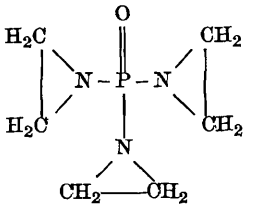
Inhibition Studies of Some Phosphoramides Against Sarcoma 180.* (19054)

S. M. Buckley, C. C. Stock, R. P. Parker, M. L. Crossley, E. Kuh, and D. R. Seeger. (Introduced by C. P. Rhoads.)
(With the technical assistance of A. T. Boryczka, F. M. Costa, and V. M. Bailey.)

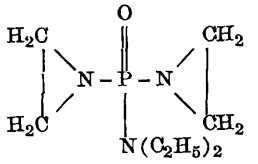
From the Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York City and Research Department, Calco Chemical Division, American Cyanamid Co., Bound Brook, N. J.

Recently, it has been shown that SK 1133, 2,4,6-tris(ethylenimino)-s-triazine, is quite effective in retarding the growth of a number of experimental tumors in mice and rats(1-7). SK 1133 has also been tried in over 150 cases of human cancer with beneficial results comparable to the nitrogen mustards and with some additional advantages(8).

Our studies with the ethylenimines have been extended by the synthesis of N-ethylene substituted phosphoramides and their subsequent testing for ability to inhibit the growth of various experimental tumors. The present report is concerned with the ability of two phosphoramides, SK 3818, N,N',N''-triethylene-phosphoramide and SK 4614, N,N-diethyl-N',N''-diethylene-phosphoramide, to inhibit the development of Sarcoma 180 in mice (Fig. 1). The relationship of dosage, toxicity, and retardation of tumor growth were determined and the picture of the peripheral blood and histopathological changes in the tumors and the organs of treated mice were studied.



I. SK 3818
N,N',N''–triethylene phosphoramide



II. SK 4614
N,N–diethyl–N',N''–diethylene phosphoramide

FIG. 1.

Materials and methods. The compounds were prepared and characterized by elementary analysis in the research laboratories of the Calco Chemical Division following the procedures of Bestian(9). In view of their high degree of reactivity the compounds were prepared as solutions with physiological saline just prior to injection. After preliminary toxicity determinations in mice(10) the compounds were tested by a standardized technic we have employed on over 5000 compounds(11) in seeking for anti-tumor activity. White Swiss mice (CFW Female and Male, RF Female and Male, 20 ± 2 g in weight) were used as experimental objects. They were implanted with Sarcoma 180 by the usual trocar method(12). The course of injections was begun 24 hours after implantation of the tumor. The compounds were adminis-

* We wish to acknowledge support of this study by funds from the American Cancer Society to the Sloan-Kettering Institute for Cancer Research.

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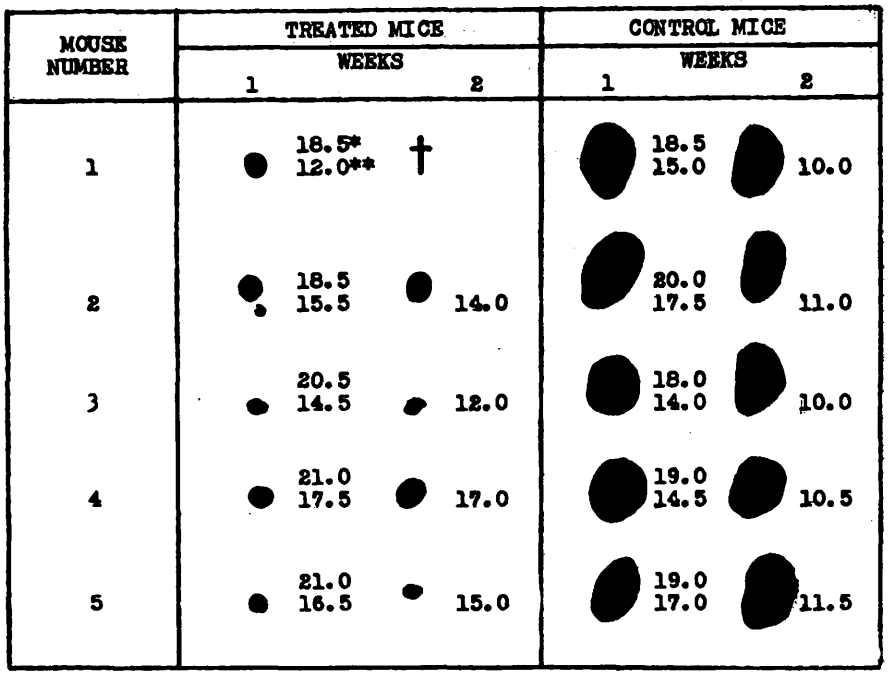
INHIBITION OF SARCOMA 180 BY SK 3818 AT 10 MG/KG/DAY



* WEIGHT OF ANIMAL AT START OF THERAPY.
** WEIGHT OF ANIMAL AT TIME OF TUMOR MEASUREMENT.

FIG. 2.

tered once daily intraperitoneally for seven days. Tumor bearing control mice were injected with corresponding amounts of physiological saline. On the eighth day after tumor implantation the tumors of the treated and of the control group were measured in two diameters by calipers. The degree of tumor inhibition was expressed as the ratio of the average diameter of the tumors of treated to those of untreated tumor bearing mice. The degree of inhibition of tumor growth was determined as follows: Inconclusive result (Inc.) = Three or more of a group of 5 mice succumbed during inhibition test. Marked Inhibition (†) = No growth of tumors in treated animals to a growth with an average diameter one-fourth that of the untreated controls. Slight Inhibition (‡) = Growth of tumors in treated animals from one-fourth to three-quarters the average diameters of the control tumors. No Effect (*) = Growth of tumors greater than three-quarters the average diameter of the control tumors.

Histopathological changes in tumors and organs of treated mice were studied in a special experiment as described previously (12). In brief, tumor pieces were implanted into the right thigh muscle of mice in groups of 8 each. Daily injections of the compounds (treated groups) and of physiological saline (control group) were started 4 days after tumor implantation and continued for 7 days. Three, 5 and 7 days after beginning of therapy mice in the different groups were sacrificed immediately after blood had been secured for counts. The tissues (tumor, thymus, spleen, axillary and mesenteric nodes, femur, sternum, stomach, intestine, lungs, heart, voluntary muscle, liver, both kidneys, adrenals and pancreas) were fixed in Zenker-formol solution and stained with hematoxylin-eosin for microscopic examination. Thus, peripheral blood and progression of possible lesions were studied 3, 5 and 7 days after beginning of therapy.

Results. 1. Relationship of dosage, toxicity, and retardation of growth of tumor. The degrees of retardation of tumor growth ob-

TABLE I. Relation of Dose to Inhibition of Sarcoma 180 by SK 3818.

Dose, mg/kg/day	No. of deaths*/ No. of mice	AWC	Tumor inhibition	
			Grading	IQ × 100
25	3/5	—	Inc.	—
20	8/30	−4.5/−1.5	‡ to Inc.	30
15	6/45	−4.5/−1.5	‡ to †	25
12.5	4/35	−3.0/−1.0	‡	35
10	1/65	−4.0/−1.5	‡	40
8	0/20	−4.0/−1.5	‡	50
7.5	6/50	−3.0/−1.5	‡	40
6	0/10	−2.5/−2.0	‡	50
5	1/45	−2.5/−1.5	‡	55
4	0/10	−2.5/−2.0	‡	60
2.5	0/55	−1.5/−1.5	§ to ‡	75
2	0/30	0 /−1.0	§ to ‡	85
1	0/25	−1.0/−1.5	§ to ‡	85
.5	0/10	−1.0/−1.5	§ to ‡	90
.1	0/5	+2.0/ 0	§	80

* Results at end of one wk inhibition test.
† Marked inhibition. ‡ Slight inhibition. § No effect.
AWC = Avg wt change, as ratio of avg change per treated mouse to that per untreated control mouse.
IQ = Inhibition quotient = ratio of avg diameter of tumors in treated mice to those in untreated control mice.
Inc. = Inconclusive (3 or more out of 5 mice died).

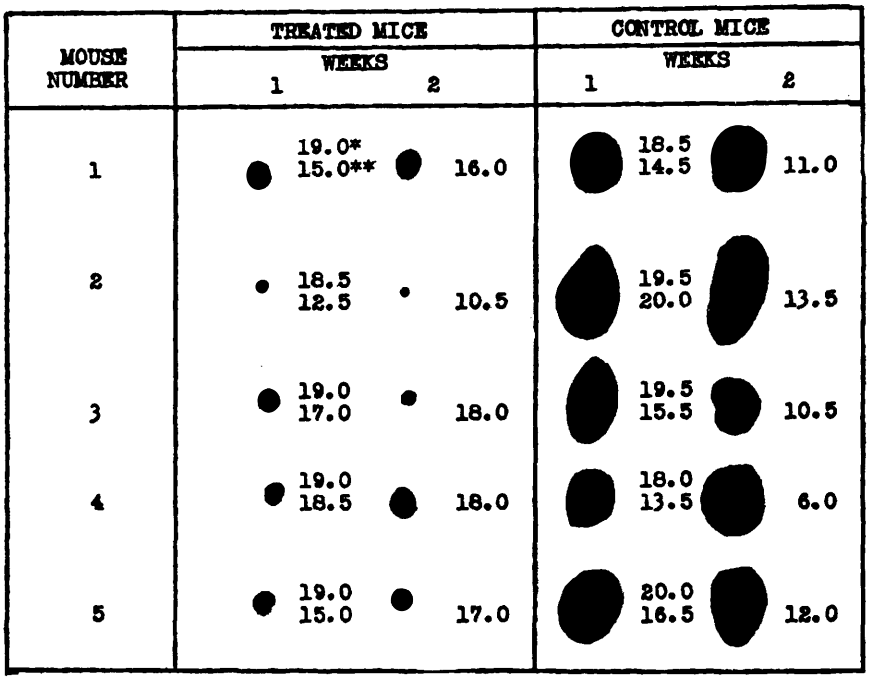
TABLE II. Relation of Dose to Inhibition of Sarcoma 180 by SK 4614.

Dose, mg/kg/day	No. of deaths/ No. of mice	AWC	Tumor inhibition	
			Grading	IQ × 100
20	7/30	−4.0/−2.0	‡ to Inc.	35
15	8/50	−3.5/−2.0	‡ to †	35
12.5	2/40	−2.5/−2.5	‡	20 to 35
10	1/60	−2.5/−1.5	‡	45
7.5	0/30	−1.5/−0.5	‡	55
5	1/40	−1.5/−1.0	* to ‡	65
3	0/40	−1.5/−1.5	* to ‡	70 to 80
2.5	0/10	−0.5/−0.5	*	90
2	0/20	−1.0/−1.5	* to ‡	75 to 85
1	0/15	0 /−0.5	*	90
.5	0/5	0 /−0.5	*	90
.1	0/5	−1.0/−0.5	*	90

* No effect. † Marked inhibition. ‡ Slight inhibition.
AWC = Avg wt change, as ratio of avg change per treated mouse to that per untreated control mouse.
IQ = Inhibition quotient = ratio of avg diameter of tumors in treated mice to those in untreated control mice.

tained at various levels of SK 3818 and SK 4614 are presented in Tables I and II. Both compounds have the ability to inhibit the growth of Sarcoma 180. The grading of a marked inhibition was observed in both instances at a dose level of 15 mg/kg/day for 7 days. The relationship of dosage, acute and delayed toxicity and degree of tumor inhibition appeared to be optimal at a dose level of 10 mg/kg/day for 7 days. Fig. 2 and 3 present area diagrams of the relative size of tumors of representative treated and untreated mice as observed at the end of the inhibition test and one week after therapy had been discontinued. Both compounds have similar inhibitory effects at the dose level employed. A comparison of the activity range, however, indicates that SK 3818 is characterized by a somewhat greater range of effective dose levels. SK 4614, for example, shows approximately a 5-fold range between the minimum effective dose and the maximum dose giving a conclusive result, whereas SK 3818 has a corresponding range of approximately 6. SK 1133, 2,4,6-tris(ethylenimino)-s-triazine, as discussed in a previous study(13), showed a

INHIBITION OF SARCOMA 180 BY SK 4614 AT 10 MG/KG/DAY



* WEIGHT OF ANIMAL AT START OF THERAPY.
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FIG. 3.

4-fold range. These results were based on data obtained in numerous experiments at the end of the course of injections. Mice in these experiments were subsequently observed for a prolonged period in order to note the appearance of delayed toxicity at the various dose levels employed. The maximum doses which cause a considerable degree of retardation of tumor growth, but no deaths due to acute or delayed toxicity are 5.0 mg/kg/day for SK 3818, 7.5 mg/kg/day for SK 4614, as compared to 0.25 mg/kg/day for SK 1133.

2. Picture of peripheral blood. Groups of 8 mice (RF Female, 20 $\pm$ 2 g) were studied during the course of injections with SK 3818 and SK 4614 (10 mg/kg/day for both compounds) 3, 5 and 7 days after beginning of therapy, corresponding to 7, 9, and 11 days after intramuscular implantation of Sarcoma 180. The red blood cell count was not altered during the course of injections. The white blood cell count of treated mice with and without tumors was characterized by a progressively increasing leucopenia as described for SK 1133(13), whereas the tumor bearing control mice showed a progressively increasing leucocytosis. The differential count varied from the one observed with SK 1133 (lymphopenia with relative increase of polymorphonuclear leucocytes) inasmuch as the granulocytes practically vanished from the picture of the peripheral blood toward the end of therapy, whereas the mononuclear cells showed a relative increase. Table III presents data obtained in treated tumor bearing mice and in the various control groups at the end of the course of the 7 injections of SK 3818 and SK 4614. Blood for counts was always collected from the base of the tail. In applying this method, the values obtained for the total white blood cell count were slightly lower in comparison to values calculated when blood was drawn from the end of the tail. The ratio as calculated from data

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TABLE III. Total and Differential Count of White Blood Cells in Mice Treated with SK 3818 and SK 4614.

Groups	Treatment, SK No.	Dose, mg/kg/day for 7 days		WBC, 1 mm³ × 10⁻³	PMN, %	MNC, %
I Normal mice without tumors	Phys. saline	—	Mean	10.2 (32)	18.5 (32)	81.5 (32)
			Range	6.2–14.2	11 –29	71– 89
II Control mice with tumors	Phys. saline	—	Mean	15.3 (12)	41.5 (12)	58.5 (12)
			Range	13.4–18	28.5–54	47– 71.5
III Treated mice without tumors	3818	10	Mean	1.3 (8)	1 (8)	99 (8)
			Range	.8– 2.4	0 – 2	98–100
IV Treated mice with tumors	3818	10	Mean	1.5 (8)	1 (8)	99 (8)
			Range	.6– 2	0 – 3	97–100
V Treated mice without tumors	4614	10	Mean	1 (8)	12 (8)	88 (8)
			Range	.8– 4.1	6 –16	84– 94
VI Treated mice with tumors	4614	10	Mean	1.9 (8)	2 (8)	98 (8)
			Range	1.1– 3	1 – 4	96– 99

WBC = White blood cell count.
PMN = Polymorphonuclear granulocytes.
MNC = Mononuclear cells, *i.e.*, lympho- and monocytes.
No. in parentheses = No. of mice.

obtained from groups of 8 normal mice (RF Female, 20 ± 2 g) is approximately 2 to 3.

3. Pathology.† (a) Administration of SK 3818 (10 mg/kg/day). Mice were immediately sacrificed and carefully autopsied 3, 5 and 7 days after beginning of therapy. During the course of injections, a considerable loss of weight occurred in both treated groups and tumor bearing control group. Diarrhea was not observed at any time in the treated mice.

Sarcoma 180. The tumors were in all instances smaller than the control tumors, especially in the groups receiving 5 and 7 injections. Histopathologically, the changes seen corresponded to the ones described for tumors of mice treated with 2,4,6-tris-(ethylenimino)-s-triazine(13) which were vacuolar degeneration of the cytoplasm, enlargement of nuclei and nucleoli, karyolysis, increased occurrence of karyorrhexis and pyknosis. The number of mitoses was decreased. All these changes were observed to some degree as early as 3 days after beginning of therapy.

Myeloid system and lymphoid system. An attempt was made to correlate the hematological data with the histopathological lesions seen in sternal and femoral marrow, spleen, thymus and lymphoid tissue of axillary and mesenteric lymph nodes and intestinal follicles. As early as 3 days after beginning of therapy, sternal and femoral marrow showed a considerable depletion. The bone marrow elements had disappeared from the sinusoids of the spleen. At the same time, the Malpighian bodies were not reduced in number, only the number of cells present was reduced. At the end of the experiment, the femoral marrow was fluid and purple red in all instances as compared to the pink and gelatinous marrow of untreated control mice. Sections revealed a severe depletion. In the spleen the myeloid elements were absent. A definite attack on the lymphoid elements was seen. Large forms of lymphocytes had disappeared during treatment. The small lymphocytes were decreased in thymus (especially in the cortical part), lymph nodes and spleen. Germinal centers were not observed. Only the intestinal follicles were relatively unaffected even after 7 injections. In summary then, a definite depletion of both systems was present at the end of therapy as reflected in the low total white blood cell count. However, SK 3818 appeared to damage the myeloid system more selectively at the dose level employed, a finding which was paralleled in the peripheral blood by granulocytopenia and relative increase of mononuclear cells.

Intestinal lesions. Parts of the gastro-in-

† We are indebted to Dr. John J. Buckley, Division of Laboratories and Research, New York State Department of Health, Sloan-Kettering Institute, for the histopathological examination.

testinal tract (stomach, duodenum, jejunum, ileum, colon ascendens, colon transversum and rectum) were carefully searched for lesions after 3, 5 and 7 injections of SK 3818. There was shortening of the villi after 7 injections. Focal areas of edema, ulceration with inflammatory reaction and occasional hemorrhages were seen in the large intestine only. The most consistent lesions were located in the glandulae intestinales(14), where cells were necrotic at the base and in the middle part of the crypts. Pathological and incomplete mitosis was observed especially in the small intestine.

Other lesions. Occasionally, there was vacuolar degeneration of tubules in both kidneys. In a few instances, the livers showed areas of necrosis and hemorrhage after seven injections of the compound; lung, heart, voluntary muscle, pancreas and both adrenals were free of lesions.

There was no difference between the findings of treated mice with tumors and treated, non-tumor bearing animals. (b) Administration of SK 4614 (10 mg/kg/day). The experimental procedures were the same as employed for the investigation of SK 3818. The histopathological changes were essentially the same as described for SK 3818, only to a lesser degree (intestine, kidney, liver). There was essentially no difference between the findings of treated mice with tumors and treated, non-tumor bearing animals.

Discussion. SK 3818 and SK 4614, two N-ethylene substituted phosphoramides, by an injection course of 7 days, were able to retard the growth of Sarcoma 180 whether started 24 hours or 4 days after implantation of tumor grafts. These results present new evidence for the observation that anti-tumor activity is associated with the presence of ethylenimine groups. The two phosphoramides are related in their reactivity to the ethylenimonium intermediates occurring during the hydrolysis of the nitrogen mustards. It was not surprising therefore to find lesions similar to the ones induced by nitrogen mustards(15), ethylenimino-s-triazine and some ethylenimine derivatives of urea(5,13,16), namely, histopathological manifestations limited to tissues characterized by a high degree of proliferation such as the hematopoietic system, the intestinal mucosa and various experimental tumors(17,18). In their greater adverse effect against the myeloid system, SK 3818 and SK 4614 resemble the ethyleneureas(13). It remains to be investigated if any clinical value will be attributed to this observation, *i.e.*, selective administration of the two compounds for cases of myelogenous leukemias rather than of lymphatic leukemias. However, it is scientifically and especially chemotherapeutically of interest to note that by changing the structure of the nucleus that contains ethylenimine groups the cytotoxic action can be varied somewhat. The lesions in the intestine correspond to the mode of action of nitrogen mustards on intestinal epithelium of the rat as studied recently(14).

Perhaps of practical value is the observation that SK 3818 has shown a greater range of effective dose levels than those observed with SK 1133, 2,4,6-tris(ethylenimino)-s-triazine and other ethylenimines that have been tested. For this reason SK 3818 might prove better than SK 1133 for maintenance therapy. Clinical trials of the phosphoramides are in progress. The results of those studies(19) will throw light on any possible clinical advantages of N,N′,N″-triethylenephosphoramide over 2,4,6-tris(ethylenimino)-s-triazine.

Summary. 1. SK 3818, N,N′,N″-triethylenephosphoramide and SK 4614, N,N-diethyl-N′, N″-diethylenephosphoramide are both capable of retarding the growth of Sarcoma 180 in mice 24 hours and four days after implantation of tumor. Among the chemotherapeutic agents tested in our laboratory SK 3818 has shown the greatest effective dose range. 2. Histopathological examination of tumors of mice treated with SK 3818 and SK 4614

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showed enlargement of cells with vacuolar degeneration of cytoplasm, enlargement of nuclei and nucleoli. Furthermore, the number of mitoses was decreased. Numerous cells showed nuclear alterations as commonly encountered during necrosis such as pyknosis, karyolysis and karyorrhexis. 3. Histopathological examination of organs of mice treated with SK 3818 and SK 4614 revealed lesions similar to the ones occurring during administration of nitrogen mustards: intestinal damage and depletion of myeloid and lymphoid system with leucopenia (granulocytopenia and relative increase of mononuclear cells) in the peripheral blood.

Received September 17, 1951. P.S.E.B.M., 1951, v78

The Central Nervous Depressive Effect of Khellin. (19055)

GRAHAM CHEN, CHARLES R. ENSOR, AND DAVID RUSSELL.
From the Research Laboratories, Parke, Davis, and Co., Detroit, Mich.

Khellin, a glucoside isolated from the plant *Ammi visnaga*(1-3) has been tested clinically in recent years as an antispasmodic agent and a coronary dilator, principally in the management of angina pectoris. Clinical results from different laboratories are varied both as to its efficacy as a smooth muscle relaxant and its toxic effects(4-8). Insomnia, somnolence, light-headedness, dizziness, nausea and vomiting have occurred in patients receiving therapeutic doses of the drug. While the pharmacological action of Khellin on the cardiovascular system and the smooth musculature has been extensively investigated (9-11), no study apparently has been made of its central nervous action. In our toxicological studies, it was observed in mice that Khellin both depresses and excites the central nervous system. Depression occurs prior to excitement, which appears only with large doses. The depression is mild but of long duration. Excitement begins with an increase in motor activity, followed by clonic convulsions indicating stimulation of nervous centers above the medullary levels. Such an action may produce some of the toxic effects of Khellin in man.

Since sedation is of value therapeutically in patients suffering from angina pectoris or from chronic bronchial asthma(12,13), the central depressive action of Khellin may be likewise effective in the relief of angina or asthmatic attacks. With such a view in mind, the central nervous depressive action of Khellin was investigated in cats, rats and mice. The results are presented in this report.

Methods and results. A. *Anti-caffeine effect.* This was determined with jiggle cages in rats receiving a stimulating dose of caffeine by the procedure of Schulte and associates(14). Activity was measured and recorded in terms of the total number of revolutions of the work

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