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Bone Marrow Toxicity of Cyclophosphazenes is Related to their Structure and the Treatment Schedule

NANNO H. MULDER,* ELISABETH G. E. DE VRIES,* HETTY TIMMER-BOSSCHA* and JOHAN C. VAN DE GRAMPEL†

**Department of Internal Medicine, Division of Medical Oncology, University Hospital, Oostersingel 59, 9713 EZ Groningen, The Netherlands and †Department of Inorganic Chemistry, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands*

Abstract—*For three aziridinyl-substituted inorganic heterocycles belonging to the cyclophosphazene group, the tendency for cumulative bone marrow toxicity was studied in mice. In a one-day regimen, one of these drugs, gem-$N_3P_3Az_4Pyr_2$ (Az = aziridinyl, Pyr = pyrrolidinyl) (AZX), led to an increased death rate after a repeated injection, the number of bone marrow stem cells (CFU_{gm}) being decreased* 5 weeks *after the second injection of this drug. In a four-day regimen two drugs, $(NPAz_2)_2$ NSOAz (Soaz) and AZX, led to an increased death rate after the second treatment course. In surviving animals leucocyte and thrombocyte counts were significantly lower in the second than in the first course. CFU_{gm} counts were decreased for both drugs. For the third drug, trans-$N_3P_3(Az)_2(NHMe)_4$ (AZP), no evidence of cumulative bone marrow toxicity was demonstrated. It is suggested that whereas cytostatic activity of these compounds seems to be comparable, their tendency to cause cumulative marrow toxicity may vary.*

INTRODUCTION

FROM aziridinyl-substituted inorganic heterocycles belonging to the class of cyclophosphazenes, cytostatic activity has been established in various test systems [1, 2]. One derivative of this group of drugs, Soaz, has been tested in three phase I studies, which have recently been published [3–5]. Two of these studies concluded that this drug has substantial cumulative bone marrow toxicity, and further clinical trials were therefore abolished.

Nevertheless, this drug was found to have some cytostatic activity in humans [5] and was furthermore well tolerated by the patients. Also, other newly synthesized derivatives have substantial activity in test systems. It seems important therefore to establish whether a variance exists in the degree of myelotoxicity between members of this group of cytostatics and whether further clinical screening of these compounds would be worthwhile.

We have evaluated three compounds from this group for their potential to induce cumulative marrow toxicity in mice.

MATERIALS AND METHODS

Drugs

Soaz, $(NPAz_2)_2NSOAz$, was a gift from Otsuka Chemical Co., Osaka, Japan. AZP, (*trans*-$N_3P_3(Az)_2(NHMe)_4$, and AZX, (gem-$N_3P_3Az_4Pyr_2$) (Az = aziridinyl, Pyr = pyrrolidinyl), were synthesized by the Department of Inorganic Chemistry, University of Groningen. Soaz has five, AZP two and AZX four aziridinyl groups (Fig. 1).

All three compounds are readily dissolvable in water. At the maximally tolerated dose, the ratio of survival of treated/survival of untreated (T/C value) for Soaz was 188%, for AZP 213% and for AZX 211% in mice bearing the L1210 tumor (determined by TNO, Rijswijk, The Netherlands). The drugs were administered intraperitoneally either by a single injection (one-day regimen) or by injections on four consecutive days (four-day regimen). The dosage used was 80% of the LD_{10} value.

Mice

Each group consisted of at least ten 6-week-old female outbred Swiss mice. Blood was obtained twice weekly by puncture of the orbital plexus. Leucocyte and thrombocyte counts were performed by conventional techniques.

Bone marrow cultures

Bone marrow was flushed with 1 ml alpha modification of Eagle's medium (Flow Laboratories, Irvine, U.K.) through the prepared femur of mice killed by cervical dislocation, for granulocytic and macrophage colony-forming units (CFU_{gm}).

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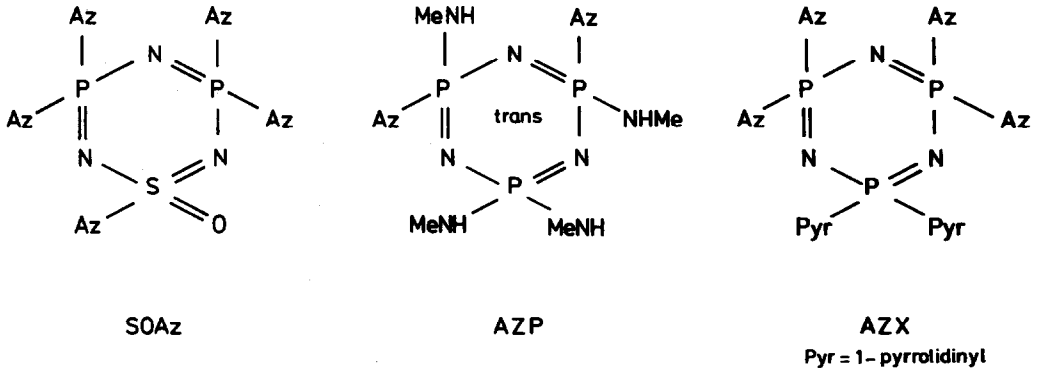

Fig. 1. Structure of three cyclophosphazene compounds.

After resuspension through a 25-gauge needle, nucleated cells were counted. One milliliter of alpha medium is supplemented with 30% fetal calf serum (FCS), 0.5% bovine serum albumen (Gibco, Grand Island, NY), 0.8% methylcellulose (Fluka A.G.), 160 U penicillin, 160 μg streptomycin, 7 μg mercaptoethanol and 10 μl of spleen-conditioned medium [6], a gift from Dr H Goris, Department of Microbiology, University of Groningen. One milliliter of this medium and 5×10^4 nucleated cells were placed in a 35-mm Petri dish. Four dishes were prepared for each experiment and incubated at 37°C with 5% CO_2. Colonies were counted after 7 days.

RESULTS

In the 1-day regimen the LD_{10} for Soaz was 275 mg/kg, for AZP this value was 35 mg/kg and for AZX 200 mg/kg. Normal values for leucocytes in these mice are 6.17×10^9/l, for thrombocytes 382×10^9/l (Table 1).

One-day regimen of Soaz

Leucocyte and thrombocyte nadirs occurred at day 14 after the first injection, and recovery had taken place by day 21. When a second injection was given after a 6-week interval, no mortality occurred. The leucocyte level at 23 days after the first course was 4.90×10^9/l, compared to 3.60×10^9/l after the second course. The thrombocyte levels were 366×10^9/l and 458×10^9/l respectively (N.S.).

One-day regimen of AZP

Leucocyte and thrombocyte nadirs were reached at day 10, with recovery on day 21. On day 23 the leucocyte count was 9.90×10^9/l, thrombocyte count 553×10^9/l. After 6 weeks the leucocyte count was 12.1×10^9/l and thrombocytes 553×10^9/l. No increase in toxicity was seen in the second course given at that time. Twenty-three days after the second injection the leucocyte count was 8.8×10^9/l and the thrombocyte count 370×10^9/l (N.S.).

One-day regimen of AZX

The leucocyte and thrombocyte nadirs of AZX were comparable to those of Soaz and AZP after the first injection. A second injection was given after 6 weeks, when 35% of the animals died. Blood counts 23 days after the first and second courses showed values for leucocytes of 5.10×10^9/l and 2.25×10^9/l and thrombocyte counts of 391×10^9/l and 351×10^9/l respectively (N.S.).

Table 1. Four-day regimen of Soaz, AZP and AZX: leucocyte and thrombocyte counts 23 days *after the first and second courses (interval between courses:* 4 weeks *for Soaz and AZP,* 8 weeks *for AZX; mean values are given plus S.E.M.)*

	Leucocytes		Thrombocytes	
	First course	Second course	First course	Second course
Soaz	2.94 (0.30)*	0.80*	309 (17.7)	152 (18.2)*
AZP	16.60 (1.10)*	7.0 (3.25)	360 (35.5)	336 (24.4)
AZX	2.49 (0.30)*	†	205 (65.2)	†
Control	6.17 (1.10)		382 (25.0)	

*Values significantly different from control values ($P < 0.001$, Student's t test).
†>90% of animals died in second course.

Four-day regimens (Table 1)

The LD_{10} levels in a 4-day regimen were for Soaz 600 mg/kg, for AZP 50 mg/kg and for AZX 500 mg/kg.

Four-day regimen of Soaz

On day 23 of the first course (day 1 is the day of the first injection) the mean leucocyte count was $2.94 \times 10^9/l$ and the mean thrombocyte count $309 \times 10^9/l$.

A second course was given after an interval of 4 or 8 weeks.

Re-injection with the same dose and schedule took place on day 28 (interval 4 weeks); 45% (5/11) animals then died within 14 days, the dying animals having leucocyte counts of $0.20 \times 10^9/l$ and thrombocyte levels of $50 \times 10^9/l$. On day 51, 23 days after re-injection, surviving animals had leucocyte counts of $0.80 \times 10^9/l$ and thrombocytes of $152 \times 10^9/l$. Two weeks later these levels were respectively 4.70 and $79 \times 10^9/l$.

Re-injection of the same dose of Soaz with an interval of 8 weeks led to a 40% death rate. Blood counts 23 days after re-injection in the surviving animals were: leucocytes, $1.60 \times 10^9/l$ and thrombocytes, $229 \times 10^9/l$.

Four-day regimen of AZP

The mean leucocyte count on day 23 was $16.60 \times 10^9/l$, mean thrombocytes $360 \times 10^9/l$.

Re-injection after 4 weeks led to two deaths in 18 animals. Twenty-three days after injection, surviving animals had a blood count of: leucocytes, $7.00 \times 10^9/l$ and thrombocytes, $336 \times 10^9/l$. When a second course was given after 8 weeks no deaths occurred, and leucocyte and thrombocyte levels were comparable.

Four-day regimen of AZX

AZX was given in a dose of 100 mg/kg per day for 4 days, total dose 400 mg/kg. On day 23 the leucocyte counts were $2.49 \times 10^9/l$, thrombocyte counts $205 \times 10^9/l$. After 8 weeks the leucocyte counts had gradually recovered to $11.10 \times 10^9/l$, thrombocytes to $392 \times 10^9/l$.

Table 2. CFU_{gm} count colonies per 5×10^4 nucleated cells: mean (S.E.M.) 5 weeks after a second course, with an interval between the first and second courses of 4 weeks

	One-day regimen	Four-day regimen
Soaz	114.58 (14.76)	35.75 (3.68)**
AZP	106.90 (19.60)	84.91 (3.95)
AZX	61.66 (17.82)*	
Control	97.50 (13.19)	97.50 (13.19)

Significantly different from control values at $*0.01 < P < 0.02$ and $**P < 0.001$ (Student's t test).

Only then, after an interval of 8 weeks, was a second course given. After this re-injection the death rate was 95%.

Bone marrow cultures for CFU_{gm} (Table 2)

CFU_{gm}s were determined in mice treated with Soaz, AZP or AZX 5 weeks after the second treatment course. The interval between first and second treatment was 4 weeks. Dosage of drugs was as described above. The mean control value and S.E.M. for CFU_{gm} per 5×10^4 nucleated cells in untreated mice was 97.50 ± 13.19.

The first group was treated with the drugs in the 1-day regimen. The CFU_{gm} level for AZX was 61.66 ± 17.82 after the second course, for AZP mean 106.90 and for Soaz 114.58. The difference between AZX treated and control mice is significant ($0.01 < P < 0.02$).

The second group was treated with 4-day courses. The CFU_{gm} level in Soaz-treated mice was 35.75, in AZP-treated mice 84.91. Treatment with Soaz led to a significantly lower number of CFU_{gm} than treatment with AZP or no treatment ($P < 0.001$).

DISCUSSION

In a phase I study in patients Soaz was found to have cumulative toxicity on the bone marrow in a 4-day regimen as well as in a weekly regimen [4, 5].

This form of toxicity in humans is difficult to predict from animal experiments [7]. However, the toxicity of Soaz given as a 4-day regimen in mice resembles that found with the same regimen in men. Three arguments are presented in this study that Soaz has cumulative bone marrow toxicity in mice: first, the increased mortality after a second exposure to the drug: in view of the blood counts of dying animals, their deaths could be related to thrombo- and leucocytopenia; second, the decreased leuco- and thrombocyte counts persisting at day 23 after re-exposure; and third, the decrease of CFU_{gm} counts 5 weeks after the second course.

Prolonging the interval between courses does not ameliorate the toxicity of the second course, indicating that the toxicity induced by the first course on the stem cell population is irreversible. A large number of other compounds derived from the same basic cyclophosphazene structure has been synthesized, and most of them have substantial activity in *in vitro* screening tests [2]. If the screening model for cumulative bone marrow toxicity described here is worthwhile, further clinical screening of these compounds should preferably be initiated using a 1-day regimen.

For AZP no clear evidence was found for increased bone marrow toxicity after a second

course, either in the 1-day or the 4-day regimen. The slightly increased death rate following the 4-day course may be due to the small therapeutic spectrum of this drug in this schedule, with an LD_{10} of 50 mg/kg and an LD_{60} of 60 mg/kg. At the dosage used small changes in weight registration could readily lead to inadvertent toxic overdosing.

As a phase I study of AZP has been started in our institution, the presumed absence of cumulative bone marrow toxicity in humans with that drug can soon be verified.

We conclude that within the group of cyclophosphazenes a spectrum of toxicity occurs as far as the bone marrow is concerned. The expression of this toxicity may be dependent on the schedules of drug administration used. It is possible that the experiments described in this study can serve as a screening test for other active compounds from the group of cyclophosphazenes.

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