<td>ASTA-Z 25</td>
<td>84.0 ± 1.7</td>
</tr>
<tr>
<td>ASTA-Z 25 + AS101 0.1</td>
<td>105.1 ± 6.0</td>
</tr>
<tr>
<td>ASTA-Z 25 + AS101 0.5</td>
<td>120.6 ± 8.4</td>
</tr>
<tr>
<td>ASTA-Z 50</td>
<td>31.5 ± 5.2</td>
</tr>
<tr>
<td>ASTA-Z 50 + AS101 0.1</td>
<td>60.3 ± 10.4</td>
</tr>
<tr>
<td>ASTA-Z 50 + AS101 0.5</td>
<td>76.7 ± 12.7</td>
</tr>
<tr>
<td>ASTA-Z 100</td>
<td>3.6 ± 1.4</td>
</tr>
<tr>
<td>ASTA-Z 100 + AS101 0.1</td>
<td>20.1 ± 2.0</td>
</tr>
<tr>
<td>ASTA-Z 100 + AS101 0.5</td>
<td>14.5 ± 1.6</td>
</tr>
</tbody>
</table>
in in vivo studies which showed them to be effective doses. These doses were demonstrated to protect mice from both chemotherapy and irradiation. Fig. 1 shows that BM GM-CFC from untreated mice were inhibited by either of ASTA-Z doses (P < 0.001 and P < 0.0002, respectively). Injection of both doses of AS101 increased the number of colonies from BM treated with either 25 or 50 µg/ml of ASTA-Z. At 25 µg/ml of ASTA-Z, the number of BM-CFC from AS101 treated mice did not differ from that of control cultures without ASTA-Z. This result was shown at both doses of AS101. At 50 µg/ml ASTA-Z, the number of colony forming units-cell increased from 75.1 ± 6 (SE) in BM from untreated mice to 171.3 ± 12 in BM from mice given injections of 10 µg AS101 and 166.3 ± 13 in BM from mice given injections of 30 µg AS101 (P < 0.002 and P < 0.001, respectively).

Effect of AS101 on Lethal Effects of ASTA-Z on HL-60 and K562 Leukemic Cells. After demonstrating that preincubation with AS101 protects bone marrow progenitors from the lethal effects of ASTA-Z, it was important to exclude the possibility that these culture conditions would also protect leukemic cells from the toxic effect of ASTA-Z. Table 2 shows a dose response for ASTA-Z on colony formation by HL-60 and K562 leukemic cells. As can be seen, ASTA-Z concentrations of 50–75 µg/ml eliminated 90–100% of K562 colony formation whereas 25 µg/ml could inhibit most of HL-60 colony formation (P < 0.001 and P < 0.002, respectively).

Because we do not know at present whether AS101 exerts its protective effects directly or indirectly through another mediator, the synthesis of which is induced by accessory cells stimulated with AS101, we performed the same experiment as described above, but instead of preincubation with AS101 we added to the leukemic cells conditioned medium obtained from BM incubated with AS101 for 24 h. As can be seen in Table 2, AS101 conditioned medium did not protect K562 or HL-60 cells from the lethal effects of ASTA-Z.

In order to demonstrate the virtual differential influence of AS101 on the protection of normal marrow CFU-GM versus leukemic cell lines, a direct mixing experiment was performed in which the effect of chemotherapy is mimicked in vitro on tumor cells infiltrating marrow. Normal BM cells were mixed with HL-60 or K562 leukemic cells in the presence of AS101 for 24 h and were then purged with ASTA-Z. The mixture of cells was seeded in soft agar in the presence of CSF. As shown in Table 3, a pronounced protective effect on CFU-GM was seen at all ASTA-Z concentrations (P < 0.01) while AS101, at both 0.1 and 0.5 µg/ml, was incubated in cultures. There was no significant change, however, in the number of K562 colonies in the presence of AS101. Similar results (not shown) have been obtained with HL-60 leukemic cells.

Table 2 Effect of AS101 on lethal effects of ASTA-Z on HL-60 and K562 leukemic cells

<table>
<thead>
<tr>
<th>Cell cultures (µg/ml)</th>
<th>Colonies/10^3 cells plated</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>K562</td>
</tr>
<tr>
<td>Control RPMI</td>
<td>215.6 ± 12</td>
</tr>
<tr>
<td>ASTA-Z 25</td>
<td>29.3 ± 3</td>
</tr>
<tr>
<td>ASTA-Z 50</td>
<td>11.8 ± 2</td>
</tr>
<tr>
<td>ASTA-Z 75</td>
<td>0</td>
</tr>
<tr>
<td>ASTA-Z 25 + AS101 0.1 CM</td>
<td>28.6 ± 2.3</td>
</tr>
<tr>
<td>ASTA-Z 25 + AS101 0.1 CM</td>
<td>27.9 ± 2.1</td>
</tr>
<tr>
<td>ASTA-Z 50 + AS101 0.1 CM</td>
<td>10.6 ± 0.6</td>
</tr>
<tr>
<td>ASTA-Z 50 + AS101 0.5 CM</td>
<td>10.9 ± 0.8</td>
</tr>
<tr>
<td>ASTA-Z 75 + AS101 0.1 CM</td>
<td>0</td>
</tr>
<tr>
<td>ASTA-Z 75 + AS101 0.5 CM</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3 Effect of preincubation with AS101 on the recovery of BM GM-CFC and K562 leukemic colonies after mixed cultures with ASTA-Z

BM and K562 cells were incubated in mixed cultures with or without various doses of AS101 for 24 h. ASTA-Z at various concentrations was added for 30 min. Cells were washed and plated in soft agar in the presence of rat spleen conditioned media as a source of CSF. Results represent mean ± SE from 3 different experiments.

In vitro cultures (µg/ml)

<table>
<thead>
<tr>
<th>Colonies/5 × 10^5 BM cells + 5 × 10^5 K562 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control RPMI</td>
</tr>
<tr>
<td>ASTA-Z 25</td>
</tr>
<tr>
<td>ASTA-Z 50</td>
</tr>
<tr>
<td>ASTA-Z 75</td>
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<tr>
<td>ASTA-Z 25 + AS101 0.1 CM</td>
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<td>ASTA-Z 25 + AS101 0.5 CM</td>
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<td>ASTA-Z 50 + AS101 0.1 CM</td>
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<td>ASTA-Z 75 + AS101 0.1 CM</td>
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<tr>
<td>ASTA-Z 75 + AS101 0.5 CM</td>
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</table>

*P < 0.01.
Effect of AS101 Preincubation on the Recovery of GM-CFC from BM Treated with Cyanamide and ASTA-Z. A number of studies have indicated that resistance to cyclophosphamide is determined by the activity levels of cellular ALDH (15). In the following experiment we wanted to determine indirectly the role of ALDH activity in causing cyclophosphamide resistance in BM cultures by measurement of the toxicity of ASTA-Z in the presence of an inhibitor of ALDH (cyanamide). We also wanted to see if AS101 in the presence of this inhibitor can increase the resistance to ASTA-Z, thus indirectly pointing to the ability of AS101 to increase ALDH activity.

We chose a concentration of cyanamide which would inhibit in the presence of ASTA-Z approximately 80% of CFU-GM. As can be seen in Fig. 2, inhibition of ALDH by cyanamide increases the toxicity of 25 µg/ml ASTA-Z. The number of CFU-GM decreases from 75.3 ± 8 to 13.8 ± 1 inhibition (P < 0.001) while preincubation with AS101 at 0.2 or 0.5 µg/ml increases the number of CFU-GM to 40 ± 3 and 37.8 ± 3, respectively (P < 0.001). At 50 µg/ml ASTA-Z, incubation with cyanamide increases the toxicity of the compound. The number of CFU-GM decreases from 41 ± 5 to 7.3 ± 1 (P < 0.02) while AS101 at 0.2 or 0.5 µg/ml decreases toxicity and increases the number of CFU-GM to 22.8 ± 1 and 20.1 ± 1, respectively.

Effect of AS101 and Cyanamide Injections in Vivo on the Resistance to Cyclophosphamide. We have recently published our findings of the ability of AS101 to protect mice from hematopoietic damage caused by cyclophosphamide. We also recently found that injection of 10 µg AS101 24 h before treatment with cyclophosphamide significantly increases the number of spleen cells and their rate of proliferation in the presence of Con A. In the present experiment we wanted to see the effect of cyanamide in vivo on the resistance of spleen cells to cyclophosphamide and the role of AS101 in the resistance to cyclophosphamide by regulating ALDH activity. Fig. 3a shows that injection of cyanamide and CTX decreases the number of spleen cells by approximately 60 and 90% in comparison to CTX alone (100 or 200 mg/kg). Injection of AS101 24 h prior to 100 mg/kg CTX and cyanamide injection restored the number of spleen cells to values of CTX alone (P < 0.001). At 200 mg/kg CTX and cyanamide, AS101 increased the number of spleen cells from 0.29 ± 0.002 to 1.9 ± 0.1 x 10^7 (P < 0.001). Cyanamide, when injected alone without CTX did not have any toxic effect.

Effect of AS101 Preincubation on recovery on recovery of GM-CFC from BM treated with Cyanamide (CTA) and ASTA-Z. BM cells were incubated for 24 h with or without 2 doses of AS101. Cyanamide (40 µg/ml) was thereafter added to the cultures. After 45 min, ASTA-Z at various concentrations was added to the cultures for 30 min. The cells were washed and seeded in agar plates. The number of CFU-GM in cultures with cyanamide alone was 184 ± 12/10^6 BM cells. Results represent mean ± SE (bars) from 3 different experiments including 6 mice/group/experiment. Each group consisted of 3 subgroups of 2 mice each. *, P < 0.02; ***, P < 0.001; #, P < 0.002.

Fig. 2. Effect of AS101 (AS) preincubation on recovery of GM-CFC from BM treated with Cyanamide (CTA) and ASTA-Z. BM cells were incubated for 24 h with or without 2 doses of AS101. Cyanamide (40 µg/ml) was thereafter added to the cultures. After 45 min, ASTA-Z at various concentrations was added to the cultures for 30 min. The cells were washed and seeded in agar plates. The number of CFU-GM in cultures with cyanamide alone was 184 ± 12/10^6 BM cells. Results represent mean ± SE (bars) from 3 different experiments including 6 mice/group/experiment. Each group consisted of 3 subgroups of 2 mice each. *, P < 0.02; ***, P < 0.001; #, P < 0.002.

Fig. 3a. Effect of AS101 (AS) and cyanamide (CYAN) injections in vivo on the resistance to CTX. Mice were given injections of 120 mg/kg cyanamide 24 h after injection of 10 µg AS101 or PBS. After 45 min, the animals were given injections of various doses of CTX. After a further 48 h, spleen cells from various groups were counted (a) and cultured for proliferation assays in the presence of Con A. Results represent mean ± SE (bars) of 3 different experiments including 4 mice/group. Each group consisted of 2 subgroups of 2 mice each. *, P < 0.001; ***, P < 0.003.

The effect of AS101 injection 24 h prior to CTX and cyanamide treatment on the proliferation of spleen cells in the presence of Con A was also tested. Fig. 3b shows that CTX at 200 mg/kg but not at 100 mg/kg decreased the rate of proliferation to 36.6% of control levels (P < 0.001). Injection of 100 mg/kg CTX and cyanamide decreased the proliferation rate to
Fig. 4. Effect of AS101 (AS) on NAD linked Al IMI activity in BM and spleen cells. BM or spleen cells were recovered from mice given injections 24 h before harvesting of AS101 (10 µg) or PBS. Activity of ALDH was compared to that of a commercial enzyme. Levels of ALDH activity in commercial ALDH were 0.5 µmol NADH formed by 10 units ALDH/min. Results represent mean ± SE (bars) of 3 experiments including 20 mice/group/experiment. *, P< 0.05.

Effect of AS101 on NAD Linked Aldehyde Dehydrogenase Activity in BM and Spleen Cells. The relative amounts of NAD linked aldehyde dehydrogenase activity in PBS and AS101 injected mouse BM cells was determined. Butyraldehyde was used as substrate in these experiments because of the relative ease of monitoring enzyme activity with this substrate. Fig. 4 shows that when enzyme activity in BM cells was compared to that of a commercial NAD linked aldehyde dehydrogenase, a significant increase was observed in BM cells from AS101 injected mice relative to control PBS injected mice. The percentage of activity increased from 30.2% in control mice to 82.5% in AS101 injected mice (P < 0.005).

Similar results were obtained when enzyme activity was studied in spleen cells. Although the level of activity in control spleen cells was much lower when compared to BM cells (17 ± 2%), a significant increase was noted in enzyme activity in spleen cells from AS101 injected mice (66 ± 7%; P < 0.05).

Effect of AS101 on the Recovery of Spleen Cells from 5-FUra Treatment. Since resistance to cyclophosphamide has been attributed in many studies to ALDH activity and since AS101 has been demonstrated to increase ALDH activity, it was important to know whether AS101 has the ability to protect the hemopoietic system from damage caused specifically by CTX or whether it can protect mice also from other chemotherapeutic agents by another mechanism of action. We tested the ability of AS101 to restore the number of spleen cells after the toxic effects of 5-FUra in vivo. As can be seen in Fig. 5, AS101 increased the number of spleen cells 24–72 h after injection of 5-FUra. The most pronounced effect was seen at 48 and 72 h after 5-FUra injection because at those points in time the toxic effect of 5-FUra was most apparent and therefore the effect of AS101 on the cellular reconstitution was more clearly marked.

The number of spleen cells decreased significantly 24–72 h after 5-FUra treatment (P < 0.02, 0.03, 0.01, respectively). AS101 injection increased the number of spleen cells 24 h after 5-FUra treatment from 18.75 ± 2 to 24.8 ± 2 x 10^7 (P< 0.02). After 24 h AS101 increased the number of cells from 17.4 ppm

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Fig. 5. Effect of AS101 (AS) on the recovery of spleen cells from 5-FUra (5-FU) treatment. Mice were given injections of 150 mg/kg 5-FUra 24 h after receiving injections of AS101 (10 µg) or PBS. Mice were sacrificed 24–72 h after 5-FUra treatment. Results represent means ± SE (bars) from 3 experiments including 6 mice/group/experiment. Each group consisted of 3 subgroups of 2 mice each. *, P < 0.02; **, P < 0.03; #, P < 0.01.
1.5 to 24.8 ± 1.7 (P < 0.02). At 72 h the number of spleen cells increased from 9.35 ± 0.6 to 17.5 ±0.9 x 10^7 (P < 0.01).

**DISCUSSION**

This study was based on our previous results concerning the ability of AS101 to protect mice from lethal and sublethal doses of cyclophosphamide. The compound was shown to increase bone marrow and spleen cellularity, CFSE secretion by bone marrow and spleen cells and the number of CFU-GM in mice given injections of cyclophosphamide (8). We show in the present study that BM from mice given AS101 injections show a similar resistance to ASTA-Z, a potent derivative of CTX, in vitro. Moreover, prior incubation of BM cells with AS101 is shown to protect CFU-GM from the toxic effects of ASTA-Z. These results might have significant clinical implications in the setting of autologous BM transplantation in which ASTA-Z is used for the purging of acute leukemic marrows. In this regard, our finding that AS101 does not protect K562 or HL-60 leukemic cell lines from the toxic effects of ASTA-Z is especially relevant. Therefore, with the use of AS101 it could be possible to provide higher doses of ASTA-Z in order to kill more efficiently leukemic cells and yet protect progenitor cells. However, two requirements must be fulfilled before the clinical use of AS101 in bone marrow purging: (a) the ability of AS101 to protect human BM cells from ASTA-Z, (b) the effect of AS101 on freshly isolated leukemic cells must be confirmed.

The successful outcome of autologous bone marrow transplantation using in vitro purging of leukemic cells by ASTA-Z is based on the ability of the treated BM to restore hematopoiesis after total body irradiation even though devoid of CFU-GM and BFU-E. However, transplantation of BM cells composed of a higher percentage of CFU-GM could possibly diminish the risk of infections of transplanted patients. Experiments using higher doses of ASTA-Z in bone marrow preincubated with AS101 should be carefully monitored for possible loss of CFU-S. It has been proposed that the selective action of cyclophosphamide depends at least in part on the balance between conversion of aldoxoribine, the product of the hydroxylated form of CTX, to active and inactive metabolites in sensitive and insensitive cells. The rate of conversion of 4-HC/aldophosphamide to the inactive metabolite, carboxyphosphamide, a reaction catalyzed by cellular NAD linked aldehyde dehydrogenase has been proposed to be a major determinant of the sensitivity of neoplastic and normal cells to ASTA-Z and in general to oxazaphosphorines (18, 19).

Target cells containing a relatively greater aldehyde dehydrogenase activity would be relatively insensitive to oxazaphosphorines since less 4-HC/aldophosphamide would be available for conversion to cytotoxic metabolites in these cells. CFU-S have been previously found to be insensitive to oxazaphosphorines because they contain the aldehyde dehydrogenase isozyme that catalyzes the oxidation of aldoxoribine to carboxyphosphamide. CFU-GM are relatively sensitive to oxazaphosphorines because relatively they lack this enzyme activity (15).

AS101 has been found in this study to increase in vivo the activity of NAD linked aldehyde dehydrogenase. The activity of the enzyme was determined for total BM cells, so that we cannot conclude that AS101 increased this activity specifically in CFU-S or CFU-GM. We have previously demonstrated that AS101 can increase self-proliferation of CFU-S when injected into mice prior to sublethal doses of irradiation. The increased activity of ALDH after AS101 injection can therefore result from increasing the CFU-S population pool containing relatively high quantities of ALDH, rather than increasing the activity of the enzyme per cell.

The genotypic basis of the acquired resistance to ASTA-Z resulting from increased ALDH activity is not known. It could be due to increased steady-state enzyme, to the production of an isozyme not normally present, or to the selection of a subpopulation of cells with relatively greater ALDH activity. The result presented by us in Table 2 in which AS101 is shown not to alter the toxicity of ASTA-Z on leukemic cells suggests that AS101 does not increase ALDH activity but rather elevates the number of early progenitor cells and thus elevates the total ALDH activity. Cells such as K562 cells, a human chronic myelogenous leukemia erythroblast, are relatively more resistant to ASTA-Z purging than other tumor cells, due to their relatively high levels of aldehyde dehydrogenase (20). The fact that AS101 does not alter this resistance suggests that no further elevation in ALDH occurs.

There are other possible mechanisms that could explain protection from CTX in vivo or ASTA-Z in vitro after injection or incubation with AS101. AS101 could be acting indirectly by initiating the production of a variety of hematopoietic stimulatory factors resulting in the earlier hematopoietic recovery observed after chemotherapy. We have previously shown that the levels of IL-1 in AS101 treated mice are much higher if compared to those of PBS treated mice; it is not altogether unlikely therefore that AS101 exerts its chemoprotective effect indirectly via this cytokine. Although we show in this study an increase in ALDH activity after AS101 injection and correlate these results with resistance to CTX, AS101 is shown here to increase the number of spleen cells after 5-FUra injection. This protection cannot be attributed to increase in ALDH activity. It is possible that other chemotherapeutic agents are less toxic to early hematopoietic precursors. Since these are stimulated toward self-renewal by AS101, more resistant cells are present in the various hematopoietic tissues.

The data presented here suggest that potentially AS101 could be useful in ex vivo purging of human bone marrow when used concomitantly with ASTA-Z at concentrations known to spare CFU-S. This would increase the proportion of committed stem cells (GM-CFC) and thus decrease the risk of infections in BM transplanted patients. Moreover, since AS101 was previously shown to stimulate CFU-S toward proliferation and self-renewal, investigations designed to quantify the possible increase in CFU-S in vivo after incubation with AS101 are currently in progress in our laboratory.

**REFERENCES**

PROTECTION OF BM BY AS101 AFTER PURGING WITH ASTA-Z


Use and Mechanism of Action of AS101 in Protecting Bone Marrow Colony Forming Units-Granulocyte-Macrophage following Purging with ASTA-Z 7557

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