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Short Communication

EFFECT OF STEREOCHEMISTRY ON THE OXIDATIVE METABOLISM OF THE CYCLOPHOSPHAMIDE METABOLITE ALDOPHOSPHAMIDE

ASIF D. HABIB,*† JILA H. BOAL,* JOHN HILTON,‡ THAO NGUYEN,‡ YOUNG H. CHANG§ and SUSAN M. LUDEMAN*‡‖

*Department of Chemistry, The Catholic University of America, Washington, DC, U.S.A.; †Department of Chemistry, University of Wisconsin Center-Waukesha County, Waukesha, WI, U.S.A.; ‡Division of Pharmacology and Experimental Therapeutics, The Johns Hopkins Oncology Center, Baltimore, MD, U.S.A.; and §Department of Chemistry, Korea Advanced Institute of Science and Technology, Taejon, Korea

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Abstract—^{31}P NMR and cell perfusion techniques were used to investigate the conversion of the individual enantiomers of aldophosphamide (AP) to carboxyphosphamide (CBP) as catalyzed by aldehyde dehydrogenase in human erythroleukemia K562 cells. *R*- and *S*-cyclophosphamides (CPs) were treated with ozone and hydrogen peroxide to yield R_p- and S_p-*cis*-4-hydroperoxycyclophosphamides (R_p- and S_p-*cis*-4-HO_2-CP); reduction of each hydroperoxide gave the corresponding enantiomer of AP [along with its tautomer 4-hydroxycyclophosphamide (4-HO-CP)]. In separate experiments, K562 cells embedded in agarose gel threads were perfused at pH 7.4, 21 ± 1°, with solutions of 1.4 mM R_p- and S_p-4-HO-CP/AP, both with and without added mesna (an acrolein scavenger). A comparison of the ^{31}P NMR spectral data derived from the experiments revealed little statistical difference (± 10–20% error limits) in the normalized intensities of the CBP peaks arising from the individual AP enantiomers [with added mesna, the ratio R_p-CBP:S_p-CBP was 1.00:1.24 ± 0.13 (average deviation); without mesna, the same ratio was 1.00:1.35]. Using conventional methods for evaluating the *in vitro* drug toxicities, CP-resistant L1210 cells were treated in separate experiments with R_p- and S_p-*cis*-4-HO_2-CP; there were no significant differences between the toxicities exhibited by the stereoisomers.

Key words: cyclophosphamide metabolism; ALDH and L1210 enantioselectivity; aldophosphamide; carboxyphosphamide; NMR and cell perfusion

CP¶ is one of the most widely used anticancer drugs with therapeutic efficacy against a broad spectrum of human cancers. As shown in Scheme 1, oxidation of this drug by cytochrome P450 gives 4-HO-CP, which then undergoes a spontaneous and reversible ring-opening reaction to produce AP. This intermediate is believed to play a pivotal role in oncostatic selectivity by partitioning between pathways that produce a toxic product (spontaneous fragmentation to PM and acrolein) and a nontoxic product (oxidation via ALDH to CBP) [1–3]. Enzymatic oxidation of 4-HO-CP to 4-keto-CP represents a second, but relatively minor, route of detoxification.

CP has an asymmetric center at phosphorus; from a chemical viewpoint, it is reasonable to assume that the relative as well as the absolute configuration at this atom is retained throughout metabolism until the production of PM [see Scheme 1 where, for the sake of simplicity, only the *R* configuration at phosphorus (R_p) is shown]. Studies of the consequences of chirality at phosphorus on CP metabolism have provided mixed results; in animals, however, the data indicate that the *S* (levorotatory) enantiomer generally exhibits a higher rate of metabolism and a greater antitumor effect [4–11]. To better understand the role of stereochemistry in biological systems, investigations of the influence of stereochemical factors on specific metabolic steps are desirable. Of particular interest as it relates to CP oncostatic selectivity and therapeutic index is the degree of enantioselectivity shown by ALDH for AP. While ALDH is an enzyme with apparently loose structural restrictions for substrate activity, substrates tested have varied more in size than stereochemistry. Furthermore, cytochrome P450 is known for its acceptance of many varied substrates, yet it differentiates, at least to some extent, between the enantiomers of CP (*vide supra*).

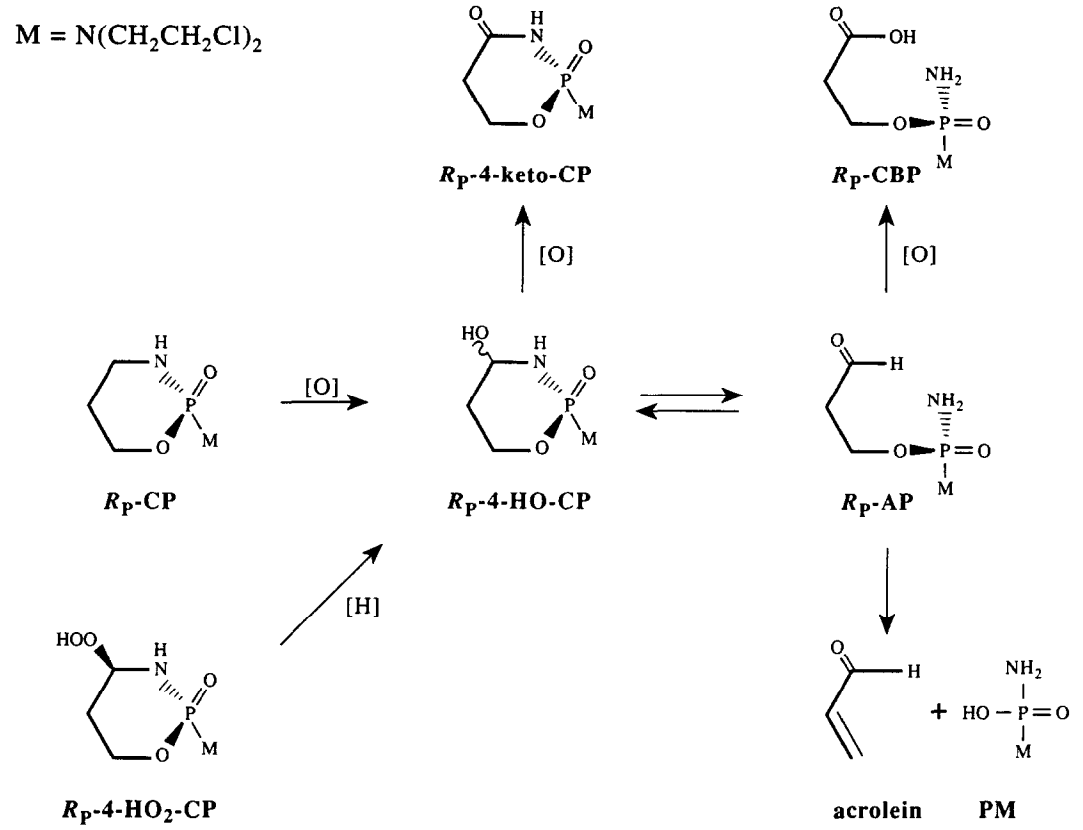
We have previously used ^{31}P NMR spectroscopy in conjunction with cell perfusion techniques to observe the intracellular conversion of racemic AP to CBP in CP-resistant K562 and CP-sensitive U937 cells [12]. This is a report on the extension of those techniques to investigations of the conversion of the individual enantiomers of AP to CBP in human erythroleukemia K562 cells. Also discussed is a study of ALDH enantioselectivity in CP-resistant L1210 mouse leukemia cells using conventional *in vitro* drug toxicity methods.

Materials and Methods

Synthesis. Ozonolyses of enantiomerically pure *R*- and *S*-CP, as previously described for *S*-CP [13], gave,

‖ Corresponding author: Susan M. Ludeman, Ph.D., The Johns Hopkins Oncology Center, ONC 1-121, 600 North Wolfe St., Baltimore, MD 21287. Tel. (410) 955-8902; FAX (410) 550-5499.

¶ Abbreviations: CP, cyclophosphamide; 4-HO-CP, 4-hydroxycyclophosphamide; AP, aldophosphamide; PM, phosphoramide mustard; ALDH, aldehyde dehydrogenase; CBP, carboxyphosphamide; 4-keto-CP, 4-ketocyclophosphamide; and 4-HO_2-CP, 4-hydroperoxycyclophosphamide.



Scheme 1.

respectively, R_p- and S_p-*cis*-4-hydroperoxycyclophosphamide [R_p- and S_p-*cis*-4-HO_2-CP; 2–3% yield; ^{31}P NMR ($CDCl_3$; 25% H_3PO_4/H_2O reference) δ 9.99].

Enantiomeric purity. Enantiomeric purity was measured using ^{31}P NMR after conversion of the enantiomeric hydroperoxides into a diastereomeric pair of 4-sulfhydryl cyclophosphamides. The resolving group used to accomplish this was the optically active thiol glutathione (*R**SH, γ-L-glutamyl-L-cysteinylglycine). While the lifetimes of such sulfhydryl adducts are variable, reaction conditions were chosen that enhanced their concentrations and stabilities [13]. As a result, individual, unstable species in complex mixtures of metabolites and stereoisomers were readily detected and identified. The same unambiguous determination of stereochemical purity could not be readily achieved by measurements of the optical rotations of the hydroperoxides because of their instability in solution.

^{31}P NMR acquisitions were done at 202.5 MHz and used a 10.2 kHz spectral window, 16,000 data points, a 45° pulse of 10 μsec, gated low power ^1H decoupling, and a pulse recycle time of 6.13 sec. Based on our previous work [14, 15], phosphorus peak heights were judged to be reliable measures of component concentration because (1) possible differential nuclear Overhauser effects were suppressed with gated decoupling, and (2) the pulse delay time was sufficient so as to compensate for differences in relaxation times among the components of interest.

In a preliminary experiment, *cis*-4-HO_2-CP (17 mg, 57 μmol), which had been made from racemic CP, was dissolved in 1 M lutidine (1.8 mL, pH 7.4) and D_2O (0.2 mL, NMR lock signal). The hydroperoxide was reduced by the addition of sodium thiosulfate pentahydrate (57 mg, 228 μmol, 4 equivalents). Following dissolution of the thiosulfate, glutathione (*R**SH, 176 mg, 570 μmol, 10 equivalents) was added, and the pH was readjusted to 7.4. ^{31}P Spectra taken of this sample at 37° revealed the growth of various adducts between glutathione and the CP metabolites, including two resonances of nearly equal intensity at δ 10.22 and 10.12, which were attributed to the *cis* isomers of 4-glutathionylcyclophosphamide (4-S*R**-CP; *R**,2*R*,4*R* and *R**,2*S*,4*S*). These assignments were based on reported shifts for 4-S*R**-CP where S*R** = *N*-acetyl-L-cysteinyl [13].

A sample of R_p-*cis*-4-HO_2-CP [i.e. (2*R*,4*R*), 4 mg, 14 μmol] was reduced and treated with 10 equivalents of glutathione as described above. In a spectrum taken after 40 min of reaction time, a relatively intense signal at δ 10.27 was observed; no other signals within ± 1 ppm were visible above the baseline. Using peak heights and considering that of the noise to be the limits of signal detection, the enantiomeric purity at phosphorus was found to be ≥ 89% (i.e. ≥ 94% R_p) based on the following equation: $|(R_p - S_p)| \div R_p + S_p$; where R_p = signal height at δ 10.27 and S_p = signal height of noise.

A sample of S_p-*cis*-4-HO_2-CP [i.e. (2*S*,4*S*), 4 mg, 14 μmol] was treated as described above for the R_p isomer. A relatively intense ^{31}P signal was observed at δ 10.15 and represented the diastereomer with the *S* configuration at phosphorus. An apparent signal also appeared as a shoulder on the downfield side of the resonance at δ 10.15; this shoulder was treated as the maximum amount of R_p-isomer present. The enantiomeric purity at phosphorus was ≥ 82% (i.e. ≥ 91% S_p) based on the equation given above with: R_p = signal height of downfield shoulder, and S_p = signal height at δ 10.15.

Cell perfusion studies. The cell perfusion/^{31}P NMR

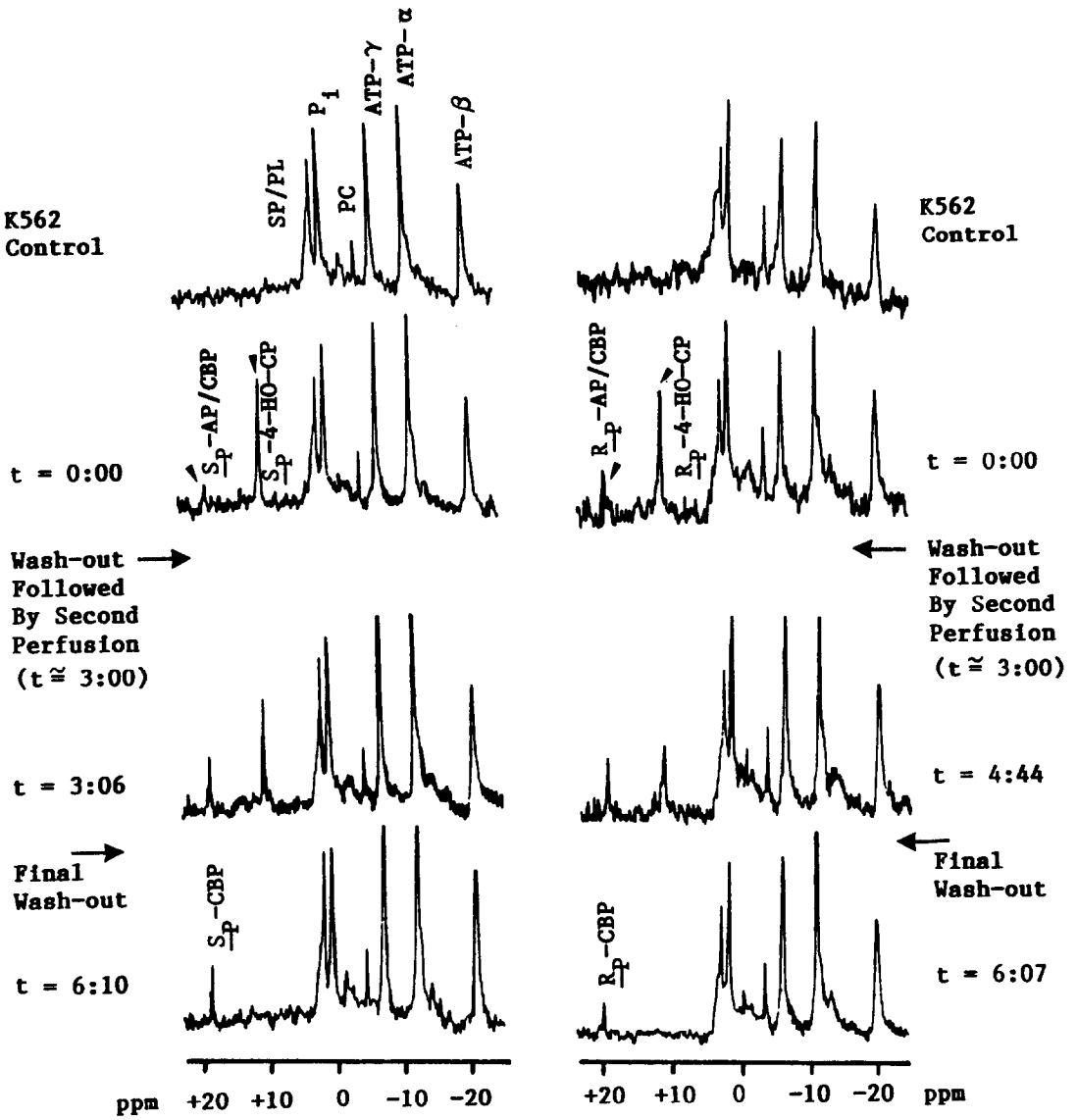


Fig. 1. ^{31}P NMR (161 MHz) spectra of K562 cells perfused with R_p-4-HO-CP/AP (right panel) and S_p-4-HO-CP/AP (left panel) with added mesna at pH 7.4, 21°, and t = hr:min. During drug perfusion, both AP and CBP contributed to the signal at δ 20.2; washing the cells with drug-free perfusate ("wash-out") removed AP but not CBP. Top: K562 cells in agarose gel threads prior to drug perfusion. Middle: spectra obtained *during* the first and second perfusions with 1.4 mM drug. Bottom: spectra acquired *after* final wash with drug-free medium. Abbreviations: SP/PL, sugar phosphates/phospholipids; P_i, inorganic phosphate; and PC, phosphocreatine. ATP-α, -β and -γ refer to the three phosphorus atoms in ATP.

experiment was performed as described previously [12]. In short, packed K562 cells (0.5 to 1.0 mL) were mixed with an agarose gel solution (37°) and then extruded through cooled tubing to create threads that were collected in a 10-mm NMR perfusion tube containing nutrient mixture (Ham's F-12 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin). During the NMR experiment, the cells were perfused with medium in a recirculating manner; solutions of the metabolites being studied were added directly to this medium. In experiments that used mesna ($HSCH_2CH_2SO_3Na$), this compound was also added to the perfusate (20% molar equivalents relative to drug).

In separate experiments, hydroperoxides R_p- and S_p-*cis*-4-HO_2-CP (0.14 mmol each) were reduced (triphenylphosphine/CH_2Cl_2) and then extracted quantitatively into sterile water (2 mL) to give solutions of the corresponding stereoisomers of 4-HO-CP and AP as interconverting tautomers [12, 13]. For perfusion, half of the metabolite solution was diluted with 50 mL medium (to give 1.4 mM 4-HO-CP/AP); the remaining half was stored in dry ice until needed for a second perfusion.

^{31}P NMR chemical shifts were referenced to extracellular (the medium) inorganic phosphate (0.0 ppm). The NMR parameters have been described [12].

Results and Discussion

Solutions of the individual enantiomers of AP (and its tautomeric form 4-HO-CP) were made by the reductions of R_p- and S_p-*cis*-4-HO$_2$-CP as synthesized from *R*- and *S*-CP. In separate experiments, K562 cells embedded in agarose gel threads were perfused at pH 7.4, 21 ± 1°, with fresh solutions of 1.4 mM R_p- and S_p-4-HO-CP/AP. Mesna (20% molar equivalents relative to drug) was added to each perfusate as an acrolein scavenger [16, 17]; this mediated the toxic effects of high drug concentrations and thereby increased cell viability [12]. For each experiment, two sequential 2.5-hr perfusions were used, and each perfusion was followed by a cell wash of 10–15 min using drug-free medium. The cell washes removed all membrane-permeable metabolites such as acrolein and unmetabolized 4-HO-CP/AP. Because the ^{31}P signal at δ 20.2 was derived from a combination of AP and CBP, the quantification by NMR of CBP required the removal of AP. Following wash-out, then, the remaining signal intensity at δ 20.2 was attributed to the membrane impermeable CBP [12].

Throughout each cell perfusion experiment, ^{31}P NMR spectra were acquired at time intervals of approximately 30 min (Fig. 1); the spectrum obtained after the final cell wash was used to determine the relative level of intracellular CBP (δ 20.2). The calculation was made by determining the ratio of the signal areas (peak height × width at half-height) for CBP and the β-phosphate of ATP (δ −19.2). The latter signal was relatively constant in viable cells and thus was suitable as an internal concentration reference with estimated error limits of ± 10–20% [12]. A comparison of the spectral data derived from the two experiments at similar time points revealed little difference in the normalized intensities of the CBP peaks arising from the individual AP enantiomers [R_p-CBP:S_p-CBP = 1.00:1.24 ± 0.13 (average deviation, 2 experiments with R_p- and 3 with S_p-4-HO-CP/AP)]. Moreover, there were no significant differences, in general, among the spectra obtained from the metabolism (in K562 cells) of 4-HO-CP made from racemic CP versus R_p- and S_p-4-HO-CP/AP [12].

The above experiments were also run in the absence of mesna. Similar results were obtained: normalized signal intensities gave the ratio R_p-CBP:S_p-CBP = 1.00:1.35 (1 experiment each with R_p- and S_p-4-HO-CP/AP).

A brief investigation of possible ALDH enantioselectivity in L1210 mouse leukemia cells was also conducted; intracellular ALDH activity in the L1210 cell lines has been shown to be an important determinant of CP sensitivity [18]. Using conventional methods for evaluating the *in vitro* drug toxicities of pre-activated CP analogs [18, 19], CP-sensitive and -resistant L1210 cells were treated in separate experiments with R_p- and S_p-*cis*-4-HO$_2$-CP (0–100 μM, 60 min exposure time, 37°). R_p- and S_p-*cis*-4-HO$_2$-CP were equitoxic to both the sensitive and resistant cells in culture. In the sensitive cells, the concentrations of the hydroperoxides required to cause a 50% loss in viability were 25 μM (R_p) and 23 μM (S_p). In the resistant cells, which overexpress ALDH, 60-min exposures to 100 μM R_p-*cis*-4-HO$_2$-CP and 100 μM S_p-*cis*-4-HO$_2$-CP produced a 21 and 19% loss in viability, respectively, compared with untreated controls.

These experiments were the first to directly test the effect of stereochemistry on the oxidative metabolism of AP. While the NMR technique was somewhat unconventional, it nevertheless allowed for the direct detection of an intracellular metabolic step in a non-invasive manner. These highly desirable outcomes were achieved, however, at some expense [relatively high drug concentrations (1.4 mM) and quantification errors (± 10–20%)]. Despite the limitations of the NMR method, valuable qualitative information was obtained. The results indicated that both enantiomers of AP were substrates for ALDH in K562 cells, and there was little, if any, stereoselectivity demonstrated. While both sets of NMR experiments (with/without mesna) indicated a slight preference of ALDH for S_p-AP (to give S_p-CBP), these results were probably on the edge of statistical significance when considering the experimental constraints of this NMR method. Similarly, no enantioselectivity was indicated by the L1210 experiments. The detoxification metabolite 4-keto-CP (δ *ca.* 9 [19]) was not detected in any NMR experiment.

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