Vol. 93, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1980 Pages 797-803

# PROTECTION BY N-ACETYLCYSTEINE OF CYCLOPHOSPHAMIDE METABOLISM - RELATED IN VIVO DEPRESSION OF MIXED FUNCTION OXYGENASE ACTIVITY AND IN VITRO DENATURATION OF CYTOCHROME P-450[1]

M.J. Berrigan, H.L. Gurtoo[2], S.D. Sharma, R.F. Struck and A.J. Marinello

Department of Experimental Therapeutics
and Grace Cancer Drug Center (MJB, HLG, SDS and AJM)
Roswell Park Memorial Institute
Buffalo, New York 14263
and
Southern Research Institute (RFS)
Birmingham, Alabama 35205

Received February 20,1980

SUMMARY:

Cyclophosphamide (CP) at high or repeated doses results in the depression of mixed function oxygenase activities of the liver. Recent studies have attributed this to the interaction between acrolein, a metabolite of CP, and sulfhydryl groups in cytochrome P-450. The present report demonstrates the protection afforded by N-acetylcysteine against acrolein-induced denaturation of cytochrome P-450 in vitro and CP-related depression of mixed function oxygenase in vivo. Co-administration of CP and innocuous chemicals that provide free sulfhydryl groups should, in the future, be useful in enhancing the therapeutic index of CP by either reducing some of the toxicity and/or by allowing the use of repeated treatment with lower but effective doses of CP.

## INTRODUCTION:

Cyclophosphamide is a widely used anticancer and immunosuppressive agent that requires activation (Figure 1) by the hepatic microsomal mixed function oxygenase system (1). High or repeated doses of cyclophosphamide in rats cause a reduction in the metabolism of various mixed function oxygenase substrates, including cyclophosphamide (2). Marinello et al (3) reported denaturation of cytochrome P-450 during incubation of rat hepatic microsomes with acrolein and 4-hydroxycyclophosphamide and suggested that the ability of 4-hydroxycyclophosphamide to denature cytochrome P-450 may be related to its degradation to acrolein during the incubation.

---

1. This work was supported by the USPHS Grants CA-23634 and CA-13038 from the National Cancer Institute, Bethesda, Maryland.

2. To whom requests for reprints should be addressed.

0006-291X/80/070797-07$01.00/0

Cytochrome P-450 is an essential component of the mixed function oxygenase system and is required for activation of cyclophosphamide and metabolism of a wide range of other therapeutic agents and xenobiotics (4). The integrity of cytochrome P-450 may, therefore, prove to be a crucial factor in determining the efficacy of certain chemotherapeutic agents in terms of their degree of activation or deactivation, as well as the production of toxic metabolites. As cyclophosphamide inhibits hepatic microsomal mixed-function oxygenase activity *in vivo* (2,3) and its metabolites denature cytochrome P-450 *in vitro* (3), it was of considerable interest to investigate the possible protection of the depression of mixed function oxygenase by co-administration of agents that might trap the metabolites responsible for cytochrome P-450 denaturation. Because cyclophosphamide metabolites may denature cytochrome P-450 by reacting with sulfhydryl groups in the active site of the cytochrome (3), N-acetylcysteine, which contains a free sulfhydryl group, was used *in vivo* to block cyclophosphamide-associated depression of mixed function oxygenase, and *in vitro*, to block acrolein-mediated denaturation of cytochrome P-450. The results of these investigations are presented in this report.

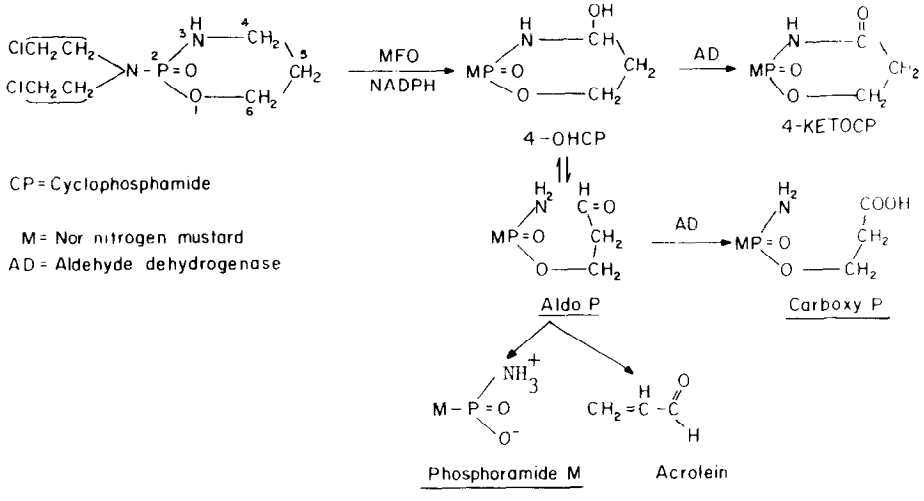


Figure 1: Metabolism of Cyclophosphamide by Hepatic Microsomal and Cytosolic Enzymes.

MFO, mixed function oxygenase; AD, Aldehyde Dehydrogenase.

## MATERIALS AND METHODS

### Chemicals:

NADP, DL-isocitrate, isocitrate dehydrogenase, dithionite, and N-acetyl-L-cysteine were purchased from Sigma Chemical Company, St. Louis, Missouri. Benzo(a)pyrene and cyclophosphamide were obtained, respectively, from Aldrich Chemical Company, Milwaukee, Wisconsin and the Drug Development Branch of the National Cancer Institute, Bethesda, Maryland.

### Animal Treatment:

Male Wistar rats (200-250 gm), obtained from Charles River Laboratories, were used. All animals were maintained under identical conditions and received food and water ad libitum. At least three animals per group were used in each experiment. All treated animals were injected intraperitoneally (5 ml/kg).

Four groups of rats were treated as follows: one group received no treatment; the second group received N-acetylcysteine in buffer (0.1 M potassium phosphate, pH adjusted to 5.0, 360 mg/kg) and isotonic saline; the third group received buffer and cyclophosphamide in saline (180 mg/kg); and the fourth group received cyclophosphamide (180 mg/kg) and N-acetylcysteine (360 mg/kg). N-acetylcysteine was given in two equally divided doses, 30 minutes before and after cyclophosphamide or saline. Corresponding controls received either the same volume of saline or buffer divided into two equal doses.

Since initial studies demonstrated that optimal depression of mixed function oxygenase activities of hepatic microsomes occurs on day 4 following the administration of this dose of cyclophosphamide (180 mg/kg), four days following treatment the rats were sacrificed by decapitation, and the livers, removed immediately, were perfused with cold isotonic saline. Minced livers were homogenized in cold 0.25M sucrose and the homogenate centrifuged for 15 minutes at 15,000 x g. The resultant supernatant was centrifuged at 105,000 x g for 90 minutes to obtain the microsomal pellet. All operations were performed in the cold (4°). The microsomes prepared in this manner were resuspended in 0.25M sucrose and their protein concentrations were determined by the method of Lowry et al (5), using bovine serum albumin as the reference standard.

### In Vitro Interaction of Acrolein and Cytochrome P-450:

A typical incubation mixture, in a volume of 1.2 ml, contained the following components: 7 μ moles acrolein, 0.2 mmole potassium phosphate buffer (pH 7.4), microsomes (6 mg protein) and in some cases, varying concentrations of N-acetylcysteine. Control incubations contained all of the above except acrolein. All reactions were carried out aerobically for 30 minutes at 37° in a Dubnoff metabolic shaker. At the end of the incubation period, the volume was adjusted to 10 ml with 0.1M potassium phosphate buffer (pH 7.4) and the spectrum of cytochrome P-450 was recorded as described in the Legend.

### Measurement of AHH Activity:

Aryl hydrocarbon hydroxylase (AHH) activity was measured as previously reported (6,7). Benzo(a)pyrene was used as the substrate and the formation of phenolic metabolites was quantitated by measurement of their fluorescence

using 396 nm wavelength for excitation and 522 nm wavelength for emission. Fluorescence was compared with a standard curve obtained with 3-hydroxy benzo(a)pyrene. AHH activity is expressed as pmole equivalent of 3-OH-benzo(a)pyrene produced per mg protein during a 10-minute incubation at 37°. An Aminco-Bowman spectrophotofluorometer employed in these studies was calibrated with a quinine sulfate standard before and during each determination.

Cytochrome P-450 Spectra:

Cytochrome P-450 was determined by difference spectroscopy on an Aminco DW-2 Spectrophotometer, following the method of Omura and Sato (8).

## RESULTS AND DISCUSSION

As shown by the difference spectra in Figure 2, incubation of hepatic microsomes with acrolein results in a substantial (40-50%) loss of cytochrome P-450. This is evidenced by the reduction in

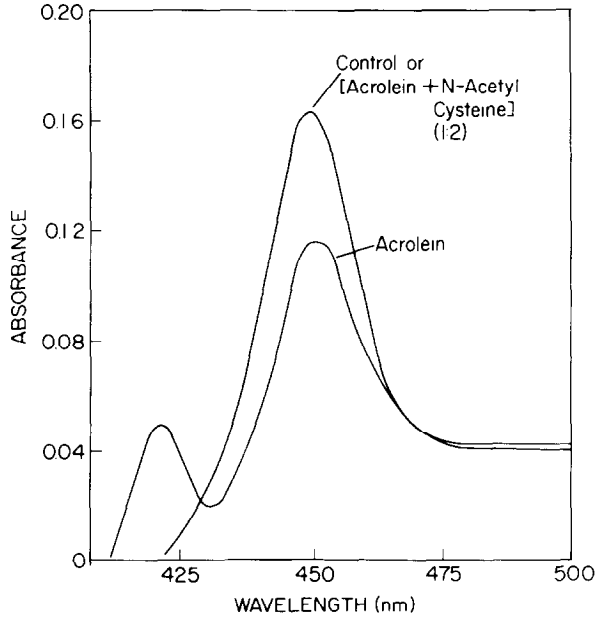


Figure 2: Protection by N-Acetylcysteine of the In Vitro Denaturation of Cytochrome P-450 by Acrolein.

Rat hepatic microsomes were incubated with acrolein in the absence and presence of varying concentrations of N-acetyl-cysteine. 3.0 ml of the treated microsomal suspension was transferred to sample and reference cuvettes. Base line of equal light absorbance was recorded and a few mg. of dithionite were then added to each cuvette. Carbon monoxide was bubbled for 30 seconds through the contents of the sample cuvette only and the spectrum was recorded.

absorbance in the 450 nm region. The inclusion of N-acetylcysteine with acrolein in the incubation at a molar ratio of 2:1 (or 3:1) protects against the acrolein-induced loss of the cytochrome.

Relative to untreated controls, administration of N-acetylcysteine (in the absence of cyclophosphamide) to the rats did not result in a depression of either AHH activity or the levels of cytochrome P-450 in the hepatic microsomes (Figures 3A and 3B). In contrast to this, administration of cyclophoshamide (in the absence of N-acetylcysteine) resulted in a 46% depression of AHH activity and a 34% depression of cytochrome P-450 levels. However, when cyclophosphamide was combined with N-acetylcysteine (N-acetylcysteine being given 30 minutes before and after cyclophosphamide), the levels of both AHH activity and cytochrome P-450 were similar to those obtained for untreated controls, suggesting therefore, that N-acetylcysteine protects against cyclophosphamide-mediated depression of mixed function oxygenase activity.

It has been reported that 4-hydroxycyclophosphamide reacts with sulfhydryl groups (9,10) and that acrolein and 4-hydroxycyclophosphamide cause cytochrome P-450 denaturation by reacting, most likely, with sulfhydryl groups in the active site of the cytochrome (3). Based on this suggestion, it was thought that the addition of a sulfhydryl containing compound, such as N-acetylcysteine, might protect against the denaturation of cytochrome P-450 by supplying a pool of free sulfhdryl groups that effectively compete for reaction with acrolein and 4-hydroxycyclophosphamide or its decomposition products.

The possibility that it may be possible to protect against cyclophosphamide-related depression of mixed function oxygenase enzymes by the use of a second agent, innocuous itself, may have clinical ramifications in that low, but effective, doses of cyclophosphamide could be used repeatedly or the dose could be increased.

It has been reported that cyclophosphamide administration causes hemorrhagic cystitis in rats (11). Cox (12) and Brock and co-workers

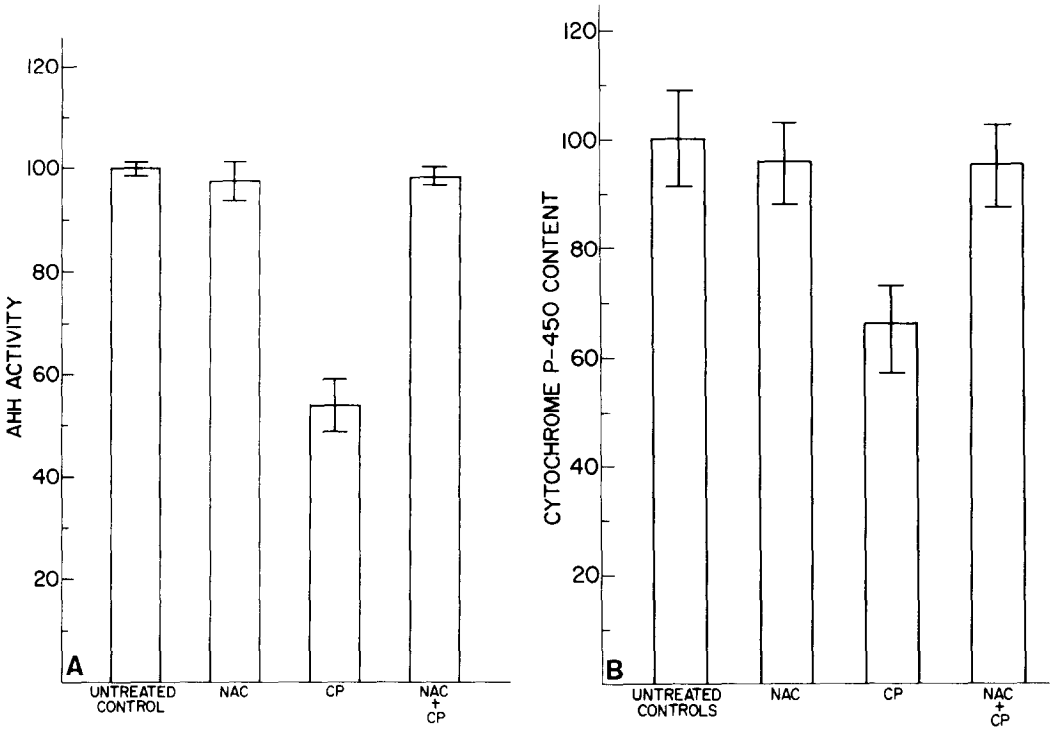


Figure 3A: Protection by N-Acetylcysteine (NAC) of Cyclophosphamide (CP)-Related Depression of Aryl Hydrocarbon Hydroxylase (AHH) Activity.

In addition to untreated controls, adult male Wistar rats were treated with CP and/or NAC. Rats were sacrificed on day 4 to isolate hepatic microsomes. AHH activity of the microsomal preparations was determined. Details are described in the text. Results are expressed as percent of untreated controls ± standard error of the mean (N = 3). AHH activity of the untreated control groups of rats was 4,446 ± 64 pmole equivalent of 3-OH-benzo(a)pyrene formed/mg protein/10 minutes. Results similar to those reported here were obtained in repeat experiments.

Figure 3B: Protection by N-Acetylcysteine (NAC) of the Cyclophosphamide (CP)-Related Depression of Cytochrome P-450 Content.

Microsomal preparations, as described in the legend of Figure 3A, were used to determine the cytochrome. The cytochrome P-450 content of the microsomes from untreated rats was 1.13 ± 0.10 nmoles/mg protein. The results are expressed as percent of untreated controls ± SEM. In a repeat experiment, results similar to those reported here were obtained.

(13) have attributed this pathological condition to the metabolite acrolein, and Cox (12) has reported that N-acetylcysteine effectively blocks this toxicity. This is in agreement with our suggestion that the noxious effects of some cyclophosphamide metabolites may be avoided by trapping

the toxic metabolites with N-acetylcysteine, as demonstrated here for cytochrome P-450. Tolley (14) has successfully used N-acetylcysteine to reduce the bladder toxicity of cyclophosphamide and has also presented some evidence suggesting that a treatment protocol of N-acetylcysteine and cyclophosphamide, given in appropriate doses and schedules, does not impair the chemotherapeutic activity of cyclophosphamide against Walker 256 carcinosarcoma in rats.

REFERECES:

1. Brock, N. and Hohorst, H.-J. Arzneim.-Forsch., 13: 1021, 1963.
2. Gurtoo, H.L., Gessner, T.,and Culliton, P. Cancer Treat. Rep., 60: 1285, 1976.
3. Marinello, A.J., Gurtoo, H.L., Struck, R.F.,and Paul, B. Biochem. and Biophys. Res. Comm., 83(4): 1347, 1978.
4. Cooper, D.Y., Levin, S., Narasimhula, S., Rosenthal, O., and Estabrook, R.W. Science, 147: 400, 1965.
5. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. J. Biol. Chem., 193: 265, 1951.
6. Wattenberg, L.W. and Leong, J.L. J. Histochem. Cytochem., 10: 412, 1962.
7. Nebert, D.W. and Gelboin, H.V. J. Biol. Chem., 243: 6242, 1968.
8. Omura, T. and Sato, R. J. Biol. Chem., 239: 2370, 1964.
9. Wagner, T., Peter, G., Voelcker, G., and Hohorst, H.-J. Cancer Res., 37: 2592, 1977.
10. Draeger, U., Peter, G. and Hohorst, H.-J. Cancer Treatment Rep., 60: 355, 1976.
11. Levy, L. and Harris, R. Biochem. Pharmac., 26: 1015, 1977.
12. Cox, P. Biochem. Pharmac., 28: 2045, 1979.
13. Brock, N., Stekar, J., Pohl, J., Niemajor, U., and Scheffler, G. Arzneim.-Forsch., 29: 659, 1979.
14. Tolley, D.A. Brit. J. Urology, 49: 659, 1977.