JCI The Journal of Clinical Investigation

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J Clin Invest. 1954;33(10):1388-1396. https://doi.org/10.1172/JCI103016.

Research Article

CLINICAL EFFECTS OF THE DICHLORO AND MONOCHLORO-PHENYL ANALOGUES OF DIAMINO PYRIMIDINE: ANTAGONISTS OF FOLIC ACID [1, 2]

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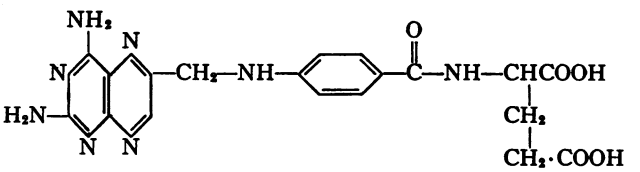
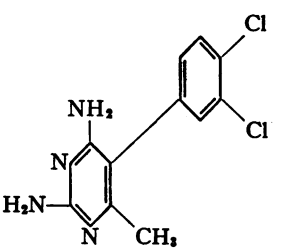
(Submitted for publication December 1, 1953; accepted June 16, 1954)

Hitchings, Elion, Vanderwerff, and Falco (1) demonstrated that certain 2,4-diamino-pyrimidines inhibited the growth of *L. casei;* this inhibition could be reversed by folic acid and citrovorum factor. The effects of these active pyrimidines are thus similar to those of the 4 amino derivatives of folic acid. But in contrast to the folic acid antagonists, the active pyrimidines are not pterins, and it appears unlikely that a rearrangement or structural alteration to a pterin ring system can occur. It is of interest, however, that high antagonist activity is associated with the presence of 2 and 4 amino groups in the pyrimidine nucleus, and a weighty substituent in the 5 position, suggesting a definite similarity in molecular configuration to the folic acid antagonists (Figure 1).

FIGURE 1



AMINOPTERIN



2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (DDMP)

Because the 4-amino derivatives of folic acid inhibit the growth of tumors and leukemias in animals and in man (2), Clarke, Buckley, Sternberg, Stock, Rhoads, and Hitchings (3) tested a series of diamino-pyrimidines for their influence on transplantable mouse tumors, and observed that one of this group, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (DDMP) inhibited the growth of the Sarcoma 180. The drug also produced inhibition in the growth of leukemia (4) and the Ehrlich ascites tumor (5) in mice, and modified the development of the chick embryo (6). At the doses tolerated under the conditions of testing, DDMP was the pyrimidine analogue most effective in inhibiting tumor growth. Since the folic acid antagonists are particularly effective

[1] This investigation was supported by research grants from the American Cancer Society, the Damon Runyon Memorial Fund for Cancer Research, the Lasker Foundation and the Grant Foundation, and by Grant C-1889 from the National Cancer Institute of the National Institutes of Health of the United States Public Health Service.

[2] We wish to express our appreciation to Dr. George H. Hitchings of the Wellcome Research Laboratories for the supply of DDMP and the Lederle Laboratories Division of the American Cyanamid Company for the supply of Leucovorin.

against acute leukemia in children, DDMP was examined for its therapeutic activity in this disease. In order to conduct a proper clinical assessment, it was also necessary to obtain information on the clinical pharmacology of DDMP, its activity in relation to the folic acid antagonists such as A-methopterin, and the protective action of Leucovorin.[8]

PHARMACOLOGY IN LABORATORY ANIMALS

The LD_{50} doses of DDMP by single intraperitoneal injection in mice and rats are 38 and 28 mg. per kg., respectively, and deaths usually are delayed, occurring within 3 to 10 days. Doses in excess of the LD_{50} cause an acute convulsive death. At the LD_{50} dose level, the delayed deaths can be prevented by the simultaneous administration of Leucovorin but not by folic acid. Leucovorin will not prevent the lethal effects of larger single doses of DDMP.

[8] Leucovorin, 5, 6, 7, 8 tetrahydro-5-formyl pteroylglutamic acid, is a synthetic product which has been shown to have one-half the microbiological activity of natural citrovorum factor (7).

The bone marrow of dogs receiving toxic doses of DDMP (1 mg. per kg. daily × 5) showed the megaloblastosis, giant metamyelocytes and hypersegmentation of the nucleus of the granulocytes (8, 9) seen with the 4-amino derivatives of folic acid.

CLINICAL RESULTS

Clinical toxicity

DDMP was administered orally to 44 patients. These patients were examined daily, and had daily hemoglobin and leukocyte determinations and weekly platelet and reticulocyte counts. In most cases the drug was given daily, but when early signs of oral or gastrointestinal toxicity or of leukocyte or platelet depression appeared, the drug was withheld temporarily. Thirty-four patients had an adequate trial of the drug, in that it was given until either signs of toxicity or a therapeutic response occurred.[4]

[4] The diagnoses and the number of adequately treated cases were: Acute leukemia, children, 19, adults, 6; single cases of rhabdomyosarcoma, chronic myelocytic leukemia, fibrosarcoma, reticulum cell sarcoma, melanoma, carcinomas of the adrenal, pancreas, rectum, lung and carcinoid of the colon.

TABLE I

Dosages in patients receiving adequate trial of DDMP

	No. of patients	No. courses of DDMP	Usual daily dose, *mg.*	Duration treatment Range *days*	Duration treatment Aver.	Total dose Range *mg.*	Total dose Aver.
Previously untreated with chemotherapy, or none within two weeks of DDMP							
Children							
Acute leukemia							
Previously untreated	9	13	2.5–5	10–33	22	40–145	78
Last dose of Amethopterin more than 2 weeks before	4	4	2.5–10	21–41	29	53–132	95
Adults							
Acute leukemia	4	6	5–10	4–39	24	35–175	108
Misc. cancers	8	8	2.5–20	8–68	33	50–635	280
Amethopterin given less than two weeks before or simultaneously with DDMP							
Children							
Acute leukemia	6	6	2.5–5	5–35	15	13–93	33
Adults							
Acute leukemia	2	4	2.5–15	4–25	13	30–163	66
Misc. cancers	2*	4	2.5–10	2–12	7	5–120	61

* These patients were also treated with DDMP alone.

The tolerated daily dose of DDMP ranged from 2.5 to 10 mg. in children and 2.5 to 20 mg. in adults (Table I). In the patients with acute leukemia, children could tolerate almost the same total dose as the adults. Many of the adults with lymphoma or carcinoma, however, tolerated larger daily and larger total doses than did the patients with leukemia. It was not possible to correlate satisfactorily, however, the absence of marrow involvement by neoplastic disease with the ability to tolerate larger doses.

A patient who received A-methopterin (2.5 mg.) together with the daily dose of DDMP (Figure 2) and the children who had received A-methopterin within two weeks prior to the onset of DDMP therapy, tolerated smaller amounts of the latter, and signs of toxicity appeared early in the course of treatment.

Toxic manifestations appeared in the gastrointestinal tract, the skin, and the hematopoietic system. Injury to the gastrointestinal tract was evidenced by nausea and vomiting, mouth ulcerations, or diarrhea (occasionally hemorrhagic). A morbiliform, erythematous, or follicular rash was noted frequently. Two adults, after prolonged therapy with DDMP, developed symmetrical, diffuse, brown pigmentation. This was most marked on the extensor surfaces of the joints of fingers and toes, and on their faces. Those children developing toxic rashes had residual brownish pigmentation

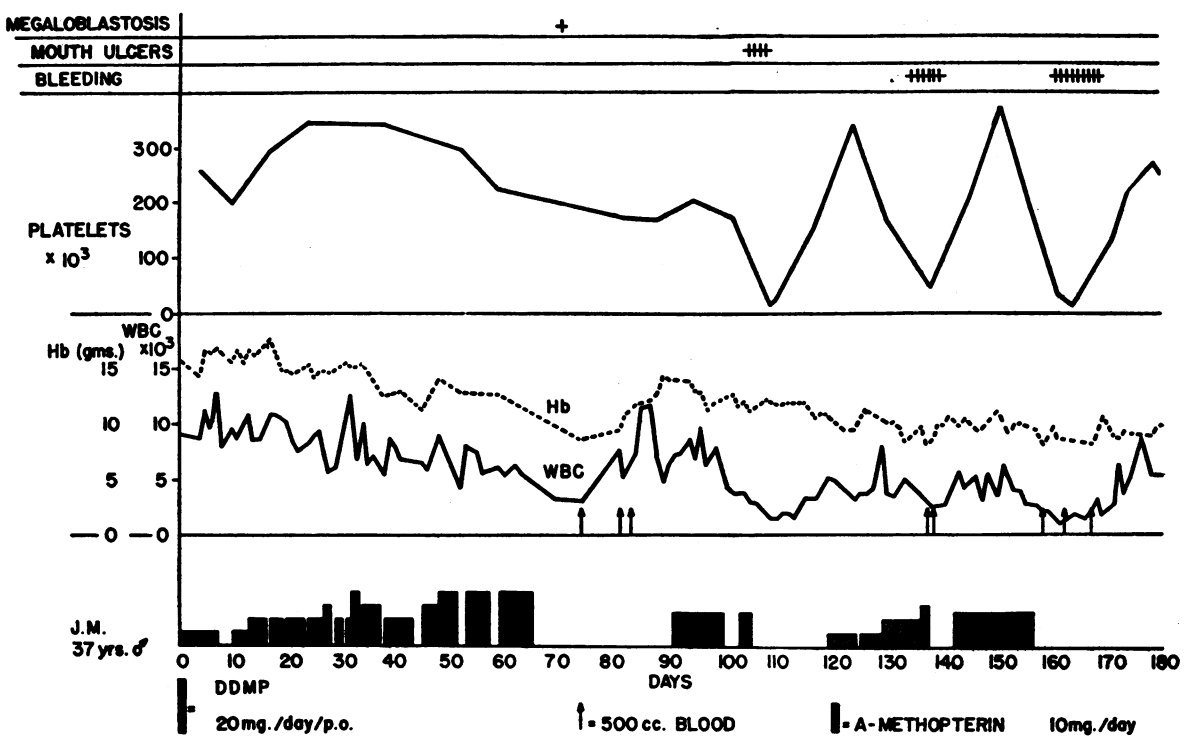


FIGURE 2

J. M., a 37-year-old colored man with carcinoma of the rectum, metastatic to the abdomen and chest, received a trial of DDMP to determine whether it had any effect on rectal cancer. To attempt to demonstrate a possible additive effect a combination of DDMP and A-methopterin was given. A total dose of 635 mg. of DDMP over a period of 65 days caused hematological toxicity with hypocellularity and megaloblastosis of the bone marrow and the drug was stopped. The abnormal blood picture returned to normal within four weeks; thereafter DDMP 10 mg. and A-methopterin 2.5 mg. daily were given for a total of 12 doses when hematological toxicity again appeared and treatment was stopped. Three weeks later, after recovery from the toxicity, A-methopterin at a total dose of 105 mg. produced an abrupt fall in the leukocyte and platelet counts. On discontinuing the drug the blood picture returned to normal. Another trial of combined therapy (DDMP—120 mg. and A-methopterin—30 mg. total dose) produced a fall in the leukocyte and platelet counts and treatment was discontinued. During the study there was no evidence of tumor inhibition and, since the disease was progressing, the patient received radiotherapy with no improvement. The patient expired, six weeks after the last dose of DDMP and A-methopterin. Permission for autopsy was not obtained.

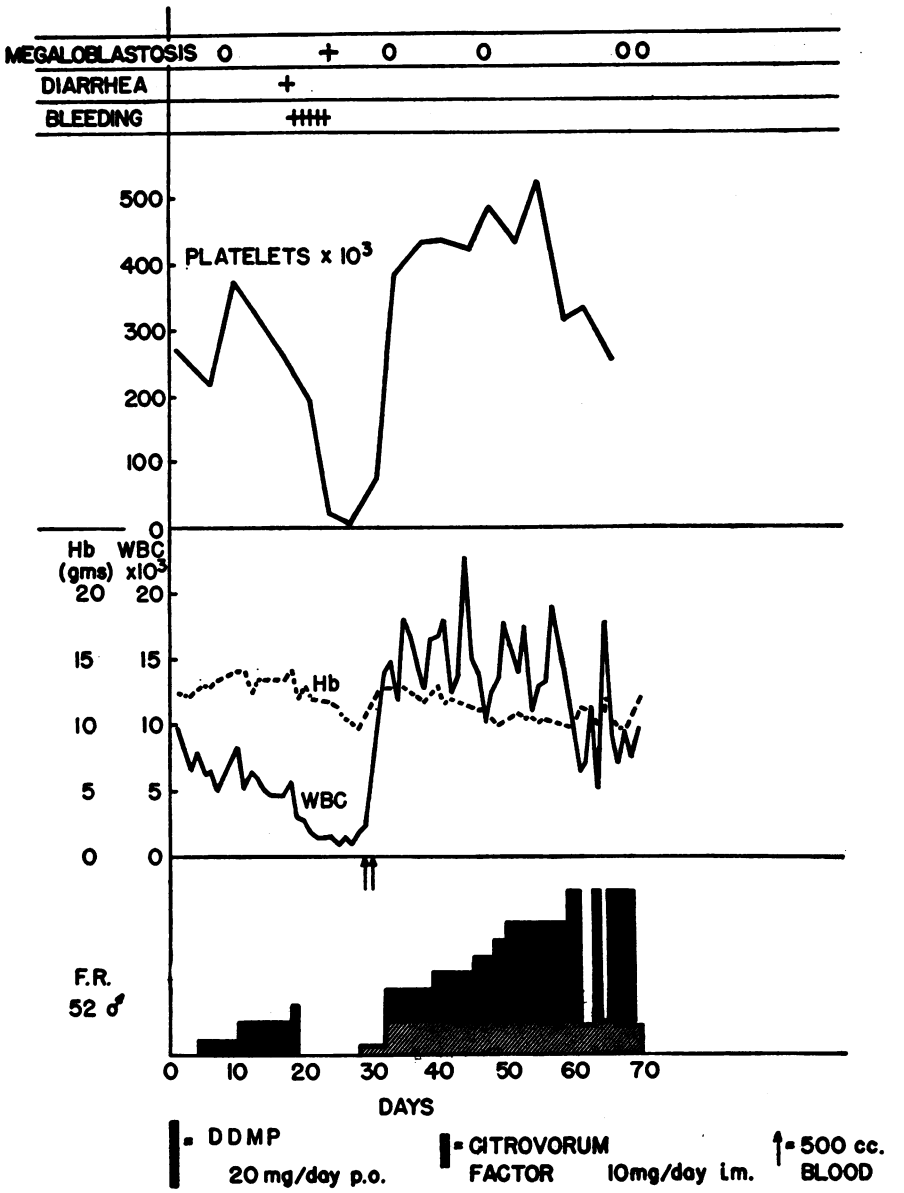


FIGURE 3

F. R., a 52-year-old man, with bronchogenic carcinoma, was treated with DDMP and toxic symptoms of leukopenia, thrombocytopenia, bleeding and rash occurred after a total dose of 125 mg. in 15 days (daily dose 5 to 15 mg.). There was no evidence of tumor regression. After the drug was stopped the blood picture improved. The patient was then restarted on DDMP together with Leucovorin. Despite increasingly large doses of DDMP (a total of 820 mg. in 38 days (10 to 40 mg. daily)), 10 mg. of Leucovorin daily protected against its hematological toxicity. Tumor regression did not occur on this regimen, and the patient expired. Permission for autopsy was not granted.

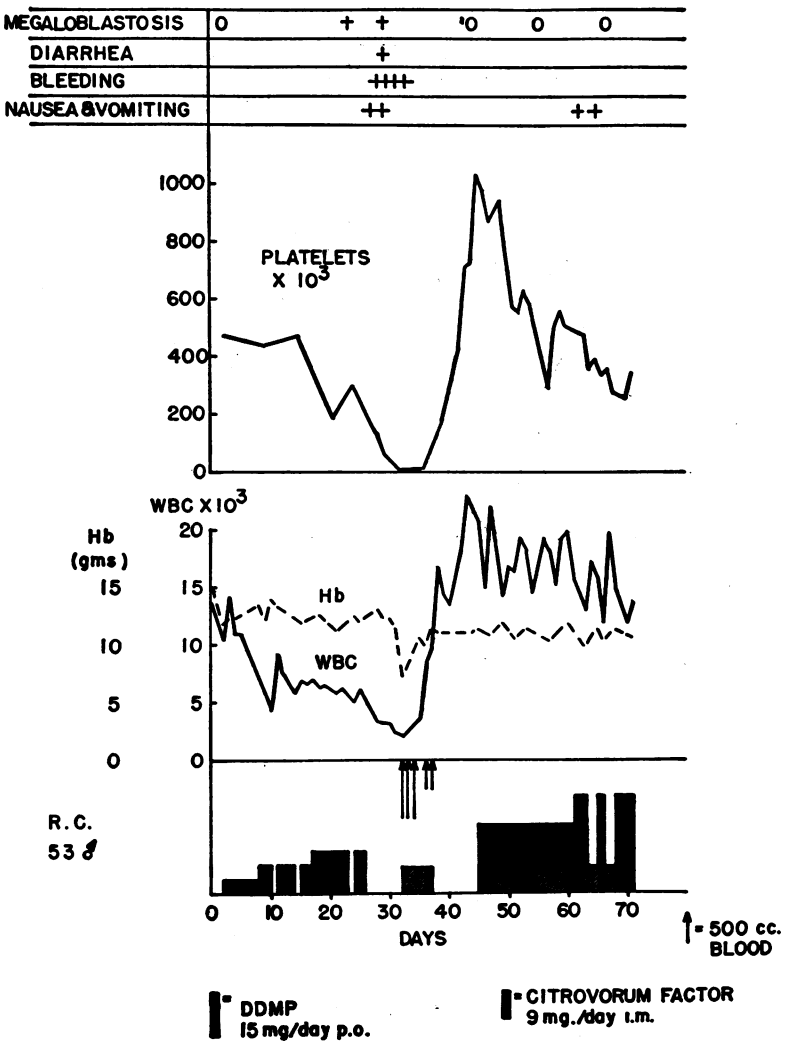


FIGURE 4

R. C., a 53-year-old man with bronchogenic carcinoma, was treated with DDMP. Toxic symptoms of nausea, vomiting, diarrhea, thrombocytopenia, bleeding, and leukopenia occurred after he received a total dose of 220 mg. in 24 days (daily dose: 5 to 15 mg.). At the time of greatest toxicity an increase in transmission of breath sounds in a previously atelectatic area was found. This probably did not indicate tumor regression, since cavitation of the tumor occurred subsequently. Hematologic findings improved when DDMP was discontinued. The patient subsequently received 415 mg. of DDMP in 26 days (15 to 25 mg. daily) simultaneously with Leucovorin,[5] without evidence of hematological toxicity, although nausea recurred.

Tumor progression continued, and the patient expired following a massive hemoptysis. Autopsy showed bronchogenic carcinoma of the right upper lobe with metastases to lymph nodes, right lower lobe, left lung, liver, right adrenal gland and bone marrow. There was cavitation of the primary tumor with massive hemorrhage and aspiration of blood into the right lower lobe and all lobes of the left lung.

[5] Microbiologic assay by Dr. Dorris Hutchison of the Leucovorin samples available for clinical use showed that there was only 10 to 20 per cent of the stated activity, so that the patient, R. C., was actually receiving 0.9 to 1.8 mg. of microbiologically active Leucovorin daily. Patient F. R. (Figure 3) had been studied 10 months earlier, and while the Leucovorin was not assayed microbiologically at that time, it is likely that this fresher preparation more closely approximated the stated activity.

at the site of the rash for as long as six months. Two children and two adults who received DDMP to the point of toxicity then developed alopecia. Skin pigmentation has been reported as occurring after prolonged administration of A-methopterin (10) and alopecia also is a previously noted complication of therapy with the 4-amino antagonists of folic acid (2).

A fall in the total leukocyte count to severely leukopenic levels frequently accompanied the other toxic manifestations. A decrease in the number of circulating platelets often occurred even in those patients whose bone marrow was essentially normal before therapy.

The bone marrow showed megaloblasts, differing slightly from those seen in pernicious anemia in that the nuclear chromatin was somewhat more granular, in 17 of the 34 patients receiving an adequate course of DDMP. Three showed normoblastic erythropoiesis. The marrow of the 14 remaining patients failed to show nucleated erythroid cells either due to the leukemic state or because, after therapy, inadequate specimens were obtained.

Giant metamyelocytes and hypersegmented polymorphonuclear leukocytes also were seen.

With certain differences, the pattern of toxicity observed resembles that seen after A-methopterin administration. Mouth ulcerations occurred in less than half of the patients adequately treated with DDMP and the ulcerations were more diffuse with generalized mucosal erythema. They usually appeared after the onset of leukopenia, rather than preceding it, as with A-methopterin, and were, therefore, not a useful premonitory sign of toxicity. Another factor making the drug more difficult to use than A-methopterin was the occasional progression of leukopenia, mouth ulcerations and gastrointestinal symptoms for as long as 7 to 10 days after DDMP was discontinued.

Evidence was obtained in two patients that the hematological toxicity of DDMP was prevented by the simultaneous administration of Leucovorin (Figures 3, 4).

Therapeutic trials

Of the 34 patients receiving an adequate trial of DDMP, a therapeutic response occurred only in children with acute leukemia. Of twelve children with acute leukemia treated initially with DDMP, three showed hematological and clinical improvement following, and presumably due to, the drug. Two of these remissions were complete, and lasted two and seven months, respectively. Hematological remission consisted of improvement in bone marrow with a decrease of the total of stem cells and lymphocytes to less than 30 per cent as well as a return of the total leukocyte and differential counts toward normal levels. The third child had a good clinical remission, but the differential count of the bone marrow smear showed a decrease of the stem cells from 98 to 55 per cent, with an increase in the erythroid precursors from 0 to 30 per cent. The drug had to be discontinued because the patient left the hospital, and it was considered too hazardous to continue on an out-patient basis.

Seven children who had been treated with A-methopterin prior to DDMP, and had developed resistance or were initially resistant to A-methopterin, showed no definite therapeutic response to an adequate trial of DDMP.

A 3-year-old child with embryonal rhabdomyosarcoma received an amount of the drug (DDMP) sufficient to cause megaloblastosis and depression of leukocytes and platelets. The tumor became necrotic but there was no significant change in size.

Fourteen adults received adequate courses of DDMP; six had acute leukemia and eight had other neoplastic diseases. Twelve showed significant leukocyte depressions, all developed other evidences of toxicity, and none showed a therapeutic response.

Comparison with Daraprim®

The phenyl-substituted members of the 2,4-diaminopyrimidine series have antimalarial activity, (11) and one of this group, 2,4-diamino-5-p-chlorophenyl-6-ethylpyrimidine (Daraprim®) has been used successfully in the suppression and prophylaxis of malaria in man (12–15). While the dichloro derivative, DDMP, is almost as active an antimalarial as the monochloro compound, Daraprim,® it has not been used because of its greater toxicity in man.

Daraprim® is also less toxic for animals (16) but, at tolerated doses in mice, no anti-tumor effect has been noted. Mice can be protected against the acute lethal convulsive seizures produced by Daraprim® by pre-treatment with sodium phenobarbital, so that larger doses can be given which

finally result in delayed deaths similar to those seen after DDMP. These larger doses did not inhibit the Sarcoma 180 (17).

Daraprim® appears to act as an antimetabolite. Hitchings and his co-workers have produced with Daraprim® (18) a syndrome resembling folic acid deficiency in the growing rat. The debilitation, hemosiderin deposits, alopecia, and leukocyte depression can be reversed by Leucovorin but not by folic acid. Liver powder and autolyzed yeast have even greater protective action than that expected on the basis of their citrovorum factor content. Whereas single doses of DDMP can produce this deficiency picture in rats, Daraprim® requires a high level and continuous administration. Daraprim® also produces megaloblastosis in dog bone marrow (8, 9).

The recommended dose of Daraprim® for suppression or prophylaxis of human malaria is 25 mg. weekly. This compound has been administered at 5 mg. daily for one year, 50 mg. twice weekly for three months (13, 14) and 25 mg. daily for 50 consecutive days (15) with no evidence of toxicity other than a transient fall in hemoglobin level and erythrocyte count in one patient.

In our studies, two patients were treated with Daraprim® at doses higher than those used in antimalarial therapy; no therapeutic effect on the neoplastic disease was observed. The toxic effects, other than nausea and vomiting, were hematologic, with platelet and leukocyte depressions, anemia, and megaloblastosis. One patient with a metastatic synovioma was given a total of 1500 mg. of Daraprim® in a 26-day period, the daily dose ranging from 50 to 100 mg. One week after therapy was stopped the platelet count fell from 250,000 to 27,000 per cu. mm. with a slight fall in hemoglobin level, but the leukocyte count remained unchanged. The platelet count remained low for three days and then rose to normal levels.

The second patient was a 49-year-old woman with widely disseminated metastatic carcinoma, probably primary in the uterus. She was markedly cachectic, had evidence of liver metastases, and a normal blood urea nitrogen. She received three daily doses of 100 mg. each of Daraprim® and the drug was then discontinued because of severe nausea and vomiting. Five days later the leukocyte count fell from the pretreatment level of 18,000 to 1,200 per cu. mm. The sternal marrow aspirate, at this time, was cellular with definite megaloblastosis, giant metamyelocytes, and an increase in plasma cells. The leukocyte count continued to fall to 500 per cu. mm., and thrombocytopenia and bleeding tendencies developed. The bone marrow reverted to a normoblastic picture, but the platelet count remained low, bleeding continued and, despite repeated blood transfusions, the patient died 18 days after the last dose of Daraprim®. Permission for autopsy was not obtained.

It is possible that the inadequate diet of this patient prior to Daraprim® therapy, as well as the pre-existent liver damage, may have increased her sensitivity to the antimetabolite, possibly because of a borderline folic acid deficiency.

DISCUSSION

It has been postulated that folic acid is essential for cellular growth and division because of its role in purine and pyrimidine metabolism. Folic acid has been implicated in the activation of such reactions as the formation of amino acids, transfer of methyl groups, methylation of purine ribosides, and closure of the purine ring. In order to carry out its functions, folic acid is converted to its effective form, the citrovorum factor. The conversion of folic acid to citrovorum factor is blocked by the 4-amino derivatives of folic acid, which explains the poor protection provided by folic acid in animals.

The available evidence indicates that DDMP, while structurally quite different, acts by interfering with the same metabolic pathways as those affected by A-methopterin, although it may act at somewhat different loci. Experimental work suggests that the 2,4-diaminopyrimidines interfere both with the conversion of folic acid to citrovorum factor and with its utilization or further transformations.

DDMP and Daraprim®, like A-methopterin, produce megaloblasts, giant metamyelocytes and hypersegmented polymorphonuclear leukocytes in the bone marrow. The diaminopyrimidines inhibit the growth of *L. casei* (1), and this is reversed competitively by folic acid. Folic acid, however, cannot reverse or prevent the inhibition of growth caused by these 2,4-diaminopyrimidines in *S. faecalis* (18) or in intact animals (19). The inhibition of bacterial growth can be prevented

competitively by Leucovorin in *L. citrovorum* and over a narrow range of concentration in *S. faecalis* (18). In mice and rats, however, although Leucovorin can prevent the lethal effects of DDMP when one to three times the LD_{50} dose is given, when the dose of the antimetabolite is raised significantly above this level, increasing the amount of Leucovorin does not prevent death. This differs from the protective effect afforded by Leucovorin against A-methopterin in mice (20). In patients, Leucovorin protected against the toxicity of DDMP, in that three to six times the total amount of the drug, which had previously produced hematologic and clinical signs of toxicity, had no toxic effect when administered simultaneously with much smaller doses of Leucovorin.

Despite the similarity in some of the effects of DDMP and A-methopterin, there are clinical differences, suggesting that these agents may have different sites of action on the metabolic pathways involving folic acid and citrovorum factor. Toxic manifestations persist longer after the discontinuation of DDMP therapy; mouth ulceration may develop concurrently with diarrhea and leukocyte depression rather than preceding them, or may be absent entirely. Thus it is not as satisfactory a warning sign as with A-methopterin. The leukocyte depression tends to be more delayed and protracted. A possible explanation is that, after an ingested dose of A-methopterin, serum levels fall and are no longer detectable in about 3 to 8 hours, and approximately 40 to 60 per cent of the dose is excreted within 12 hours (21); whereas there is indirect evidence based on Daraprim® studies (22) suggesting that DDMP may disappear much more slowly. The postulated prolonged maintenance of serum levels of DDMP does not necessarily explain this difference in toxicity, however, since A-methopterin has been demonstrated in measurable amounts in an unchanged form (as shown by microbiological assay and paper chromatography) in mouse liver at least eight months after the administration of this compound (23). The tendency of A-methopterin to persist in the tissues may account for the synergistic effect of A-methopterin and DDMP.

DDMP may be expected to produce transient hematological remissions in about one-fourth of the children with acute leukemia, and its therapeutic activity thus parallels, to some extent, that of A-methopterin. It was not therapeutically effective in children who were resistant, or who had responded and then become resistant, to A-methopterin, and its greater toxicity and difficulty in safely controlling dosage prevents it from being a useful substitute for for A-methopterin.

SUMMARY

1. The compound 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine, DDMP, is an antagonist of the folic acid system although it is structurally quite different. In man it produces toxic effects similar to the 4-amino analogues of folic acid. These effects are principally bone marrow depression with megaloblastosis, mouth ulcerations, digestive disturbances and rash. DDMP differs from A-methopterin, however, in that it appears to have a more prolonged toxic effect, and mouth lesions are less conspicuous and less frequent. DDMP produced hematological improvement in 3 of 12 children with acute leukemia, but it was ineffective in children resistant to A-methopterin. Leucovorin protected against large doses of DDMP in two patients. A-methopterin and DDMP were found to have additive toxic effects, and DDMP was more toxic in children who had received A-methopterin within two weeks prior to DDMP therapy.

2. The antimalarial, Daraprim® (2,4-diamino-5-p-chlorophenyl-6-ethylpyrimidine), which is structurally related to DDMP, produced simliar toxicological effects in two patients when given in large doses by weight.

3. The clinical data support the view that a pyrimidine, DDMP, acts as an antimetabolite in the folic acid-citrovorum factor system. While DDMP may act in inhibiting the transformation of folic acid to citrovorum factor, as do the 4-amino derivatives of folic acid such as A-methopterin, its loci of action are not entirely the same.

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