Anti-Cancer Drugs, 4, pp. 505–510

Tumor inhibition and hematopoietic stimulation in mice by a synthetic copper–ATP complex

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The hematologic effect of $[Cu_3(ATP)_2\ 6H_2O]^{2-}$, a synthetic copper–ATP complex (Cu–ATP) having antitumor activity, was investigated in normal and Ehrlich ascites carcinoma-bearing mice. Cu–ATP (25mg/kg) induced appreciable tumor inhibition and prolonged host survival which were accompanied by elevated levels of hemoglobin, platelet and lymphocytes while total WBC count and bone marrow cellularity remained unaffected. In normal mice the compound elicited marrow and splenic hypercellularity with a greater number of granulocyte progenitors and elevated levels of peripheral WBC, RBC and platelets. In addition, the total number of CFU-S of these treated animals was increased and these puripotent stem cells differentiate preferentially towards granulocyte lineage. The results indicate that Cu–ATP does not adversely affect hematopoieses while it inhibits tumor growth; on the contrary, it has a stimulatory effect on murine granulocytopoiesis.

Key words: Cu–ATP complex, hematopoietic stimulation, mice, tumor inhibition.

Introduction

$[Cu_3(ATP)_26H_2O]^{2-}$ (Cu-ATP) is a novel synthetic metal nucleotide complex having significant antitumor activity against experimental tumors in mice.[1] Marked inhibition of tumor growth and concomitant increment in hosts' life span have been observed following Cu–ATP treatment of mice bearing transplantable lymphoma,[1] carcinoma[2] and sarcoma.[3] Tumor inhibition was augmented when Cu–ATP was administered in combination with other anticancer agents.[1] Although the results are encouraging for further therapeutic trials in experimental and clinical setting, it seems necessary to take into account the host response elicited by this compound when adminstered at a pharmacologic dose. This is particularly important as a majority of cancer chemotherapeutic agents including cisplatin, another metal complex with routine use in cancer treatment, are hematotoxic to the recipients thereby limiting their use in optimal dose schedules.[4] Accordingly, we have investigated the hematological response of normal and tumor-bearing mice following i.p. administration of Cu–ATP at a dose that yielded maximal antitumor effect. It was observed that Cu–ATP induces considerable tumor inhibition and concomitant stimulation of hematopoiesis at the level of pluripotent stem cells.

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Materials and methods

Animals

Closed colony bred male Swiss mice, 6–7 weeks of age and weighing 20–22 g, were used throughout. The animals, obtained from the Institute's own vivarium, were maintained in metal cages (five mice per cage) with alternate light and darnkess (12 h each). Food (standard mouse pellet, Hind Lever, Bombay, India) and water were given *ad libitum*.

Tumor

Ehrlich ascites carcinoma (EAC) was maintained by serial i.p. transplantation of 1×10^6 viable tumor cells suspended in 0.2 ml of sterile phosphate buffered saline (PBS, pH 7.4). Control animals received 0.2 ml of PBS only.

Drug and treatment schedule

Cu–ATP complex was received as a gift from Dr RG Bhattacharya and Dr KK Nayak (Department of Chemistry, Jadavpur University, Calcutta, India). The compound was dissolved in PBS immediately

before use and was injected i.p. at a dose of 2.5 mg/kg body weight/day for 10 consecutive days into groups of normal and EAC-bearing mice. In the case of the latter, injection started 24 h after tumor transplantation. Control animals received equal volumes (0.2 ml) of PBS. The present dose schedule was selected for its optimal antitumor effect.[1]

Tumor growth response

Effect of Cu–ATP treatment on tumor growth was determined by assessing viable tumor cell count and percentage increase in hosts' life span (%ILS) following standard procedure.[5]

Hematological studies

Routine hematological studies were done from freely flowing tail vein blood by standard procedures.[6]

Assessment of erythropoiesis

The rate of red cell production was assessed by measuring the 72 h RBC ^{59}Fe incorporation[7] after i.v. administration of 1.0 μCi $^{59}FeCl_3$ (specific activity 3–20 mCi/mg Fe, Amersham, UK). Blood samples (0.5 ml) of ^{59}Fe-injected mice were collected at 72 h after isotope administration and the radioactivity was measured in a gamma counter. RBC ^{59}Fe incorporation was expressed as percentage of injected radiactivity assuming the total blood volume as 7% of body weight.

Bone marrow and splenic cellularity

The animals were killed by cervical dislocation and the femurs and spleens were removed. Marrow cells were flushed from the femoral shaft into ice-cold RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco). A single cell suspension was prepared by sequentially passing the cell suspension through 20, 22 and 26 gauge needles. Single cell suspension of the spleen was prepared in ice-cold RPMI 1640 plus 10% FCS by using a sterile steel wire mesh. Total nucleated cell count was done by a Neubauer counting chamber under light microscope after destroying the non-nucleated cells in the suspension by 2% glacial acetic acid treatment. Differential distribution of hematopoietic cells in the marrow and spleen was assessed from Leishman-stained smears of bone marrow and spleen cells.[8]

CFU-S assay

Pluripotent hematopoietic stem cells (CFU-S) in control and treated mice were assayed following the spleen colony technique of Till and McCulloch.[9] The assay mice were lethally irradiated with 8.1 Gy from a ^{137}Cs source at a dose rate of 0.75 Gy/min. Within 2 h of irradiation the animals were injected i.v. with 1.0×10^5 bone marrow or 5.0×10^5 spleen cells collected from the donor mice. The spleen of the recipients were dissected out 10 days later and were fixed in Bouins fluid. Macroscopic colonies at the surface of the spleen were scored as CFU-S. Thereafter the spleens were processed for histology. Microscopic colonies in hematoxylin & eosin-stained sections (5 μm thickness) were identified and classified according to the criteria of Curry and Trentin.[10]

Statistical analysis

The results were analyzed by Students t-test and statistical significance was assigned when $p < 0.05$.

Results

Cu–ATP treatment of EAC-bearing mice resulted in significant tumor inhibition as evident from 85% and 75% reduction ($p < 0.001$) of tumor cell count on day 12 and day 16 post-transplantation, respectively (Table 1). The life span of the treated

Table 1. Effect of Cu–ATP treatment on tumor cell count (mean ± SE) and hosts' life span

Group	n	Tumor cell count ($\times 10^7$)		%ILS
		day 12	day 16	
EAC (control)	12	21.3 ± 2.3	39.4 ± 4.5	
EAC + Cu–ATP[a]	16	3.1 ± 0.7[c] (−85)[b]	9.7 ± 1.7[c] (−75)	70.1

[a] Cu–ATP was injected i.p. at a dose of 2.5 mg/kg/day for 10 consecutive days. Injection started 24 h post-tumor transplantation.
[b] % changes over control are in parentheses.
[c] $p < 0.001$ when compared with matched controls.
n, number of animals started.

Table 2. Hematological changes (mean ± SE) in normal and EAC-bearing mice following Cu–ATP treatment

	Normal mice			EAC (day 11)		EAC (day 14)	
	control	treated		control	treated	control	treated
		day 11	day 14				
Hemoglobin (g/dl)	16.0 ± 0.2	16.5 ± 0.2	16.5 ± 0.3	15.0 ± 0.3	16.2 ± 0.4[a]	14.8 ± 0.2	15.5 ± 0.2
RBC ($\times 10^6/\mu l$)	5.4 ± 0.2	5.7 ± 0.3	6.0 ± 0.2[a]	4.9 ± 0.1	5.3 ± 0.3	4.7 ± 0.2	5.0 ± 0.3
Platelets ($\times 10^5/\mu l$)	9.4 ± 0.1	13.5 ± 0.4[a]	11.3 ± 1.2[a]	10.2 ± 0.4	15.1 ± 0.5[a]	11.3 ± 1.0	13.2 ± 1.2[a]
WBC ($\times 10^3/\mu l$)	7.9 ± 0.8	12.1 ± 1.3[a]	13.0 ± 1.4[a]	24.7 ± 1.9	28.0 ± 2.1[a]	36.2 ± 2.5	35.2 ± 1.5
Neutrophil ($\times 10^3/\mu l$)	3.1 ± 0.4	7.3 ± 0.3[a]	9.1 ± 0.3[a]	16.3 ± 0.2	13.7 ± 0.2[a]	24.7 ± 0.9	21.5 ± 0.5[a]
Eosinophil ($\times 10^3/\mu l$)	0.3 ± 0.02	0.5 ± 0.01	0.4 ± 0.02	0.5 ± 0.06	0.8 ± 0.01[a]	0.3 ± 0.04	0.3 ± 0.2
Lymphocyte ($\times 10^3/\mu l$)	4.4 ± 0.09	4.0 ± 0.06	3.4 ± 0.06[a]	7.4 ± 0.09	12.7 ± 0.08[a]	10.1 ± 0.04	12.7 ± 0.09[a]
Monocyte ($\times 10^3/\mu l$)	0.1 ± 0.001	0.3 ± 0.04	0.1 ± 0.01	0.5 ± 0.02	0.8 ± 0.02	1.1 ± 0.03	0.7 ± 0.04

A total of 15 mice were studied in each group. Cu–ATP was injected at a dose of 2.5 mg/kg/day for 10 consecutive days. In the case of tumor-bearing mice, treatment started 24 h post-EAC transplantation.
[a] $p < 0.05$ when compared with respective controls.

hosts was prolonged by 70% when compared to that of matched controls.

Growth of EAC tumor in Swiss mice was accompanied by a decline in hemoglobin and RBC values, and appreciable improvements were recorded following Cu–ATP treatment (Table 2). Cu–ATP treatment of normal mice also resulted in elevated RBC and hemoglobin levels ($p < 0.05$, Table 2), suggesting perhaps a mild stimulatory effect of this synthetic compound on murine red cell indices. This possibility was substantiated further by the observation of stimulated erythropoiesis, as evident from elevated RBC ^{59}Fe incorporation, in the treated groups (Table 3). However, the increment in erythropoiesis was statistically significant ($p < 0.05$) in the treated tumor hosts only.

Like the red cell and hemoglobin values, Cu–ATP treatment resulted in elevated levels of circulating platelets and leukocytes. Since the marked neutrophilic leukocytosis was a consistent finding in untreated EAC-bearing mice, Cu–ATP-induced neutrophilia was noticeable in treated normal mice only (Table 2). However, drug treatment was followed by an appreciable increment in the absolute number of circulating lymphocytes in the tumor hosts.

Table 3. Effect of Cu–ATP treatment on 72 h RBC ^{59}Fe incorporation (%, mean ± SE) of normal and EAC-bearing mice

Group	72 h RBC ^{59}Fe incorporation (%)[a]
Normal control	42.1 ± 3.5
Normal + Cu–ATP	43.8 ± 3.2
EAC control	35.4 ± 1.6
EAC + Cu–ATP	48.5 ± 3.1[b]

[a] Percentage of injected radioactivity. A total of 10 animals were studied in each group.
[b] $p < 0.05$ when compared with EAC control.

While the total number of nucleated cells in the femoral marrow was unaffected, splenomegaly and splenic hypercellularity were observed in Cu–ATP-treated normal mice group. Like neutrophilic leukocytosis, splenomegaly with increased number of nucleated cells paralleled EAC tumor growth. Cu–ATP treatment had only a mild suppressing effect on these values (Table 4).

Although the nucleated cellularity of the femur was relatively unchanged, Cu–ATP treatment was followed by a sharp fall in the percentage distribution of erythroid and lymphoid cells with concomitant elevation of myeloid cells, especially the neutrophils (Table 4). Differential count of bone marrow cells in the femur of untreated EAC-bearing mice resembles largely with that of treated normal mice group and Cu–ATP treatment of these tumor hosts had little effect on this marrow differential count (Table 4).

Cu–ATP treatment caused a marked reduction ($p < 0.01$) in the relative distribution (%) of lymphocytes but increments in myeloid and erythroid cells in the spleen of the normal mice group. In contrast, Cu–ATP treatment and resultant tumor regression had a stimulatory effect on the frequency (%) of erythroid and lymphoid cells of the hosts' spleen (Table 4).

Since peripheral blood cells and hematopoietic cells of all the lineages in mice originate from a common pluripotent stem cell (CFU-S), the effect of Cu–ATP treatment on CFU-S was investigated.

Table 4. Effect of Cu–ATP treatment on total and differential count of bone marrow and spleen cells of mice (results are mean ± SE)

	Normal mice		EAC-bearing	
	control	treated day 11	control day 11	treated day 11
Femoral marrow				
nucleated cells ($\times 10^6$)	24.9 ± 0.8	26.5 ± 1.2	20.7 ± 0.7	19.8 ± 0.6
erythroid (%)	22.1 ± 1.2	8.2 ± 0.5[a]	5.2 ± 0.3	8.8 ± 0.6
myeloid (%)	59.2 ± 2.8	86.5 ± 2.9[a]	88.2 ± 3.3	85.9 ± 3.2
lymphoid (%)	18.6 ± 1.0	5.2 ± 0.4[a]	6.3 ± 0.4	5.1 ± 0.3
megakaryocytic (%)	0.1 ± 0.04	0.1 ± 0.03	0.3 ± 0.1	0.2 ± 0.05
Spleen				
nucleated cells ($\times 10^7$)	16.5 ± 0.9	28.8 ± 1.2[a]	26.9 ± 0.6	20.6 ± 1.2
erythroid (%)	3.0 ± 0.3	7.9 ± 0.8	6.9 ± 0.6	12.8 ± 1.9[a]
myeloid (%)	2.1 ± 0.2	42.5 ± 3.2[a]	23.6 ± 1.7	15.1 ± 1.5[a]
lymphoid (%)	94.8 ± 2.3	49.5 ± 2.9[a]	69.4 ± 2.8	72.0 ± 2.8
megakaryocytic (%)	0.01 ± 0.005	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
spleen weight (mg)	105 ± 4	194 ± 11[a]	267 ± 31	262 ± 29

A total of 15 animals were studied in each group.
[a] $p < 0.05$ when compared to respective controls.

It is evident from Table 5 that the drug in the present dose schedule has no adverse effect on the absolute number of femoral marrow CFU-S. A mild stimulatory effect on marrow CFU-S number was observed in drug-treated normal mice and a 2-fold increase in the number of splenic CFU-S was recorded in this group (Table 5). The number of splenic CFU-S of treated EAC-bearing mice was reduced in comparison to their matched controls, but the number was still greater than that of normal animals.

Histological evaluation of CFU-S-derived colonies revealed that Cu–ATP treatment of normal mice resulted in stimulated differentiation of bone marrow and splenic CFU-S towards granulocyte lineage. Similarly, megakaryocytopoietic stimulation was observed in the bone marrow CFU-S of both normal and EAC-bearing mice following Cu–ATP treatment (Table 6).

Discussion

Myelosuppression resulting in leukopenia and thrombocytopenia is a frequent and major complication of cancer chemotherapy.[4] Thus, peripheral leukocyte and platelet counts are usually monitored during antineoplastic therapy.[11] The present study was designated in this perspective to evaluate the effect of Cu–ATP, a new synthetic antitumor agent, on blood and hematopoietic tissues of mice. Cu–ATP was administered i.p. at a tumor-inhibitory dose of 25 mg/kg body weight. At this dose level we could find no evidence of any adverse

Table 5. Relative distribution and absolute number (mean ± SE) of bone marrow and splenic CFU-S following Cu-ATP treatment

	Normal		EAC	
	control	treated (day 11)	control (day 11)	treated (day 11)
CFU-S/10^5 femoral marrow cells	30.3 ± 1.5	35.1 ± 1.8[a]	20.2 ± 0.6	22.5 ± 1.3
Total CFU-S/femur ($\times 10^3$)	7.5 ± 0.9	9.3 ± 1.1[a]	4.2 ± 0.6	4.4 ± 1.2
CFU-S/10^6 spleen cells	28.2 ± 1.3	31.5 ± 0.9[a]	44.4 ± 2.0	37.0 ± 1.8[a]
Total CFU-S/spleen ($\times 10^4$)	4.6 ± 0.8	9.1 ± 1.2[a]	11.9 ± 1.6	7.6 ± 1.3[a]

Six donor and assay animals were used for each experimental group.
[a] $p < 0.05$ when compared with controls.

Table 6. Histological evaluation of CFU-S-derived colonies formed in irradiated mouse spleen (results are mean ± SE)

Source of CFU-S	Colony type (%)			
	erythroid	granulocyte	megakaryocyte	mixed/undifferentiated
Femoral marrow				
normal control	59.4 ± 2.3	21.7 ± 1.7	5.7 ± 0.9	13.2 ± 0.8
normal + Cu–ATP	50.2 ± 1.9[a]	28.6 ± 1.2[a]	11.2 ± 0.6[a]	10.0 ± 0.9
EAC control, day 11	20.2 ± 0.8	55.6 ± 0.9	12.2 ± 1.3	12.0 ± 0.6
EAC + Cu–ATP	22.8 ± 1.6	50.1 ± 1.6	17.3 ± 0.7[a]	9.8 ± 0.7
Spleen				
normal control	26.5 ± 2.2	50.2 ± 1.8	10.3 ± 2.3	13.0 ± 0.7
normal + Cu–ATP	27.2 ± 2.8	66.1 ± 1.2[a]	4.6 ± 0.6[a]	2.1 ± 0.2[a]
EAC control, day 11	25.5 ± 3.3	44.4 ± 2.6	15.4 ± 1.1	14.7 ± 1.1
EAC + Cu–ATP	26.3 ± 1.8	50.1 ± 2.5	17.2 ± 0.4	6.4 ± 0.5[a]

Six donor and assay animals were used for each experimental group.
[a] $p < 0.05$ when compared with respective controls.

effect on circulating leukocyte and platelet counts of both EAC tumor-bearing and tumor-free normal mice. In fact, in the latter group we observed a 1.5-fold increase in total leukocyte count with a 2- to 3-fold rise in absolute number of neutrophils. Circulating platelet level of these animals was also elevated. Thus, the results indicate that Cu–ATP has a stimulatory, rather than inhibitory, effect on the number of peripheral leukocytes and platelets.

Another interesting observation following Cu–ATP treatment was a mild but consistent rise in hemoglobin and RBC values, particularly in the tumor hosts. This observation assumes significance as anemia is a common finding in malignancy[12] and chemotherapy often aggravates the situation because of the suppressive effect of a great majority of anticancer agents on erythropoiesis.[11] The observed increments in hemoglobin and RBC levels of Cu–ATP-treated EAC-bearing mice may be attributed to stimulated erythropoiesis as evident from RBC ^{59}Fe incorporation studies. However, it is difficult to delineate whether the increase in red cell production was secondary to tumour regression or the result of the action of Cu–ATP itself. The latter assumption seems unlikely because Cu–ATP failed to stimulate erythropoiesis significantly ($p > 0.05$) in normal mice.

Although the underlying mechanism of Cu–ATP-induced tumor inhibition is yet to be clearly established, intercalation of this compound with the DNA has been reported.[1] We were interested, therefore, to evaluate the action of this agent on bone marrow hematopoietic cells. We have also included the spleen in this study because the murine spleen is an important hematopoietic organ along with the marrow.[13] The data presented here clearly showed that Cu–ATP has no suppressive effect on the number of nucleated cells in the femoral marrow and spleen. In fact, the compound has stimulatory action on splenic cellularity of normal mice. A study of the progenitor cells in the marrow and spleen clearly indicates stimulation of granulocytopoieses, particularly in the spleen of the treated normal mice group. An apparent absence of this effect in tumor-bearing mice may be explained by the already stimulated granulocytopoietic activity following tumor transplantation, making further expansion of hematopoiesis towards this lineage difficult.

The stimulatory effect of Cu–ATP on hematopoiesis in general and granulocytopoiesis in particular is further evidenced by an increased number of CFU-S and their preferential differentiation towards granulocyte lineage as evident from CFU-S-derived spleen colony studies. Assuming that one femur represents approximately 6.5% of total bone marrow in mice,[14] the total number of CFU-S in treated normal mice was increased to 152.1×10^3 (143.0×10^3 in bone marrow plus 9.1×10^3 in spleen) from a control value of 120.0×10^3. Thus, the treatment resulted in 26.7% increment in the CFU-S population. Since blood cells of all the lineages originate ultimately from CFU-S, the Cu–ATP-induced increase in peripheral blood cell counts may be attributed to the stimulatory effect of this antitumor agent at the level of CFU-S.

It is thus evident from the present investigation that Cu–ATP does not affect hematopoiesis while it acts against tumor cells; on the contrary, it stimulates granulocytopoiesis in mice. This char-

acteristic activity of Cu–ATP as an antitumor agent in this tumor system seems to have important bearing on future clinical trials, particulary in combination with myelosuppressive anticancer drugs as hematotoxicologic studies in rodents accurately predict the effects in man.[15]

Conclusion

The results presented here demonstrate that Cu–ATP inhibits tumor growth and concomitantly elevates peripheral blood cell counts. The increase in CFU-S population and their altered differentiation in favor of granulocytopoiesis strongly suggests primary involvement of the hematopoietic stem cells in the onset of Cu–ATP-mediated changes in the number of blood cells particularly granulocytes. Recently, cytokines have been used to protect the tumor hosts from chemotherapy-induced severe and sometimes protracted myelosuppression.[16] However, the major disadvantage of most of these biologicals is their efficacy to promote tumor growth as well.[17] On the basis of the present observation it is tempting to conclude that Cu–ATP may be considered for combination chemotherapy to protect the host from hematotoxicity as well as to supplement the tumoricidal efficacy. However, these findings need to be substantiated by evaluating the effect of Cu–ATP on committed granulocyte stem cells. Furthermore, systemic toxicity of Cu–ATP, if any, needs proper evaluation. It is encouraging in this context that we did not observe any hepato- or renal toxicity in mice following Cu–ATP treatment.[3]

Acknowledgments

The authors are thankful to Dr (Mrs) J Roy Chowdhury, Director, Chittaranjan National Cancer Institute, Calcutta, for providing facilities, and also to Professor RG Bhattacharyya and Dr KK Nayak, Chemistry Department, Jadavpur University for providing us with the Cu–ATP complex.

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(Received 3 June 1993; accepted 10 June 1993)