The technique was extended to include two further materials differing widely in solubility, paracetamol and phenacetin. The results from experiments using these materials are shown in Figs. 3 and 4. Again, good agreement between these measurements can be seen.

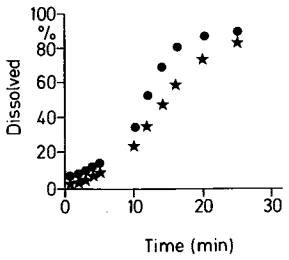

Fig. 3 *In vitro* dissolution of a tablet containing a paracetamol – $^{111}InCl_3$ coprecipitate. See Fig. 1 for explanation of symbols.

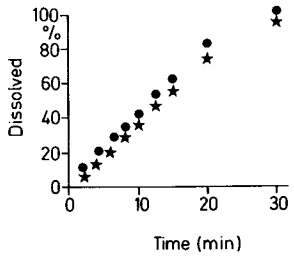

Fig. 4 *In vitro* dissolution of a tablet containing a phenacetin – $^{111}InCl_3$ coprecipitate. See Fig. 1 for explanation of symbols.

Conclusion

The ability to monitor the dissolution rate of a drug *in vivo* is important in pharmaceutical research and development. A combination of P.A.C. studies and gamma scintigraphy appears to offer one approach to achieving this goal. This paper has examined the potential of monitoring drug dissolution by P.A.C. studies. By precipitating the drug in the presence of the radionuclide, the radionuclide is associated with the drug crystals in a way that its release rate reflects that of the drug. It may be argued that the process involved changes the original physical properties of the drug, but this can be easily ascertained, and the process altered accordingly. Current novel approaches to the administration of drugs given as solids often require precise release rates or almost immediate dissolution followed by controlled release of solution. A combination of P.A.C. studies with suitably labelled drug material and gamma scintigraphy give the possibility of following these processes *in vivo*.

Dissolution studies using P.A.C. measurements are not without certain limitations. The *in vivo* P.A.C. dissolution studies performed to date were limited to dosage forms that dissolved within a confined anatomical region, i.e., the stomach or rectum. Dosage forms that spread throughout the gastrointestinal tract while undergoing dissolution may be technically difficult to measure by P.A.C. because of geometric problems with the detectors. In addition, free ^{111}In may bind to specific components of mucous secretions resulting in alterations in the observed anisotropy value. It has been shown that this type of binding does not occur in the stomach (2), but does occur in the rectum (3). Further experiments are needed to determine if such binding can occur throughout the remainder of the gastrointestinal tract. Newer approaches currently under study that employ a single-detector system may circumvent some of the shortcomings of the present detection system.

References

(1) Wilson, C. C., Hardy, J. G. (eds.) (1982) Radionuclide Imaging in Pharmaceutical Research, Croom Helm, London.
(2) Beihn, R. M., Digenis, G. A. (1981) J. Pharm. Sci. 70, 1325–1328.
(3) Jay, M., Beihn, R. M., Snyder, G. A., McClanahan, J. S., Digenis, G. A., Caldwell, L., Mlodozeniec, A. (1983) Int. J. Pharm. 14 343–347.
(4) Mullins, J. W. (1961) Crystallization., Butterworth, London 128–133.

The Interaction of Cyanonaphthyridinomycin with DNA

Milton J. Zmijewski, Jr.[1,2] and Marcia Mikolajczak[1,3]

Received: August 1, 1984; accepted: October 20, 1984.

Abstract: The characteristics of the *in vitro* interaction of cyanonaphthyridinomycin (CYANO) with DNA are described. Unlike naphthyridinomycin (NAP), CYANO is extremely dependent on reductive activation with dithiothreitol (DTT) to bind DNA. The reaction of CYANO with DNA is kinetically slower than that observed for NAP and is still linear after six hours incubation at room temperature. The extent of binding is pH dependent with acidic pH being inhibitory. CYANO, as with NAP, appears to bind to dG:dC base pairs in the minor groove of double stranded DNA. Studies using [C^3H_3:^{14}CN] CYANO demonstrated that the cyanide group is lost when the drug binds to DNA. In the absence of DNA but in the presence of DTT, cyanide is still released from CYANO and the extent of release is also inhibited by acid pH conditions. These results suggest that the cyanide group comes off prior to binding of the antibiotic to DNA. The rate limiting step in the reaction of CYANO with DNA would appear to be the release of cyanide from the drug molecule.

[1] University of Utah, College of Pharmacy, Department of Medicinal Chemistry, Salt Lake City, Utah 84112
[2] Address all correspondence to this author: Eli Lilly and Company, 307 E. McCarty Street, Indianapolis, Indiana 46285
[3] Current address: 478 Neptune Avenue, Encinitas, California 92024

Naphthyridinomycin (NAP) is an antitumor antibiotic produced by *Streptomyces lusitanus* (1). We have previously demonstrated that this compound inhibits DNA and RNA synthesis in susceptible organisms by apparently binding to dG:dC base pairs in the minor groove of DNA (2–4). The rate of the *in vitro* interaction is enhanced by the presence of reducing reagents suggesting that NAP is a bio-reductively activated antitumor agent (4). The drug-DNA adduct is presumably formed through the α-carbinolamine of the antibiotic and the N-2 nitrogen of guanine in the minor groove of DNA (4) as has also been proposed for the pyrrolo[1,4]benzodiazepine antibiotics (5) and saframycin A and S (6, 7).

The extreme reactivity of the α-carbinolamine of NAP to nucleophiles was also demonstrated by our finding that this functionality could be masked by reacting the drug with cyanide in an aqueous medium to generate cyanonaphthyridinomycin (CYANO) (8). This antibiotic and its quinone altered derivatives (9) also have antitumor activity in animal model systems.

Recently another semi-synthetic antibiotic naphthocyanidine has been reported (10), and CYANO (also called cyanocycline A) has been found to be a natural product of *Streptomyces flavogriseus* (11).

In a very recent study, Hayashi et al. (12) have shown that CYANO inhibits DNA and RNA synthesis in a tumor cell line and *E. coli*. Using an indirect assay, these same authors present data which suggest that CYANO inhibits DNA and RNA synthesis by binding to DNA (12). They propose that CYANO binds to DNA by a mechanism different from NAP. This was proposed because they could not detect enhanced reactivity of CYANO in the presence of dithiothreitol (DTT) nor could they detect loss of the cyanide group from acid treated CYANO.

In this report, we utilized a double radioisotope labeled CYANO prepared from our biosynthetic studies (13) to show that the antibiotic does bind *in vitro* to DNA. The reaction occurs with loss of cyanide from the antibiotic-DNA adduct. The loss of cyanide from the molecule is not promoted by acidic pH conditions but is stimulated by the presence of DTT. The apparent rate limiting step in the interaction of this antibiotic with DNA is the release of the cyanide to generate the reactive α-carbinolamine of NAP.

Material and Methods

All solvents used in the purification and analysis of the antibiotics were reagent grade and distilled prior to use. DTT, spermine, calf thymus DNA, *Clostridium perfringens* and T_4 DNA and poly[dG]•poly[dC] were purchased from Sigma Chemical Company. Poly[dG] was purchased from P.L. Biochemicals, Inc. Radioisotope labeled compounds used were L-[methyl-^{3}H]methionine (80 Ci/mmole, New England Nuclear) and potassium [^{14}C] cyanide (50 mCi/mmole, Amersham). Nucleic acid concentrations were calculated as described previously (4).

Preparation of [C^3H_3]naphthyridinomycin and [C^3H_3, ^{14}CN]cyanonaphthyridinomycin

[C^3H_3]Naphthyridinomycin (NAP) was prepared biosynthetically by the addition of L-[methyl-^{3}H]methionine to production cultures of *S. lusitanis* and purified as described previously (3). The doubly labeled cyanonaphthyridinomycin (CYANO) was prepared in a similar fashion. [C^3H_3] NAP in the fermentation broth was treated with 1 M sodium cyanide and stirred at room temperature for one hour. [C^3H_3] CYANO was extracted with methylene chloride and purified over an aluminium oxide column. [^{14}CN] CYANO was prepared with potassium [^{14}CN] cyanide. The two labeled species were combined to obtain [C^3H_3;^{14}CN] CYANO having a tritium to ^{14}C ratio of 1/1.

Kinetics of binding of NAP and CYANO to DNA

In a typical kinetics experiment, the radioisotope labeled antibiotic (0.057 μmole/ml), calf thymus DNA (0.42 μmole of nucleotide base pairs/ml) and dithiothreitol (DTT, 1 μM/ml) were incubated in the appropriate buffer at the desired pH and room temperature. At prescribed times, samples were removed to determine the extent of binding. In order to rapidly separate bound from unbound antibiotic, the DNA drug adducts were precipitated with spermine (14). To one ml samples of the incubation medium 110 μl of a 0.1 M spermine solution (dissolved in water) was added. After 15 min at 4 °C, samples were centrifuged at low speed for 10 min to pellet the DNA. The buffer was pipetted off and the DNA washed with an additional amount (0.5 ml) of buffer containing spermine. After centrifugation, the buffer was again pipetted off. One ml of the appropriate buffer containing 0.5 ml sodium chloride was added to the DNA, and the samples were incubated at 4 °C overnight to redissolve the DNA. The next day samples of each solution were analyzed for DNA (U.V. at 260 mm) and radioactivity as described before (3). Routinely, this method allowed for the recovery of between 70 and 94 % of the original DNA present. In control experiments where the DNA-drug adducts were prepared by dialyzing out unbound antibiotic, this method recovered 76 % of the DNA and 76 % of the radioisotope labeled antibiotic. Kinetic studies carried out with this method and a butanol extraction method (4) gave comparable results (data not shown).

Dialysis of DNA-Drug Adducts. Dialysis of samples containing DNA-drug adducts were performed as described previously (3).

Results

Comparative binding kinetics of NAP and CYANO to DNA

Under the conditions employed (Fig. 1), NAP and CYANO exhibited

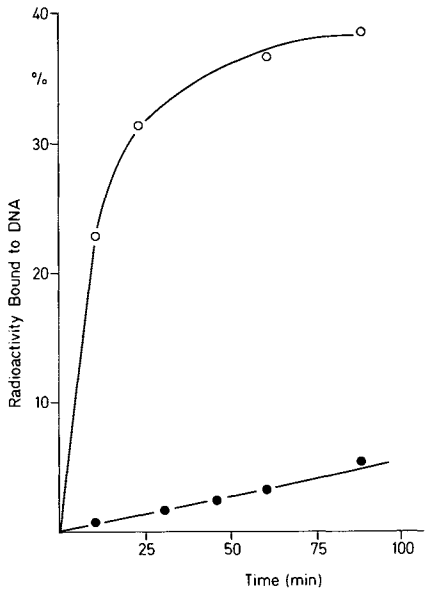

Fig. 1 Comparative *in vitro* reactivity of [C^3H_3] NAP (o) and [C^3H_3] CYANO (•) with calf thymus DNA at pH 7.8 and in the presence of 1 mM Dithiothreitol (DTT).

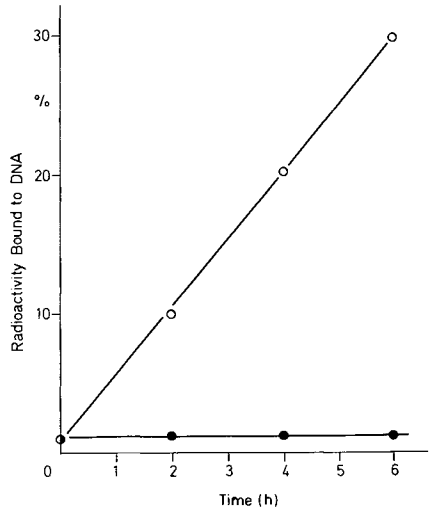

Fig. 2 Percent of [C^3H_3:^{14}CN] CYANO bound to DNA over several hours at pH 7.8 and in the presence of 1 mM DTT. (o) ^{3}H; (•) ^{14}C.

very different reaction kinetics toward calf thymus DNA. The DTT promoted binding of NAP to DNA reached saturation rapidly while CYANO reacted much more slowly. Utilizing doubly labeled [C^3H_3:^{14}CN] CYANO, it was found (Fig. 2) that the kinetics were still linear after 6 hours and that little of the DNA bound antibiotic retained the ^{14}C labeled cyanide.

Effect of pH on binding

Decreasing the pH of the incubation medium caused a marked depression of the reactivity of CYANO toward DNA (Table I). At pH 5.0 (plus DTT), CYANO reacted with C.T. DNA about one-third as well as at pH 7.2. Little radioactivity from the ^{14}C-cyanide was found associated with the DNA at any pH value. In the absence of DTT, a small amount of reaction took place as judged by the higher percent of tritium bound in comparison to the ^{14}C label present. Depending on the pH, DTT enhanced binding of CYANO to DNA between three and ten fold.

Table I. pH dependent binding of [C^3H_3:^{14}CN] CYANO to calf thymus DNA.

	% radioactivity bound to DNA[a]			
	+ DTT[b]		− DTT	
pH	^{3}H	^{14}C	^{3}H	^{14}C
5.0	10.1	1.1	3.5	1.0
5.5	23.3	1.8	4.1	1.0
6.0	29.7	1.4	3.2	0.5
7.2	34.3	1.9	3.2	1.3
7.8	29.1	1.4	2.1	1.1

[a] As measured after 6 h incubation at room temperature under standard kinetic conditions (see methods).
[b] Final concentration of 1 mM.

Release of ^{14}CN from [C^3H_3:^{14}CN] in the absence of DNA

The extent of release of cyanide from CYANO in the absence of DNA was monitored as a function of pH and the presence or absence of DTT (Table II). The percent increase in the ^{3}H/^{14}C ratio of the antibiotic after four hours was indicative of a loss of cyanide from CYANO. The loss of cyanide as a function of pH followed the same pattern observed for the reactivity of the drug toward DNA. Acid pH inhibited release of cyanide while neutral pH promoted release. The presence of DNA also enhanced the amount of ^{14}CN lost from the reaction mixture. The absence of DTT from the incubation mixture resulted in no or little change in the ^{3}H/^{14}C ratio of the antibiotic under the conditions employed.

Table II. Release of [^{14}C] cyanide from [C^3H_3:^{14}CN] CYANO in the presence and absence of DTT.

pH	+DTT[a]	−DTT[a]
5.0	+ 5	0
5.5	+12	0
6.0	+28	+5
7.2	+26	0
7.8	+28	0
7.2 + DNA[b]	+36	N.E.

[a] Measured after four hours incubation at room temperature. The reaction mixture of 3 ml buffer contained 80 µg of [C^3H:^{14}CN] CYANO [^{3}H/^{14}C=1/1] in the presence or absence of 1 mM DTT. Nitrogen was bubbled through the solution during the entire incubation period. The positive numbers above represent the percent increases in the ^{3}H/^{14}C ratio of the antibiotic after four hours. The higher the positive number the greater the loss of ^{14}CN from the molecule.
[b] 0.42 µmole/ml
N.E. Not examined

Specificity of binding of CYANO to DNAs

[C^3H_3] CYANO was tested for its specificity in binding to certain DNAs (Table III). This antibiotic displays the same preference for DNA as NAP (4); binding well to double stranded dG:dC containing DNA but not to single stranded poly dG. Minor groove specificity is also indicated by the antibiotic's absence of preference for *Cl. perfringens* DNA over T_4 DNA which is 100 % glucosylated in the major groove (15).

Table III. The specificity of binding of [C^3H_3] CYANO for various DNAs.

DNA Source	D.P.M[a]
calf thymus	2301
Poly dG:Poly dC	2320
Cl. perfringens	1374
T_4	1014
Poly dG	99

[a] Each incubation contained 2.5 A units of DNA and 80 µg of CYANO in a total volume of 2.0 ml Tris-HCl (pH 7.2) containing 1.0 M DTT. After 8 h at room temperature, each incubation was dialyzed for 48 h at 4 °C against two changes (250 ml, each) of Tris-HCl at pH 7.2.

Discussion

This preliminary investigation into the characteristics of the binding of CYANO to DNA has demonstrated that this antibiotic does bind to DNA and that this binding is promoted by DTT. The kinetics of binding for CYANO are very different from that found for NAP. The rate of reaction of CYANO under our standard conditions was very slow with less than 10 % of the available antibiotic binding in 90 min. After 6 h, the reaction rate was still linear and approximately 30 % of the antibiotic was bound to the DNA. The base pair specificity of CYANO would appear to be the same as NAP since both antibiotics bind to dG•dC base pairs and to the minor groove of double stranded DNA.

The release of cyanide from CYANO in the presence of DTT (but in the absence of DNA) follows the same pH profile as the drug's reactivity toward DNA. This result suggests that the antibiotic does not bind well to the DNA until the cyanide is released. Saframycin A (7) has also been shown to release cyanide upon binding to DNA but unlike CYANO, the binding of saframycin A was enhanced by acidic pH conditions (6). Our results hint that at pH 5.0 CYANO has a group (or groups) which is (are) partially protonated and this partial protonation is interfering with the release of cyanide from the molecule. Preliminary pKa determinations indicated that CYANO does have at least one protonatable group (presumably an amino group) with a pKa between 4.6 and 5.0 (data not shown). Hayashi et al. (12) suggested that CYANO (cyanocycline A) differs from saframycin A and NAP in its binding mechanism toward DNA. From this study, the difference appears to be less dramatic than they suggest but certainly real differences do exist.

References

(1) Kluepfel, D., Baker, H. A., Piattoni, G., Sehgal, S. N., Sidorowicz, A., Singh, K., Vezina, C. (1975) J. Antibiotics 28, 497–502.
(2) Singh, K., Sun, S., Kleupfel, D. (1976) Develop. in Indus. Microb. 17, 209–221.
(3) Zmijewski, M. J., Miller-Hatch, K., Goebel, M. (1982) Antimicrob. Ag. Chemo. 21, 787–793.
(4) Zmijewski, Jr., M. J., Miller-Hatch, K., Mikolajczak, M. (1984) Accepted for publication, Chemico-Bio. Interactions.

(5) Petrusek, R. L., Anderson, G. L., Garner, T. F., Fannin, W. L., Kaplan, D. T., Zimmer, S. G., Hurley, L. H. (1981) Biochem. 20, 1111–1119.
(6) Lown, J. W., Joshua, A. V., Lee, L. S. (1982) Biochem. 21, 419–428.
(7) Ishiguro, K., Takahashi, K., Yazawa, K., Sakiyama, S., Arai, T. (1981) J. Biol. Chem. 256, 2162–2167.
(8) Zmijewski, Jr., M. J., Goebel, M. (1982) J. Antibiotics 35, 524–526.
(9) Zmijewski, Jr., M. J., Mikolajczak, M. (1983) J. Antibiotics 36, 1767–1769.
(10) Itoh, J., Omoto, S., Inouye, S., Kodama, Y., Hisamatsu, T., Niida, T., Ogawa, Y. (1982) J. Antibiotics 35, 642–644.
(11) Hayashi, T., Noto, T., Nawata, Y., Ikazaki, H., Sawada, M., Ando, K. (1982) J. Antibiotics 35, 771–777.
(12) Hayashi, T., Okutomi, T., Suzuki, S., Okazaki, H. (1983) J. Antibiotics 36, 1228–1235.
(13) Zmijewski, Jr., M. J. Mikolajczak, M., Viswanatha, V., Hruby, V. J. (1982) J. Amer. Chem. Soc. 104, 4969–4971.
(14) Subirana, J. A., Vives, J. L. (1981) Biopolymers 20, 2281.
(15) Erickson, R. L., Szyblaski, W. (1984) Virology 22, 111–113.

Drug Distribution and Biliary Excretion Pattern of a Cyclic Somatostatin Analog: A Comparison of ^{14}C Labeled Drug and a ^{131}I Iodinated Drug Analog

Larry J. Caldwell[1,4], Alan Parr[2], Robert M. Beihn[3], Bushra J. Agha[2], Arthur R. Mlodozeniec[1], Michael Jay[2], and George A. Digenis[2,3]

Received: August 6, 1984; accepted: October 3, 1984

Abstract: A cyclic somatostatin analog was compared to an iodinated analog of the same compound with respect to organ distribution and biliary excretion in the rat. The cyclic hexapeptide was radiolabeled with either ^{14}C or ^{131}I (tyrosine). Organ distribution of the iodinated compound as a function of time was nearly identical to that observed for the non-iodinated compound. Results indicated a rapid uptake by the liver and subsequent rapid excretion of the intact peptide in bile. Activity in other organs examined tended to fall off in a manner similar to the activity in blood with sequential samples. Because of the similarity in the *in vivo* behavior of the two compounds, the iodinated analog was deemed a suitable model for less invasive distribution studies, and was further examined in the dog using external gamma scintigraphy. In the unanesthetized dog the iodine activity was rapidly taken up by liver and collected in the gallbladder, thus exhibiting a similar rapid excretion pattern to that observed in the rat.

There has been an increasing interest in recent years concerning somatostatin and compounds which display somatostatin-like activity. This is primarily because of the powerful effects which somatostatin exerts on many diverse organ systems within the mammalian body, and the potential of using this type of compound to treat a variety of disease states.

One cyclic hexapeptide analog of somatostatin[5], [cyclo(L-*N*-methylalanyl-L-tyrosyl-D-tryptophanyl-L-lysyl-L-valyl-L-phenylalanyl)], A, displays resistance to enzymic degradation and at the same time is far more potent than somatostatin in eliciting certain physiological responses (1). This compound is in fact so resistant to degradation *in vivo*, that after i.v. injection, most of the compound is excreted intact in the feces. This implies a remarkable resistance to enzymic attack in blood, within the liver, and within the intestinal tract itself.

During the process of trying to better understand the *in vivo* disposition of A, it became desirable to find a suitable means to non-invasively monitor the fate of this compound after i.v. injection. The objective of the work described herein was to determine the feasibility of using an iodinated analog of A as a model compound to study the disposition of such polypeptides using external gamma scintigraphy (2). In this case we compared the organ distribution in the rat of ^{14}C-A to a ^{131}I-iodinated analog at various times after i.v. injection. Subsequently, the biliary excretion profile of both compounds was more carefully examined in the rat. Finally, the liver uptake and biliary excretion of the iodinated analog was quantitated in unanesthetized dogs using external gamma scintigraphy as a non-invasive technique.

Experimental

Preparation of iodinated drug analog

The cyclic hexapeptide A was radioiodinated [^{131}I] by a modification (3) of the procedure of Hunter and Greenword (4). $Na^{131}I$ was used as the source of the radionuclide and chloramine-T as the oxidizing agent. In a typical experiment the hexapeptide (5 µl, 1 mg/ml H_2O) was added to a test tube containing 50 µl of sodium phosphate buffer (pH 7.6), $Na^{131}I$ (10 µl, 1 mCi) was added followed by Chloramine-T (10 µl, 0.5 mg/ml H_2O) and the reaction mixture was gently shaken for 20 sec. To stop the reaction, sodium thiosulfate was added and gently mixed at room temperature. Bovine serum albumin (BSA) was added to the reaction mixture before subjecting the latter to Sephadex filtration.

[1] INTERx Research Corporation (Subsidiary of Merck & Co., Inc.), Lawrence, Kansas 66044
[2] College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536
[3] Department of Nuclear Medicine, University of Kentucky Medical Center, Lexington, Kentucky 40536
[4] Correspondence to be addressed to Larry J. Caldwell, INTERx Research Corporation, 2201 West 21st Street, Lawrence, Kansas 66044

[5] Merck Sharp & Dohme Research Laboratories