Immunosuppressive Properties of Cyclophosphamide Analogues (36275)

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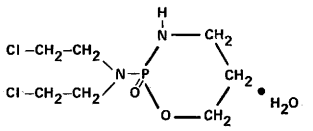
Several reports on the immunosuppressive properties of cyclophosphamide (CY) have appeared encompassing a wide range of experimental test models (1–9). CY was synthesized by Arnold and Bourseaux (10, 11) in an attempt to make a less toxic cyclic nitrogen mustard that could be activated following intracellular transport. Ideally, the inactive form of the drug would be absorbed, transported, and preferentially activated by phosphatases or phosphamidases present in neoplastic cells. The series of compounds synthesized, which included CY, hydrolyzed slowly in saline solution and were well tolerated when injected into animals (12). Adequate activating enzymes were anticipated since Gomori (13) had earlier reported high phosphatase and phosphamidase activity in certain tumors and epithelial tissue. Brock and Hohorst have postulated that CY is transformed into primary activated metabolites by liver microsomes by a process involving mixed function oxidation and *O*- and *N*-dealkylation. This activation, however, was not detected in experimental rat tumor tissue. They suggest, therefore, that the somewhat selective antineoplastic activity of CY may be the result of a greater accumulation of activated metabolites in tumor cells due to a permeability phenomenon or to a mode of action of these metabolites which differs from that of other nitrogen mustard compounds. The possibility of differences in cellular permeability between normal and cancer cells appears the more plausible of the two proposed hypotheses.

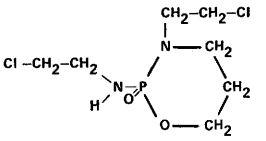
Recently, Brock (14) reported on two new analogues of CY which show significant antineoplastic activity and hopefully better therapeutic indices. A comparison of some of the immuno-suppressive properties of CY and these new analogues is the subject of this report.

Materials and Methods. Drugs. Cyclophosphamide (CY) (Cytoxan tablets, Mead Johnson) was furnished by Mead Johnson Laboratories. Ifosfamide (IF) and trofosfamide (TR) were supplied as crystalline compounds by Asta-Werke AG, Chemische Fabrik, Brackwede, West Germany. The chemical structures are shown in Fig. 1. CY tablets were ground in a mortar and suspended in distilled water. Placebo tablets were added to IF and TR to provide suspensions of similar composition. All drug suspensions were prepared fresh daily, and the animals were dosed orally by intubation.

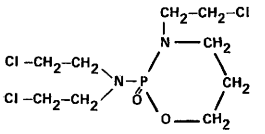
Antibody-producing spleen cells (APSC) response. Male Sprague-Dawley rats, 150–200



CYCLOPHOSPHAMIDE (CY)



IFOSFAMIDE (IF)



TROFOSFAMIDE (TR)

FIG. 1. Chemical structures.

g body weight, were (5, 7, 16) injected iv with 1 ml of a 10% suspension of washed sheep red blood cells (SRBC) and were sacrificed at periodic intervals. The plaque-forming cell culture technique of Jerne, *et al.* (17, 18) as modified by Kaplan, *et al.* (19) and Merchant (20), was used to enumerate the spleen leukocytes that produce SRBC antibodies. Blood samples were withdrawn by cardiac puncture for serological testing prior to removal of the spleen for APSC assay. All plaque counts were standardized to 10^6 viable spleen cells. Two animals were used for each plating interval for all drugs and antigen controls examined.

Serological methods. Hemagglutination (HA) antibody titer of the serum samples was determined by the microtiter (Cooke Engineering Co.) technique (21, 22) using a 0.5% suspension of washed SRBC as antigen. Microtiter plates were incubated at 37° for two hours then held at room temperature overnight before the HA titer was recorded.

Mercaptoethanol treatment. The HA titers of IgM and IgG immunoglobulin antibodies in the sera were differentiated by the 2-mercaptoethanol (2-ME) treatment procedure of Baum, *et al.* (23). Sera were diluted 1:5 with physiological saline and equal volumes of 0.1 *M* 2-ME in physiological saline were added. The mixtures were incubated at 37° for 30 min and the HA titer reassayed. The 2-ME-sensitive titers indicate primarily the levels of total immunoglobulins (IgM, IgG) while the 2-ME-resistant titers are representative of the IgG immunoglobulins (24).

Treatment schedules. Two separate treatment schedules were used in comparing the effect of the drugs dosed at 20 mg/kg/day on the APSC after antigen insult: (1) Treatment was started immediately after the intravenous injection of the antigen. It was continued once daily for a total of five consecutive days. (2) In the second regimen, treatment was delayed until the third day post antigen injection. It was likewise continued once daily for a total of five consecutive days. Antigen controls (untreated) were included in each treatment schedule. When the 40 mg/kg/day dose levels were investigated, only the second regimen was used.

Inhibition of humoral antibody production to TAB vaccine. Male albino mice (Swiss-Webster strain) weighing 18–25 g were used to determine the effect of the drugs on production of humoral antibodies to TAB (typhoid–paratyphoid A and B, Eli Lilly) vaccine according to the procedure of Berenbaum and Brown (25). The mice were injected with 0.2 ml of vaccine intraperitoneally and two days later, each mouse was given a single oral dose of drug. Seven days after drug treatment, the mice were sacrificed, exsanguinated, and the sera collected. The flagellar antibody titers of the sera were determined by a conventional twofold tube dilution technique using the microtiter equipment (22, 23). The flagellar antigen was prepared by combining all three strains of bacteria used in the vaccine.

Anaphylactic shock in rats. Male Sprague-Dawley rats weighing approximately 200 g were sensitized by injecting 25 mg ovalbumin and 0.5 ml *Hemophilus pertussis* vaccine intraperitoneally (26). Ten rats were used for each level of drug tested. Drug treatment started on the day of antigen insult and continued on alternate days for a total of seven doses. CY was tested at 2.5, 5, and 10 mg/kg. As the two CY analogues appeared to be less active in other test models, the dose levels were increased to 5, 10, and 20 mg/kg. Four hours after the last dose of drug, each rat was challenged with 1 mg of ovalbumin intravenously. Percent survival was determined 24 hr after antigen challenge. The results obtained with the treated groups were compared with untreated sensitized controls.

Results. ASPC response and HA titers. When treatment began immediately following antigen insult, all three compounds significantly suppressed the proliferation of APSC (Table I). CY treatment appeared to be slightly more effective in reducing the plaque counts than either IF or TR.

As expected, HA titers were likewise suppressed with this drug treatment schedule (Table II). There was a slight increase in HA titer with the terminal samples (day 7) in the group treated with TR. Both IF and CY suppressed the HA titers to less than a

TABLE I. Comparison of the Effect of Cyclophosphamide and Its Analogues on Antibody-Producing Spleen Cells when the Initiation of Treatment Was Varied in Relation to Antigen Insult.

Days post antigen insult	Mean $\log_{10}$ number of plaques per 10^6 spleen cells ± SE						
	Untreated antigen control	Treatment[a] started at time of antigen insult[b]			Treatment[a] started 3 days post antigen insult[b]		
		CY[c]	IF[d]	TR[e]	CY	IF	TR
0	1.4 ± 0.12						
1	1.72 ± 0.15	0.11 ± 0.11	0.70 ± 0.25	1.02 ± 0.24			
2	2.98 ± 0.09	0.56 ± 0.20	1.31 ± 0.10	0.61 ± 0.27			
3	4.15 ± 0.11						
4	4.86 ± 0.07	0.22 ± 0.22	1.25 ± 0.13	1.44 ± 0.33	3.01 ± 0.04	4.15 ± 0.09	4.02 ± 0.05
5	4.44 ± 0.13				1.32 ± 0.44	2.46 ± 0.52	3.15 ± 0.11
7	2.84 ± 0.13	0.79 ± 0.29	1.54 ± 0.14	1.39 ± 0.34	1.43 ± 0.13	1.88 ± 0.05	1.62 ± 0.33
8	2.96 ± 0.32						
10	3.07 ± 0.03				1.05 ± 0.11	1.73 ± 0.13	1.57 ± 0.12

[a] All drugs dosed 20 mg/kg (1 × day for 5 days) by oral intubation.
[b] Antigen—10% washed sheep RBC; [c] Cyclophosphamide; [d] Ifosfamide; [e] Trofosfamide.

1:2 dilution throughout the observation period.

When the 20 mg/kg/day treatment was delayed, CY significantly reduced the number of APSC compared to the antigen controls (Table I). Both IF and TR likewise reduced the numbers of APSC but not to the same extent as that observed with CY. When

TABLE II. Comparison of the Effect of Cyclophosphamide and Its Analogues on Total and 2-ME Resistant HA Titers when the Initiation of Treatment Was Varied in Relation to Antigen Insult.

Days post antigen insult	Mean $\log_2$ number of HA titer reciprocal ± SE							
	Untreated antigen control	Oral treatment[a] started at time of antigen insult[d]			Antigen control (no treatment)	Oral treatment[a] started 3 days post antigen		
		CY[e]	IF[f]	TR[g]		CY	IF	TR
1	<1.0[b] (<4.32)[c]	<1.0	<1.0	<1.0	<1.0 (<4.32)			
2	1.33 ± 0.21 (<4.32)	<1.0	<1.0	<1.0	1.0 ± 0 (<4.32)			
3	8.66 ± 0.66 (<4.32)	<1.0	<1.0	<1.0	7.3 ± 0.21 (<4.32)	8.1 ± 0.40	8.1 ± 0.54	6.83 ± 0.16
4	>12.0 (<4.32)	<1.0	<1.0	<1.0	>12.0 (<4.32)	10.5 ± 0.34	10.0 ± 0	9.5 ± 0.22
5					11.8 ± 0.16 (<4.32)	9.6 ± 0.21 (<4.32)	8.8 ± 0.16 (<4.32)	10.1 ± 0.16 (<4.32)
7	>12.0 (8.32)	<1.0	0.5	2.5	10.5 ± 0.42 (4.32)	6.5 ± 0.22 (<4.32)	9.0 ± 0 (<4.32)	7.83 ± 0.16 (<4.32)
10					9.16 ± 0.98 (6.32)	6.8 ± 0.40 (<4.32)	4.3 ± 0.33 (<4.32)	5.83 ± 0.16 (<4.32)

[a] All drugs dosed 20 mg/kg (1 × day for 5 days) by oral intubation.
[b] Total HA titer.
[c] 2-mercaptoethanol-resistant HA titers.
[d] Antigen—10% washed sheep RBC; [e] Cyclophosphamide; [f] Ifosfamide; [g] Trofosfamide.

TABLE III. Comparison of the Immunosuppressive Properties of Cyclophosphamide and Its Analogues at Higher Drug Levels.

Days post antigen insult	Mean $\log_{10}$ number of plaques per 10^6 spleen cells ± SE				Mean $\log_2$ number of total and 2-ME-resistant HA titer reciprocal ± SE			
	Untreated antigen control	Oral treatment[a] started 3 days post antigen insult			Untreated antigen control	Treatment[a] started 3 days post antigen insult		
		CY	IF	TR		CY	IF	TR
0	1.4 ± 0.12				1.4 ± 0.12[b] (<4.32)			
3	2.57 ± 0.33				2.33 ± 0.33 (<4.32)			
4	2.78 ± 0.10	2.34 ± 0.10	2.89 ± 0.11	2.57 ± 0.13	7.16 ± 0.16 (<4.32)	8.33 ± 0.55 [c]	8.16 ± 0.16	8.00 ± 0.25
5	3.57 ± 0.10	1.52 ± 0.11	2.07 ± 0.16	1.88 ± 0.09	7.50 ± 1.31 (5.32)	4.83 ± 0.54	8.16 ± 0.40	6.83 ± 0.30
7	3.55 ± 0.10	2.04 ± 0.12	1.83 ± 0.15	1.72 ± 0.22	10.16 ± 0.16 (7.32)	2.00 ± 0.44	2.50 ± 0.22	4.00 ± 0
10	3.47 ± 0.13	[d]	1.58 ± 0.14	1.73 ± 0.38	7.16 ± 0.54 (5.82)	[d]	2.50 ± 0.67	<1.0 ± 0

[a] All drugs dosed 40 mg/kg (1 × day for 5 days) by oral intubation.

[b] Total HA titer, (2-ME-resistant HA titer).

[c] All 2-ME-treated samples had values of <4.32 for all treatment groups.

[d] Both animals died before date of sacrifice.

TABLE IV. Effect of Cyclophosphamide and Its Analogues on the Production of Humoral Antibodies in Mice after Insult with TAB Vaccine.

Compound	Dose[a] (mg/kg)	Mean $\log_2$ number of agglutination titer $\pm$ SE	% Reduction compared to antigen control
IF	100	6.1 ± 0.20	19
	50	6.6 ± 0.37	<15
TR	100	6.0 ± 0.50	20
	50	7.3 ± 0.16	<15
CY	100	3.1 ± 0.30	58
	50	6.2 ± 0.36	18

[a] Oral administration.

the dose was increased to 40 mg/kg/day, the APSC were significantly reduced with all three compounds. The 40 mg/kg/day level reduced the APSC population more rapidly than the lower dose (Table III); however, the terminal populations (10 day) obtained with both dose levels were of the same magnitude. CY appeared to be not as well tolerated by the animals at the 40 mg/kg/day level since both animals died between the seventh and tenth day post antigen insult. Animals treated with IF and TR tolerated this treatment.

HA titers obtained when treatment was delayed for three days appear to be more sensitive to increased drug intake. All three drugs lowered the HA titers to a lower level when treated at the 40 mg/kg/day drug level (Tables II and III). The data suggest that CY lowers the titer more rapidly than either IF or TR, but decreased drug tolerance was evident with CY at the higher drug level. TR appears to be slightly more effective in depleting the HA titer than IF at the 40 mg/kg/day dose level.

Mercaptoethanol treatment. HA titers observed during the initial phase of immunity are predominantly the IgM immunoglobulins and are inactivated by 2-ME. The degree of inactivation can be correlated with a reduction in HA titer. The 2-ME-resistant HA titers represent the levels of IgG immunoglobulin present. The data reported here are in general agreement with the literature (23) on 2-ME inactivation of IgM as determined by the HA test (Tables II, III). Since IgG appears later in the immunization process, the IgG (2-ME-resistant) HA titers increased to detectable ($\log_2 \Rightarrow 4.32$) levels starting with day 7 in the untreated antigen control animals.

All three compounds are effective in blocking the formation of IgG immunoglobulins to SRBC in our test system. A 20 mg/kg/day dose was adequate in suppressing 2-ME-resistant HA titers for all three drugs tested.

Antigenic response to TAB vaccine. Neither IF nor TR demonstrated significant activity in blocking the formation of antibodies to TAB vaccine. CY at 50 mg/kg gave a response that closely paralleled that obtained with a 100 mg/kg dose of the two CY analogues (Table IV). The results obtained agree with those found in our other models; namely, that higher doses of IF and TR are required to effect a comparable response to CY. Potel (26) reported comparable gradations of activity (CY>IF>TR) with these same compounds in an experimental immunosuppressive model using rats sensitized with Brucella antigen.

Anaphylactic shock in rats. All three compounds demonstrated good protection against terminal anaphylactic shock produced by challenge of rats sensitized to ovalbumin. On the basis of a dose-response curve, our data indicate that CY was slightly more active than either CY analogue (Table V). All three compounds dosed at 10 mg/kg afforded complete protection to the challenged rats. Some of the untreated antigen control rats (20%) survived the terminal challenge with

TABLE V. Effects of Cyclophosphamide Analogues on Anaphylactic Shock in Rats Sensitized with Ovalbumin and *H. pertussis* Vaccine.

Drug	Oral dose (mg/kg)	After terminal antigen challenge: Survivors/ no. tested	(%)
CY	2.5	2/10	20
	5	7/10	70
	10	10/10	100
IF	5	4/10	40
	10	10/10	100
	20	10/10	100
TR	5	6/10	60
	10	10/10	100
	20	10/10	100
Untreated sensitized controls	0	2/10	20

antigen. This is inherent in the test model since Rosenthale, *et al.* (27) reported 40–50% survival in their untreated controls after terminal challenge. These investigators likewise found CY treatment to be effective in preventing anaphylaxis in rats.

Discussion. Santos (28), in discussing the pharmacology of immunosuppressive drugs, addressed himself to the need for timing drug administration in relationship to the antigen stimulus. He proposed three classes of immunosuppressants. One class of agents including X-ray and L-phenylalanine mustard produces optimum activity if administered prior to antigen stimulus. A second class of agents represented by 6-mercaptopurine and 5-fluorouracil is most active when given after the stimulus. The final class includes CY which exerts immunosuppressive activity when administrered either before or after the immune stimulus. IF and TR can be placed in the same class as CY based on our data and the findings of Santos (personal communication) in which drug treatment was started before antigen insult. HA titers were suppressed when IF and TR treatment was started simultaneously with antigen stimulus, and likewise the titers were decreased when IF and TR treatment was delayed until the third day post antigen stimulus. CY and the two analogues were effective in suppressing both IgM and IgG immunoglobulin production.

With respect to relative activities of the three compounds tested, rodents tolerated higher dose levels of IF and TR than CY. When all three compounds were dosed on an equivalent weight basis, CY consistently showed a slightly better immunological response than either IF or TR. This slight advantage was carried through all test models employed: namely, APSC response, HA titers, inhibition of humoral antibodies to TAB vaccine and anaphylactic shock in rats. Overall, the data presented here for CY are in general agreement with those reported by other investigators.

Summary. The immunosuppressive effects of cyclophosphamide and two of its analogues, ifosfamide and trofosfamide, on the cellular and humoral antibody responses in rodents were studied. We conclude that both analogues possess significant immunosuppressive activity through slightly less than that observed with CY: however, IF and TR appeared to be tolerated at higher dose levels better than CY.

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