Cytotoxic Activity Relative to 4-Hydroxycyclophosphamide and Phosphoramide Mustard Concentrations in the Plasma of Cyclophosphamide-treated Rats

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ABSTRACT

Male rats were given cyclophosphamide (50 mg/kg, i.p.), and the plasma concentrations of 4-hydroxycyclophosphamide and phosphoramide mustard were determined. Apparent plasma half-lives were 30 and 55 min, respectively. Area under the plasma concentration-time curve values were 1.5 and 2.5 mm·min, respectively. Since the plasma half-life of cyclophosphamide in rats is about 37 min, it may be that the actual plasma half-lives of the two metabolites are substantially shorter than the apparent plasma half-lives that were observed following cyclophosphamide administration. This is because of the identity of the metabolite in question would greatly aid in the design of experiments intended to determine the basis for the oncotoxic specificity of cyclophosphamide.

Specifically, experiments were designed to determine whether, in rats given cyclophosphamide, the pharmacokinetic behavior of either of these candidates in plasma was consistent with the cytotoxic activity found there.

INTRODUCTION

Cyclophosphamide is one of the most widely used antineoplastic agents. This is because of its relatively high oncotoxic specificity (2, 3, 8). Although the subject of numerous investigations, the basis for this specificity remains to be determined.

The major pathways of the metabolism of cyclophosphamide have apparently been identified, and the ultimate cytotoxic species appears to be the aziridinium zwitterion of phosphoramide mustard (8, 11, 16). The identity of the circulating metabolite that enters cells and ultimately gives rise to the cytotoxic species is still controversial. Most investigators favor 4-hydroxycyclophosphamide:aldophosphamide (2–4, 6, 7, 13, 16) as being of greatest importance, but not all of the evidence is consistent with this conclusion, and phosphoramide mustard has been offered as a candidate in an alternative scenario (9, 12, 29, 30).

The objective of the present investigation was to gain additional evidence with regard to the existing controversy since the identity of the metabolite in question would greatly aid in the design of experiments intended to determine the basis for the oncotoxic specificity of cyclophosphamide.

Specifically, experiments were designed to determine whether, in rats given cyclophosphamide, the pharmacokinetic behavior of either of these candidates in plasma was consistent with the cytotoxic activity found there.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats, 180 to 210 g, were purchased from the Bio-Lab Corp., White Bear Lake, Minn., and were housed in hanging wire cages. Female C57BL × DBA/2 F₁ (hereafter called BDF₂) mice, 18 to 22 g, were purchased from the Rat and Mice Laboratory (Ralston Purina Co., St. Louis, Mo.) and were housed in plastic cages filled with ground corn cob bedding. Water and Purina laboratory chow were available ad libitum except during the period when blood samples were collected.

Commercially available Cytoxan was used as the source of cyclophosphamide; the presence of NaCl in the commercial preparation was taken into account. 4-Hydroperoxycyclophosphamide and phosphoramide mustard (cyclohexylamine salt) were supplied by Dr. A. Takam-
izawa, Shionogi and Co., Fukushima, Osaka, Japan; and Dr. H. B.
Wood, Jr., Drug Development Branch, Division of Cancer Treatment,
National Cancer Institute, Bethesda, Md., respectively.

4-Hydroperoxycyclophosphamide was used in parts of this investi-
gation as a substitute for 4-hydroxycyclophosphamide. This was justi-
fied on the bases that: 4-hydroperoxycyclophosphamide is much more
stable and, thus, easier to work with; 4-hydroperoxycyclophosphamide
serves as a precursor to 4-hydroxycyclophosphamide, giving rise to it
very quickly in biological fluids as judged by various kinetic and
metabolism studies; and the 2 are, with reference to cytotoxic activity,
especially equipotent and equispecific both in vivo and in vitro (2, 16,
31, 32, 35).

Drug Administration

Cyclophosphamide was dissolved in 0.85% NaCl solution and was
injected i.p. in a volume equivalent to 2.5 ml/kg (rats) or 25 ml/kg
(mice). It was placed into solution immediately before injection.

Assay for Plasma 4-Hydroxycyclophosphamide

A fluorometric assay (1, 33) was used as described previously (16, 26)
to determine plasma concentrations of 4-hydroxycyclophospa-
hamide.* The assay is relatively specific for compounds that give rise to
acrolein following the deproteinization of plasma, e.g., 4-hydroper-
oxycyclophosphamide, 4-hydroxycyclophosphamide, and aldophos-
phamide; acrolein present in the plasma prior to deproteinization ap-
parently reacts quickly with various macromolecules contained therein
and thus does not give rise to falsely high values for plasma 4-hydrox-
cylocyclophosphamide concentrations (26).

Assay for Plasma Phosphoramide Mustard

Plasma phosphoramide mustard was quantified fluorometrically fol-
lowing isolation by thin-layer chromatography. The assay is based on
the formation of a stable fluorescent product resulting from the alkyla-
tion of nicotinamide by nitrogen mustards, e.g., phosphoramide mus-
tard, followed by condensation of N'-alkylated nicotinamide with cyclo-
hexanone (19, 20).

Isolation of Phosphoramide Mustard

Prior to, and at various times following, cyclophosphamide administra-
tion, 140 /il of rat tail vein blood were immediately transferred into 250-ml
microcentrifuge tubes containing 15 /il of 2.06 w semicarbazide, pH 7.4,
and were mixed. Semicarba-
A bioassay using cultured W256 tumor cells was developed to
quantify the cytotoxic activity contained in the plasma of cyclophos-
phamide-treated rats. Rats were given cyclophosphamide i.p. at a dose of
50 mg/kg; control animals received i.p. injections of 0.85% NaCl
solution, 2.5 ml/kg. At various times following drug administration,
groups of 5 animals each were sequentially anesthetized with ether,
and blood was collected from the aorta into heparin-coated syringes,
pooled into centrifuge tubes sitting in ice, and immediately centrifuged
at 16,000 x g for 2 min at 2-4°. The resultant plasma was filtered by
passage through a Millipore Swinnex-25 syringe adapter fitted with a
Millipore 0.45-ju filter and then sterilized by passage through a sterile
Millipore Swinnex-25 syringe adapter fitted with a Millipore 0.22-ju
filter. Collection was into sterile test tubes which were subsequently
placed in ice.

Starter cultures of W256 tumor cells growing in asynchronous
logarithmic growth were diluted to a concentration of 8 x 10^4 cells/ml

* The name 4-hydroxycyclophosphamide as used in this paper is meant to
denote both 4-hydroxycyclophosphamide and its hemiaminal, aldophosphame,
since the assay used cannot distinguish between them. However, the presence
of aldophosphamide in biological fluids has never been conclusively demon-
strated, probably because of its very short half-life (16, 28).

* The R, for authentic phosphoramide mustard placed in semicarbazide-treated rat plasma was 0.15 ± 0.00 (n = 60). R, values for authentic 4-hydroperoxycyclophosphamide, 4-hydroxy-
cyclophosphamide, carboxyphosphamide, bis(2-chloroethyl)amine, al-
dophosphamide, 4-ketocyclophosphamide, and cyclophosphamide
placed in rat plasma were all >0.50. This method also dissociates and separates protein-bound metabolites,
shortly after which acrolein was detected in the plasma residue on the
peaks were all >0.50. This method also dissociates and separates protein-bound metabolites,
since no alkylating material was detected in the plasma residue on the
peaks of these areas of these plates. Plate sections containing authentic
phosphoramide mustard were placed into solution immediately before injection.

Assay for Plasma 4-Hydroxycyclophosphamide

A fluorometric assay (1, 33) was used as described previously (16, 26)
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tard, followed by condensation of N'-alkylated nicotinamide with cyclo-
hexanone (19, 20).

Isolation of Phosphoramide Mustard

Prior to, and at various times

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n = 9); calculations were based on the fluorescence value obtained for the
day-to-day standard. Day-to-day variability was not due to degradation of phosphoramide mustard, since the stability of this metabolite during
the isolation procedure was established in preliminary experiments.

Quantification of Cytotoxic Activity in the Plasma of Cyclophospha-
mide-treated Rats

A bioassay using cultured W256 tumor cells was developed to
determine the cytotoxic activity contained in the plasma of cyclophos-
phamide-treated rats. Rats were given cyclophosphamide i.p. at a dose of
50 mg/kg; control animals received i.p. injections of 0.85% NaCl
solution, 2.5 ml/kg. At various times following drug administration,
groups of 5 animals each were sequentially anesthetized with ether,
and blood was collected from the aorta into heparin-coated syringes,
pooled into centrifuge tubes sitting in ice, and immediately centrifuged
at 16,000 x g for 2 min at 2-4°. The resultant plasma was filtered by
passage through a Millipore Swinnex-25 syringe adapter fitted with a
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Starter cultures of W256 tumor cells growing in asynchronous
logarithmic growth were diluted to a concentration of 8 x 10^4 cells/ml

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with Roswell Park Memorial Institute-1640 media supplemented with 10% horse serum; 2.5 ml of the diluted starter culture were transferred into sterile screw-capped centrifuge tubes. Sterile plasma obtained from cyclophosphamide-treated rats was diluted to various concentrations with sterile plasma obtained from 0.85% NaCl solution-treated rats and was added to the cultured tumor cells in a volume of 2 ml. Control conditions received 2 ml of sterile plasma obtained from 0.85% NaCl solution-treated rats. Sterile water or aqueous drug solution (0.5 ml) was then added, and the loosely capped centrifuge tubes were incubated for 1 hr at 37°C under an atmosphere of 5% CO₂ in air. At the end of this incubation period, cells were immediately diluted with 20 ml of fresh culture medium and harvested by low-speed centrifugation. The supernatant was removed via aspiration, and cell pellets were resuspended with 10 ml of fresh culture medium to arrive at a starting cell concentration of approximately 2 × 10⁵ cells/ml. Duplicate 4-ml samples of each condition were transferred into 25-sq cm sterile culture flasks and incubated at 37°C under an atmosphere of 5% CO₂ in air. Determination of bioassay cytotoxicity was by the back-extrapolation assay described previously (18, 23). Using a plasma sample size of 2 ml, the lowest plasma concentrations of 4-hydroperoxycyclophosphamide and phosphoramide mustard that caused measurable cytotoxicity were 0.375 and 6.25 μM, respectively, indicating a lower limit of sensitivity of 0.75 and 12.5 nmol for 4-hydroperoxycyclophosphamide and phosphoramide mustard, respectively. Preliminary experiments established that cell survival decreased in an approximately log-linear manner over a 1-hr period when cultured cells were exposed to 4-hydroperoxycyclophosphamide or phosphoramide mustard (data not presented).

Data Analysis

Regression analysis was as described by Sokal and Rohlf (27).

RESULTS

Plasma concentrations of 4-hydroxycyclophosphamide and phosphoramide mustard in male rats given cyclophosphamide (50 mg/kg i.p.) are presented in Chart 1. Apparent half-lives were 30 and 55 min, respectively. AUC⁶ values were 1.5 and 2.5 mm·min, respectively. An AUC value for 4-hydroxycyclophosphamide of 1.9 mm·min was obtained previously in slightly older and heavier rats of the same strain given cyclophosphamide (50 mg/kg i.p.) (26). The apparent half-life of 4-hydroxycyclophosphamide in those studies was 37 min. Plasma concentrations of 4-hydroxycyclophosphamide and phosphoramide mustard in female mice given cyclophosphamide (65 mg/kg i.p.) are presented in Chart 2. Apparent half-lives were 17 and 29 min, respectively. AUC values were 2.3 and 1.5 mm·min, respectively.

The next series of experiments was designed to determine the relative contribution of each of these circulating metabolites to the overall antitumor action of cyclophosphamide in rats. An in vitro biological assay that utilized cultured W256 tumor cells was used for this purpose. These experiments were based on the knowledge that the magnitude of the response, namely, cell death effected by an alkylating agent, is dependent on the potency of the drug as well as on the concentration of, and contact time with, the drug, i.e., the AUC value.

Initially, the cytotoxic action of plasma obtained from cyclophosphamide-treated rats was quantified. Results of a typical experiment are presented in Chart 3. This figure presents the composite growth curves of cultured W256 tumor cells after exposure to various amounts of plasma obtained 15 min after rats were given cyclophosphamide. Plasma from 0.85% NaCl solution-treated control rats had no effect on the growth of cultured W256 tumor cells. Inclusion of increasing proportions of plasma from cyclophosphamide-treated rats resulted in a concentration-dependent increase in cytotoxic activity; the decrease in surviving fraction was log linear.

All of the growth curves displayed the typical 4 phases of recovery observed for cultured W256 tumor cells following exposure to nitrogen mustards (18). This was taken as presumptive evidence that the species in the plasma effecting the cytotoxic action was an alkylating agent, presumably a metabolite of cyclophosphamide, since compounds that are not alkylating agents do not induce such growth patterns. Plasma cytotoxic activity was also determined at 30, 60, and 120 min following cyclophosphamide administration; cytotoxic activity per ml plasma at each time point was then determined from the resultant concentration/response curves (Chart 4). Cytotoxic activity was greatest 15 min following cyclophosphamide administration, the earliest sample time, and declined to near-negligible levels by 120 min.

Under bioassay conditions, 4-hydroperoxycyclophosphamide is approximately 20 times more potent than is phosphoramide mustard as indicated by the observation that concentrations of 1.5 and 30 μM, respectively, were required to kill 99.9% of the tumor cells (Chart 5). This information, together with the experiment quantifying the cytotoxic activity of plasma, allowed the calculation of the concentration of 4-hydroperoxycyclophosphamide.

⁶ The abbreviations used are: AUC, area under the plasma concentration-time curve; IC₉₉₉₉, concentration of drug required to kill 99.9% of the tumor cells.

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Chart 2. Plasma concentrations of 4-hydroxycyclophosphamide and phosphoramide mustard in cyclophosphamide-treated mice. Mice were given injections of cyclophosphamide (65 mg/kg i.p.). Blood was collected from the tail vein at the times indicated and was assayed for its 4-hydroxycyclophosphamide or phosphoramide mustard content as described in “Materials and Methods.” Points, mean of values obtained in 3 animals; bars, S.E. Plasma concentrations of the 2 metabolites were not determined in the same animals. Samples were obtained at all time points from each mouse when 4-hydroxycyclophosphamide concentrations were determined; because of the limited blood volume, samples were obtained at only 3 of the time points from each mouse when phosphoramide mustard concentrations were determined. Logarithmic plasma decay curves (inset) were obtained by linear regression analysis.

Chart 3. Example of results obtained with the bioassay used to quantify the cytotoxic activity of plasma obtained from cyclophosphamide-treated rats. Rats were given i.p. injections of cyclophosphamide (50 mg/kg). Blood was collected 15 min later from the aorta of 5 animals and was then pooled. Cultured W256 tumor cells were incubated under bioassay conditions as described in “Materials and Methods” for 1 hr with 0 (●), 0.15 (△), 0.30 (▲), 0.45 (○), or 0.60 (□) ml of plasma obtained from rats treated with cyclophosphamide. Tumor cells were then harvested and resuspended in fresh culture media; surviving fractions were determined by the back-extrapolation method described in “Materials and Methods.” Concentration/response curve (inset) was obtained by linear regression analysis.

The following considerations further support the contention that 4-hydroxycyclophosphamide, not phosphoramide mustard, is the therapeutically important circulating metabolite when cyclophosphamide is given to rats bearing W256 tumor cells. Plasma AUC values for 4-hydroxycyclophosphamide increase, as expected, in an approximately linear fashion with increasing doses of cyclophosphamide given i.p. to rats or mice (data not presented). A similar observation has been made when the plasma AUC values for 4-hydroxycyclophosphamide or phosphoramide mustard were determined in rats given cyclophosphamide at doses of 50 mg/kg or 65 mg/kg (Chart 1).

Chart 4. Cytotoxic activity in rat plasma following cyclophosphamide administration. Rats were given i.p. injections of cyclophosphamide (50 mg/kg). Blood was collected from the aorta at various times following cyclophosphamide administration and was then pooled. Cytotoxic activity was determined by the bioassay described in “Materials and Methods” and Chart 3. It is expressed in log cell reduction units. These were obtained by multiplying log surviving fraction units by −1. The concentration of 4-hydroxycyclophosphamide or phosphoramide mustard that would have to be present in the plasma to produce the cytotoxic activity found there is also shown. Points, values obtained when the blood of 5 animals was pooled.

The concentrations of 4-hydroperoxycyclophosphamide equivalents that would have to be present in the plasma to produce the cytotoxic activity found there (Chart 4) are of a magnitude similar to the concentrations of 4-hydroperoxycyclophosphamide equivalents actually found in the plasma following the administration of an identical dose of cyclophosphamide, namely, 50 mg/kg (Chart 1). In contrast, the concentrations of phosphoramide mustard that would have to be present in the plasma to produce the cytotoxic activity found there (Chart 4) are much greater than the concentrations of phosphoramide mustard found in the plasma following the administration of cyclophosphamide (50 mg/kg) (Chart 1).

The following considerations further support the contention that 4-hydroxycyclophosphamide, not phosphoramide mustard, is the therapeutically important circulating metabolite when cyclophosphamide is given to rats bearing W256 tumor cells. Plasma AUC values for 4-hydroxycyclophosphamide increase, as expected, in an approximately linear fashion with increasing doses of cyclophosphamide given i.p. to rats or mice (data not presented). A similar observation has been made when the plasma AUC values for 4-hydroxycyclophosphamide or phosphoramide mustard were determined in rats given cyclophosphamide at doses of 50 mg/kg or 65 mg/kg (Chart 1).
with various concentrations of phosphoramide mustard or 4-hydroperoxycyclophosphamide against cultured W256 tumor cells under bioassay conditions. W256 tumor cells were incubated with phosphoramide for 1 hr at 37° in culture media made 40% in rat plasma; the atmosphere was 5% CO₂ in air. Tumor cells were then harvested and resuspended in fresh culture media. Surviving fractions were determined by the back extrapolation method described in "Materials and Methods." Concentration/response curves were obtained by linear regression analysis.

made in humans (37). The plasma AUC value was 1.5 µM·min when cyclophosphamide (50 mg/kg i.p.), was administered to rats (Chart 1). Cyclophosphamide (2 mg/kg i.p.), given to rats harboring W256 cells in the i.p. ascites fluid, kills 99.9% of the tumor cells (5). This dose of cyclophosphamide can be calculated (2/50 × 1.5) to produce a plasma AUC value for 4-hydroxycyclophosphamide. The IC₉₉.₉ value is 1.5 µM when cultured W256 cells are exposed to 4-hydroperoxycyclophosphamide for 60 min in vitro; the AUC value is thus 0.09 µM·min. In contrast the plasma AUC value for phosphoramide mustard was 2.5 µM·min when cyclophosphamide (50 mg/kg i.p.) was administered to rats (Chart 1). Assuming that plasma AUC values for phosphoramide mustard also increase in an approximately linear fashion with increasing doses of cyclophosphamide given i.p. to rats, the AUC value for phosphoramide mustard can be calculated (2/50 × 2.5) to be 0.10 µM·min when a dose of cyclophosphamide, namely, 2 mg/kg i.p., that kills 99.9% of ascites W256 cells in vivo is given. The IC₉₉.₉ value was 30 µM when cultured W256 cells were exposed to phosphoramide mustard for 60 min in vitro; the AUC value is, thus, 1.8 µM·min.

Finally, 50% inhibition of W256 tumor growth i.m. was achieved when cyclophosphamide (0.69 mg/kg) was given i.p. (21). When phosphoramide mustard was used, 2.5 mg/kg had to be given i.v. to achieve the same amount of inhibition (19). Thus, even if all of the cyclophosphamide ultimately reached the plasma as phosphoramide mustard, there would not be enough to account for its cytotoxic action against W256 cells in vivo.

**DISCUSSION**

The identity of the relevant circulating metabolite with regard to the anti-tumor action of cyclophosphamide has been the subject of considerable debate. We and others interpret the available evidence to favor 4-hydroxycyclophosphamide as being the most important (2–4, 6, 7, 13, 16). Some investigators favor phosphoramide mustard, largely on the basis of several pharmacokinetic considerations (9, 12, 29, 30).

The present investigations demonstrate that the circulating metabolite of major importance in the rat with regard to the cytotoxic action of cyclophosphamide against W256 cells is 4-hydroxycyclophosphamide, even though, following cyclophosphamide administration to this species, the AUC value for phosphoramide mustard somewhat exceeds that for 4-hydroxycyclophosphamide. This is because W256 cells are considerably more sensitive to the latter.

The general relationship between AUC values and cytotoxic potencies of these 2 metabolites as it pertains to determining which of the 2 is the important circulating metabolite can be expressed as

\[
\frac{[AUC:IC_{99.9}]_\text{HCp}}{[AUC:IC_{99.9}]_\text{roA}} = 12
\]

In this relationship, HCP is 4-hydroxycyclophosphamide: aldophosphamide, PDA is phosphoramide mustard, and IC₉₉.₉ is the concentration of drug required to effect a 99.9% cell kill in a fixed amount of time; other end points could, of course, be used, but in all cases for the relationship to remain valid, the amount of cell kill must be determined during a time period in which cell survival decreases in an approximately log-linear manner during the period of drug exposure.

When the value for this relationship greatly exceeds 1.0, the important circulating metabolite is 4-hydroxycyclophosphamide. Conversely, when the value is substantially less than 1.0, phosphoramide mustard would be the important circulating metabolite. In the present investigation, the value is

\[
\frac{[1.5/1.5]_\text{HCp}}{[2.5/30]_\text{roA}} = 12
\]

In both the present and other (12, 34) investigations, the AUC value for 4-hydroxycyclophosphamide exceeded that for phosphoramide mustard in mice given cyclophosphamide. In humans given cyclophosphamide, AUC values for phosphoramide mustard appear to exceed those for 4-hydroxycyclophosphamide by a factor of about 2 to 4 (37).

In vitro, tumor cells are generally more sensitive to 4-hydroxycyclophosphamide than they are to phosphoramide mustard. For example, P388, L1210, Yoshida sarcoma, and Eagle KB cells are approximately 17, 8, 25, and 5 times, respectively, more sensitive to 4-hydroxycyclophosphamide than they are to phosphoramide mustard (2, 29). Moreover, in vitro multiplication of cells obtained from the bone marrow of normal rats is approximately 25 times more sensitive to inhibition by 4-hydroperoxycyclophosphamide than it is to inhibition by phosphoramide mustard. Some tumor cell lines, e.g., human epidermoid carcinoma, are, however, approximately equipotent to the cytotoxic action of the 2 metabolites (29). We know of none that are more sensitive to the cytotoxic action of phosphoramide mustard.

As reflected by its high therapeutic index relative to that of other antitumor agents, cyclophosphamide exhibits selective toxicity towards a number of experimental tumors, e.g., W256 and Yoshida sarcoma. This selectivity is apparently due, almost exclusively, to 4-hydroxycyclophosphamide, since this metabolite also exhibits a comparable therapeutic index, whereas phosphoramide mustard does not (2, 3). There is no tumor...
system known to us in which phosphoramide mustard exhibits a comparably favorable therapeutic index.

On the basis of the foregoing considerations, we contend that 4-hydroxycyclophosphamide is the therapeutically important circulating metabolite when cyclophosphamide therapy is of benefit to cancer patients.

Apparent plasma half-lives for 4-hydroxycyclophosphamide and phosphoramide mustard following i.p. cyclophosphamide administration to male rats were 30 and 55 min, respectively. When cyclophosphamide was given i.p. to slightly older and heavier male rats of the same strain, the plasma half-life for the parent compound was 37 min, and the apparent plasma half-life for 4-hydroxycyclophosphamide was also 37 min (26). Apparent plasma half-lives of about 150 to 350 and 250 to 750 min have been observed for 4-hydroxycyclophosphamide and phosphoramide mustard, respectively, in ‘normal’ humans given cyclophosphamide i.v. (14, 15, 36, 37). The plasma half-life of cyclophosphamide in ‘normal’ humans given the drug i.v. is usually within the range of 140 to 600 min (10, 14, 15, 25, 37).

These observations suggest that the actual plasma half-lives of 4-hydroxycyclophosphamide and phosphoramide mustard are substantially shorter than are the apparent half-lives obtained for these metabolites following cyclophosphamide administration, i.e., that the major determinant with regard to the apparent plasma half-lives of 4-hydroxycyclophosphamide and phosphoramide mustard in rats or humans is the rate of cyclophosphamide hydroxylation rather than their rate of removal. Juma et al. (14, 15) reached the same conclusion.

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