^{31}P Nuclear Magnetic Resonance Spectroscopic Observation of the Intracellular Transformations of Oncostatic Cyclophosphamide Metabolites

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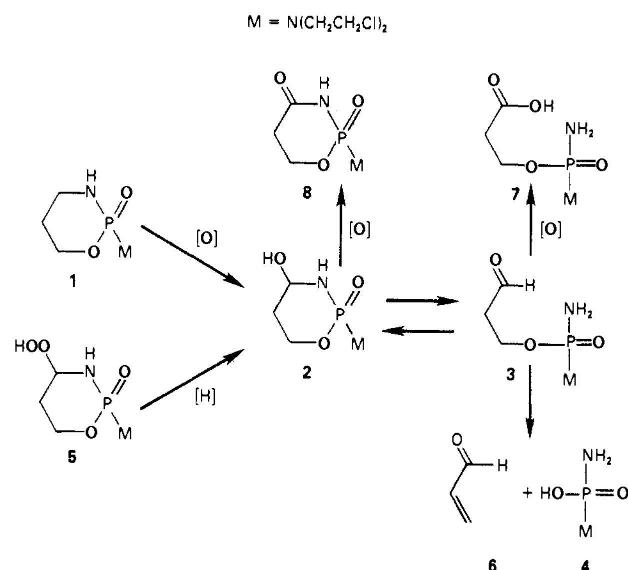
^{31}P NMR spectroscopy was used to directly monitor, for the first time, the intracellular chemistry of the ultimate active metabolite of cyclophosphamide, namely, phosphoramide mustard. These NMR studies utilized a human histiocytic lymphoma cell line (U937), embedded in agarose gel threads, and perfused with medium containing synthetically derived metabolites (4-hydroxycyclophosphamide (**2**), aldophosphamide (**3**), and phosphoramide mustard (**4**)). Metabolites **2** or **3** or both readily crossed the cell membrane; in contrast, the membrane was relatively impermeable to **4**. Intracellular concentrations of **4** could, therefore, be attributed primarily to the intracellular fragmentation of **3**. Signals suggestive of either carboxyphosphamide or 4-ketophosphamide were not detected. Spectral data were used to calculate a rate constant of $(5.4 \pm 0.3) \times 10^{-3}$ min^{-1} for the intracellular disappearance of **4** at 23 °C. The intracellular pH was determined to be 7.1 from the chemical shift of the internal inorganic phosphate signal.

The generally recognized, primary metabolic transformations of the anticancer drug cyclophosphamide (1) are shown in Scheme I.[1-3] The interactions of the metabolites 4-hydroxycyclophosphamide (**2**), aldophosphamide (**3**), and phosphoramide mustard (**4**) with cellular components are believed to determine cell toxicity; specific causal relationships between this chemistry and the oncostatic selectivity of 1 have been investigated[4-14] but have not been established. To more fully understand selectivity mechanisms, direct measurements of metabolite reactivities in biological systems are needed.

The utility of nuclear magnetic resonance (NMR) spectroscopy as a noninvasive tool for the study of intracellular physiology and chemistry has been significantly enhanced though the use of cell perfusion techniques.[15] For studies necessitating rapid changes in cellular external environment, the perfusion system described by Cohen and colleagues[16-18] is particularly useful. Using this perfusion system, we have been able to *directly* observe the intracellular transformation of the pharmacologically active cyclophosphamide metabolite, phosphoramide mustard. We now report our results.

Scheme I



Results

Packed U937 human histiocytic lymphoma cells[19] (0.5 mL; ca. 1.5×10^8 cells) were incorporated into agarose gel threads, as described.[15-18] The cell-containing threads were perfused, in a recirculating fashion,[16-18] with Ham's F-12 medium (pH 7.4) supplemented with 10% of fetal calf serum. The ^{31}P NMR spectra of the cells embedded in the gel threads were virtually identical with those obtained initially for dense cell suspensions (cell suspension data not shown; spectra of cells in gel threads may be seen in Figures 1 and 2). The ^{31}P NMR spectra of the U937 cells, after being in a dense suspension for several hours, displayed a number of changes, presumably the result of cell anoxia and starvation; these changes were typical of dying cells, i.e., loss of the adenosine triphosphate (ATP) and sugar phosphate signals, and the buildup of the inorganic phosphate signal.[15-18] In contrast, ^{31}P NMR spectra of the cells in the gel threads were unchanged over long periods of time; indeed, we were able to maintain the cells unchanged (at least as regards their ^{31}P NMR spectra) for several days (data not shown). We were thus assured that cell changes, other than those due to drug effects, were not taking place during the course of our experiments (ca. 12 h).

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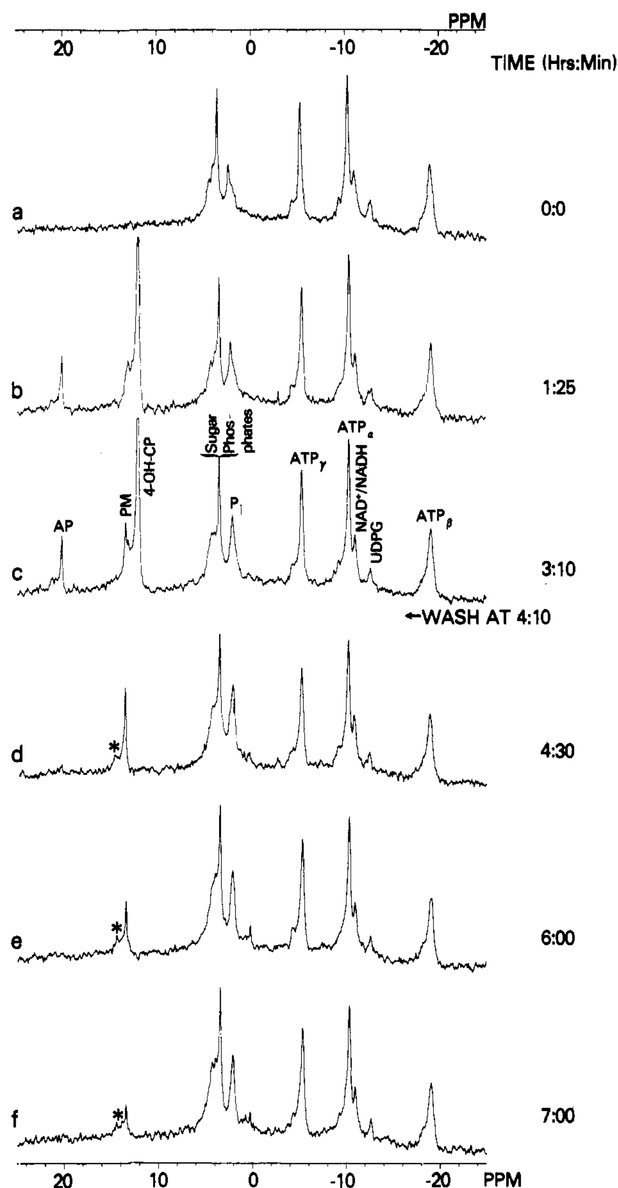

Figure 1. ^{31}P NMR spectra (161 MHz) of U937 cells as a function of time (cf. text) and in the presence of added 4-hydroxycyclophosphamide and after washing cells with fresh perfusate. Abbreviations: AP = aldophosphamide, PM = phosphoramide mustard, 4-OH-CP = 4-hydroxycyclophosphamide, P_i = inorganic phosphate, ATP = adenosine triphosphate, $NAD^+/NADH$ = oxidized and reduced nicotine adenine dinucleotide, UDPG = uridine diphosphoglucose(galactose). An asterisk (*) denotes an unknown impurity (which was not observed in other runs).

An intracellular pH of 7.1 (±0.1) was determined from the position of the intracellular inorganic phosphate resonance.[20] Signals for intra- and extracellular phosphate were distinguished on the basis of the titration behavior of the external phosphate peak (altering the pH of the perfusing medium; data not shown) as well as the disappearance of the extracellular peak following the addition of $MnCl_2$ to the perfusate (vide infra). Manganous ions, being paramagnetic, broaden the extracellular phosphate peak beyond detection; the residual resonance in the phosphate region is attributable to internal inorganic

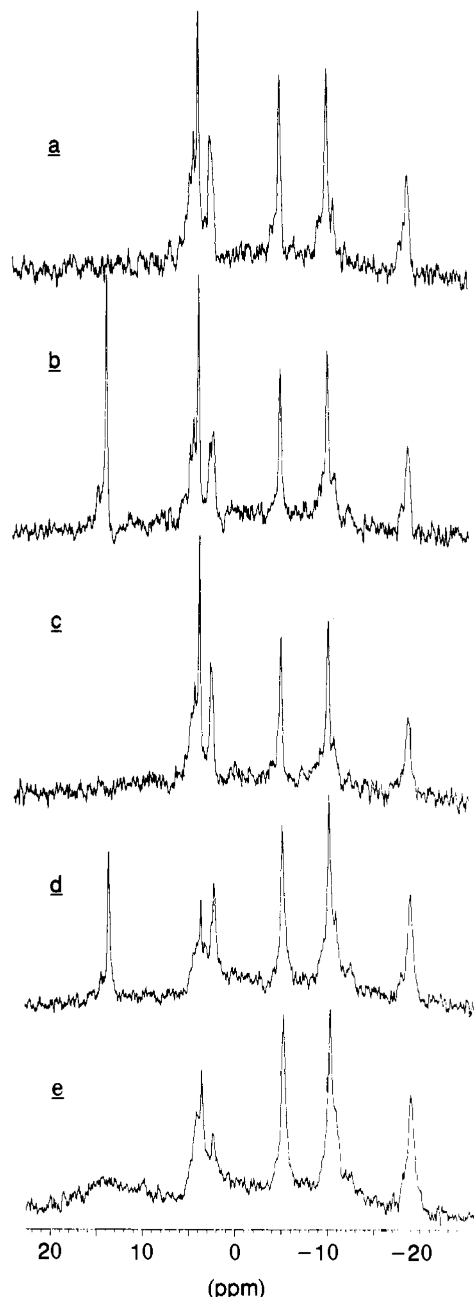

Figure 2. ^{31}P NMR spectra (161 MHz) of U937 cells: (a) control spectrum, (b) phosphoramide mustard (3 mM) added to perfusion media and perfused for 3 h, (c) following perfusion with phosphoramide mustard and washing (15 min) with fresh medium, (d) U937 cells with phosphoramide mustard added to the perfusion media, (e) as in (d) but with added (1 mM) manganese chloride. Both experiments utilized U937 cells derived from the same source; concentrations of sugar phosphates were noted to vary.

phosphate. Since the intracellular resonances were not broadened, it appears that the manganous ions do not readily enter the cell. The NMR-determined intracellular pH value is an average for a distribution of cells over the time taken for the measurement.

Access to **2**–**4** is conveniently provided through the deoxygenation of 4-hydroperoxycyclophosphamide (**5**).[21] Reduction of **5** yields **2**/**3**, and subsequent fragmentation of **3** gives **4** and acrolein (**6**); see Scheme I. Representative ^{31}P NMR spectra of cells perfused with metabolite-laden medium are shown in Figure 1b,c; a spectrum of the cells, prior to the introduction of metabolites, is shown in Figure

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1a. [Freshly prepared **2**/**3** was added to the perfusion media at time = 1:15; the acquisition of the spectrum shown in Figure 1b was begun at time = 1:25. The total acquisition time for each spectrum was 23 minutes.] The ^{31}P chemical shifts of signals for **2** (δ 12.1), **3** (δ 20.3; it is likely that **3** exists, in solution, predominantly as a hydrate[21]), and **4** (δ 13.4) were the same (±0.1 ppm) as those observed previously by us in buffered solutions.[21] Under conditions where only a minimal filtering of the free induction decay signal was employed, separate signals for *cis*- and *trans*-**2** were observable. In the early stages of metabolite interconversion, the observed concentration of **4** was less than **3**; at later times, however, the 3:4 ratio was approximately constant, indicating that the overall rates of phosphoramide mustard production and loss were comparable.

Following an approximately 2 h 50 min period of perfusion with the metabolites, the cells were washed (ca. 10 min) with fresh media. Spectral accumulation was then begun (at time = 4:30). A comparison of spectra acquired before (Figure 1c) and after (Figure 1d) washing revealed that the signal intensity for **4** was little changed; in contrast, within the limits of spectrometer sensitivity, **2** and **3** could no longer be detected. That **4** was "trapped" intracellularly was demonstrated by the observation that its signal was not affected by the addition of $MnCl_2$ (data not shown), whereas, when cells were perfused with medium containing only **4**, addition of $MnCl_2$ caused the disappearance of this resonance (Figure 2d,e). Moreover, a signal for **4** was *not* retained in spectra that were washed with fresh medium subsequent to 3-h perfusion with authentic **4** (≅3 mM; Figure 2b,c); a "control" spectrum is presented in Figure 2a. [Figure 2 is a composite of separate experiments; spectra a–c and d–e were obtained on two separate occasions.] In the presence of added $MnCl_2$, the extracellular inorganic phosphate resonance is broadened beyond detection (see Figure 2d,e, ca. 2 ppm), revealing the internal phosphate peak, from which the intracellular pH was estimated. These data are collectively interpreted as follows: (i) **2** or **3** or both are readily transported across the cell membrane, (ii) the cell membrane is relatively impermeable to **4**, (iii) intracellular concentrations of **4** result predominantly from the intracellular fragmentation of **3**.

The intracellular disappearance of **4** (see Figure 1d–f) followed a first-order decay law. Least-squares fitting of the intracellular concentration of **4**, as a function of time, to a simple first-order process provided a rate constant (k) of $(5.4 \pm 0.3) \times 10^{-3}$ min^{-1} ($\tau_{1/2} \cong 125$ min; $T = 23$ °C). The least-squares fit (solid line) of the data (two separate determinations, represented in the figure as ovals and triangles) is presented in Figure 3. Rate data obtained on other occasions yielded a similar value for k.

During the time course of these kinetic measurements (ca. 8 h), there was no discernible change in the various cellular resonances. That is, the amounts of ATP, ADP, NAD^+/NADH, UDPG, and sugar phosphates remained high and unchanged, while the amount of inorganic phosphate remained steady and low; see Figure 1. Since all spectra were recorded in an absolute intensity mode, they are strictly comparable. Therefore, cell death or lysis or both were not occurring to any considerable (>10%) extent; a signal loss in excess of 10% would have been discernible. Additionally, these spectral observations show that the enzyme systems responsible for directly or indirectly maintaining these levels of intracellular phosphate esters are not being interfered with by the various cyclophosphamide metabolites, even at the high concentrations

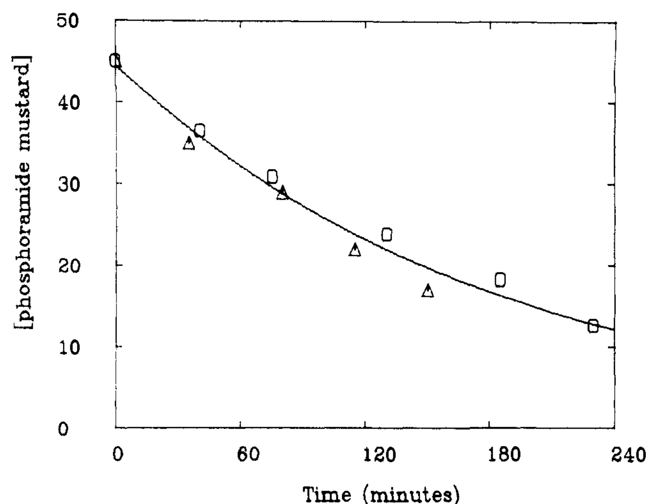

Figure 3. ^{31}P NMR derived time course for the intracellular disappearance of phosphoramide mustard (two separate experiments are represented in the plot and correspond to triangles and ovals). The concentrations are given in arbitrary units, and the times are relative to the start of the data acquisition. The smooth curve was calculated by a least-squares fitting of the experimental data points to a first-order decay expression.

employed in this study. Signals suggestive of either 4-ketocyclophosphamide (**7**) or 4-carboxycyclophosphamide (**8**) were not observed.

In a series of related experiments, the hydroperoxide **5** was added directly to the perfusing media (50 mg in 50 mL; 3 mM); **5** has been regarded as being functionally equivalent to **2**.[22] We were able to observe: (i) the reduction of **5** to **2**, (ii) the subsequent conversion of **2** to **3** and **4**, (iii) the washing out of all metabolites, with the exception of **4**, on switching to a fresh perfusing media, (iv) the intracellular transformation of **4**. The rate constant for the transformation of **4** was, to within experimental error, the same as quoted above. Unexpectedly, however, we also observed changes in cell metabolite levels, viz., loss of ATP with a concomitant increase in the intensity of the sugar phosphate resonances; the NAD^+/NADH, inorganic phosphate, and UDPG levels remained approximately constant. The observed changes were rapid and irreversible. Apparently, pathways involving cell metabolism were being altered. These changes in metabolite levels were, however, not specific to **5**, as we were able to duplicate the pattern of metabolite changes using simple alkyl peroxides. The susceptibility of this cell line to peroxides is not overly surprising, as it has been reported[19] that the U937 cells have very low levels of peroxidases. Low levels of **5** (<10 mg/50 mL) had no observable effect on cellular metabolite levels.

Discussion

The present study has provided the rate constant for the intracellular transformation of phosphoramide mustard, presumably to form the aziridinium species.[21,23] This is the first time that cyclophosphamide chemistry has been directly observed intracellularly. Considering variations in conditions, the intracellular half-life that we obtain (125 min) compares favorably with that reported by Voelcker et al.,[24] for **4** in a 70 mM phosphate buffer (pH 7.0, 37 °C), 48 min; allowing a factor of ca. 2 in rate for every 10 degrees in temperature, the adjusted half-lives are essentially

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the same. The value for the half-life of 4 in Tris buffer (pH 7.0, 37 °C) determined by Engle et al.[23] is somewhat short (14 min) relative to that found intracellularly, indicating that medium effects can be moderately significant. The rate constant for the decomposition of phosphoramide mustard is also sensitive to medium pH,[23] a 20% change in rate constant having been observed on changing the medium pH from 7.0 to 7.4. Since it is unlikely that all of the U937 cells have an identical internal pH value (which we estimated to be 7.1), our measured rate constant is a composite.

Our study has also shown that 4 does not readily cross the cell membrane—in either direction; in contrast, 2/3 were able to cross the membrane in both directions. These observations are in accord with those of Lenssen and Hohorst,[25] who, using a silicone oil technique, were able to show that at 1 °C 4 was not able to permeate Erlich ascites tumor cells, whereas 1 and 5 could. It is not overly surprising that, in the absence of specific transport mechanisms, phosphoramide mustard, being almost completely ionized at pH 7 ($pK_a = 4.75$),[23] does not readily cross the cell membrane. Additionally, we have been able to show that 3 can readily fragment to form 4 inside the cell; were this not the case, then we would not have been able to observe relatively high intracellular levels of 4. It is this ability of 2/3 to cross the cell membrane and 3 to fragment intracellularly that reconciles the generally held hypotheses that while 4 is the ultimate antitumor species,[26] it is the serum concentrations of 2/3, rather than 4, that are of chemotherapeutic interest.[27,28] Unfortunately, we have not been able to directly measure the rate constant for the intracellular conversion of 3 to 4 (because we are unable to distinguish intra- from extracellular 3), and, therefore, cannot comment about possible enzyme-catalyzed[29] conversion of 3 to 4.

It is interesting to note that, following the administration of cyclophosphamide metabolites to the perfusion mixture, the cellular levels of ATP and inorganic phosphate remained constant to within experimental error (see Figure 1). This finding may be contrasted to that of Naruse et al.,[30] derived from an NMR tomographic study of rats following a high-dose cyclophosphamide treatment for an induced neuroblastoma, wherein ATP levels rapidly and markedly decreased while inorganic phosphate levels increased. The extent to which cell metabolism and physiology are disrupted, as well as the point in the metabolic path where an interruption can occur, should vary from cell line to cell line and may be the cause of the above difference in results.

Further investigations of the intracellular transformations of 2–4 and their analogues [e.g., mafosfamide (Asta-Werke AG, Degussa Pharma Gruppe)] in various normal and tumor cells, including those that are resistant to the action of cyclophosphamide metabolites (the U937 cell line is sensitive[31]), are currently underway, and the results from these studies will be reported upon completion.

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Experimental Section

^{31}P NMR spectra (161 MHz) were obtained on a JEOL GX-400 NMR spectrometer; chemical shifts are expressed in parts per million relative to external inorganic phosphate. Intracellular phosphate ester metabolites (such as ATP) were assigned on the basis of their chemical shifts. Ham's F-12 powder with L-glutamine and without sodium bicarbonate was purchased from Flow Laboratories and was reconstituted with sterile, nonpyrogenic water (Abbott Laboratories). Low-melting-point agarose (electrophoresis grade) was obtained from Bethesda Research Labs, Inc. All solutions were kept in presterilized (by autoclaving) flasks stoppered with cotton-gauze plugs. Phosphoramide mustard, as the cyclohexylammonium salt, was a gift from the National Cancer Institute, National Institutes of Health.

Perfusion and Gel-Thread Apparatus. The apparatus and technique used to produce the cell-embedded gel threads, as well as the NMR tube used for the perfusion, have been described.[15-18] Prior to each experiment, the apparatus was cleaned sequentially with solutions of (1) sodium citrate/sodium bisulfite (25 mg each in 100 mL of distilled water), (2) 95% ethanol, (3) penicillin (or ampicillin) and streptomycin (50 mg each in 100 mL of distilled water, and (4) a solution of Ham's F-12 medium.

Agarose Gel Solution. Low-melting-point agarose (180 mg) was added to 10 mL of a buffer containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 2.6 g/L), $CaCl_2 \cdot 2H_2O$ (0.132 g/L), $MgCl_2 \cdot 6H_2O$ (0.1 g/L), KCl (0.2 g/L), and NaCl (8 g/L). To dissolve the agarose, the mixture was heated in a microwave oven (until boiling commenced), and the resultant solution was placed in a water bath (37 °C).

Cell Nutrient Medium. The cell nutrient medium was prepared by dissolving 5.31 g of Ham's F-12 powder (with L-glutamine and without sodium bicarbonate), 0.07 g of penicillin (or ampicillin), and 0.10 g of streptomycin in sterile water (450 mL). Fetal calf serum (50 mL) was added and the pH adjusted with 1 M NaOH to a final value of 7.4.

Cell-Embedded Gel Threads. A 500-mL suspension of U937 cells was centrifuged at 1500 rpm in an International Model PR-2 refrigerated centrifuge for 10 min at 4 °C. The supernatant was removed by decantation, and the packed cells were resuspended in nutrient medium (6 mL) and transferred to a 15-mL conical centrifuge tube. The suspension was centrifuged for 1–2 min in a table-top centrifuge and the supernatant was removed. The packed cells (0.5–1.0 mL) were mixed (using a vortex mixer) with warm (37 °C) agarose gel solution (10% excess by volume) and then extruded through narrow-bore (0.5 mm) Teflon tubing into a 10-mm NMR tube containing nutrient medium. A specially designed insert[15] was then plunged into the NMR tube, compacting the cell-embedded threads into a total volume of ca. 2 mL. The cells were perfused for 20–30 min with nutrient medium which was directed to waste and then perfused with medium in a recirculating fashion.

Reduction of 4-Hydroperoxycyclophosphamide. Triphenylphosphine (357 mg, 1.36 mmol, 4 equiv) was added to a solution of 4-hydroperoxycyclophosphamide (100 mg, 0.34 mmol)[21] in CH_2Cl_2 (10 mL) at 5 °C. After, the mixture was stirred for 10 min at 5 °C, sterile water (2 mL) was added and the mixture was vortexed for 5 min. The CH_2Cl_2 layer was then removed at ambient temperature on a rotary evaporator. The aqueous layer was removed from the residual solids (unreacted triphenylphosphine and its oxide) and added to 50 mL of cell nutrient medium, which was then used in a recirculating fashion to perfuse the cell-embedded threads. The extraction procedure was ca. 50% efficient and the resultant perfusate, therefore, was ca. 3 mM in aggregate metabolite concentration.

^{31}P NMR Spectra. ^{31}P NMR (161 MHz) spectral conditions included 20-kHz spectral window, 4096 data points, 1-s pulse repetition time, 90° (18 μs) observation pulse. The magnetic field was shimmed using the ^1H free-induction-decay (FID) signal of

(31) Hilton, J.; Ludeman, S. M., unpublished findings.

the water in the perfusing sample. A Lorentzian-shaped water signal with half-height width of ca. 20 Hz was generally obtained. Proton decoupling was applied only during the acquisition period in order to suppress sample heating as well as possible differential nuclear Overhauser effects. Prior to Fourier transformation, the averaged FID (2000 transients were averaged) signal was multiplied by an exponential function so as to result in an additional 15-Hz line broadening in the frequency domain spectrum and then twice zero filled (to 16K data points). Spectra were obtained at ambient probe temperature (ca. 23 ± 1 °C).

Acknowledgment. This work was supported, in part, by a Public Health Service grant (CA-21345) to S.M.L. from the National Institutes of Health. We thank Joseph Rogers (Food and Drug Administration) for maintaining the U937 cell line, Drs. Chi-Wan Chen and Richard Knop (National Cancer Institute, National Institutes of Health) for their assistance in initially setting up the perfusion system, and Dr. Gerald Zon (Food and Drug Administration) for helpful discussions.

Registry No. 1, 50-18-0; 2, 40277-05-2; 3, 35144-64-0; 4, 10159-53-2; 5, 39800-16-3.

Structure–Activity Relationship of Novel Oligopeptide Antiviral and Antitumor Agents Related to Netropsin and Distamycin

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A group of oligopeptides have been synthesized that are structurally related to the natural antiviral antitumor agents netropsin and distamycin. Cytostatic activity against both human and murine tumor cell lines as well as their in vitro activity against a range of viruses is reported. The biological activity of these agents is discussed both in terms of their structural differences and, in particular, in relation to their observed base- and sequence-dependent minor-groove binding to duplex oligonucleotides.

The current dearth of clinically effective antiviral agents demands the exploration of new structural types of potential agents of this class. While the more familiar antiviral agents belong to the nucleoside class,[1] there exists a family of naturally occurring oligopeptides[2] including netropsin (1),[3] distamycin (2),[2,4] anthelvencin,[5] amidinomycin,[6] kikumycin B,[7] and norformycin,[8] some examples of which display antiviral and anticancer activities. The most active of these, netropsin and distamycin, are too toxic for clinical use,[1,2] and yet relatively few reports exist of the examination of variants of the basic pyrrolo oligopeptide structure.[9-17] Netropsin and distamycin bind within the minor groove of DNA where they demand binding sites consisting of four and five A·T base pairs, respectively.[18] This firm and site-specific binding, which seems to underlie the biological activity,[1,2] is the net result of specific hydrogen-bonding, electrostatic attraction, and van der Waals interactions.[19] Analysis of the structural and spatial requirements for this specific binding led to the prediction that replacement of one or more pyrrole moieties by imidazole should alter the strict A·T base preference, leading to G·C preferences.[20,21] A recent study requiring the synthesis of several novel oligopeptides showed that this prediction is, in fact, borne out.[20]

In the present study we report the synthesis of structurally modified oligopeptides including those bearing quaternary ammonium end groups in place of the guanidine moiety in netropsin. These latter compounds, together with the free amino derivative, permit an assessment of the role of the nature and size of the charged end group on binding to DNA and in biological activity. We report biological data on these altered-base preferential minor-groove binding and other structurally modified oligopeptides and attempts to relate their biological properties to their specific interactions with DNA.

Synthesis

The structure of the antibiotic netropsin (1) was established in 1963 by Julia and Prêau-Joseph.[3] Since that time, syntheses of both the parent antibiotic and distamycin[22-24] as well as certain homologues and analogues[9,10,13-17] have

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