Covalent Binding of Isomeric 7-(2,3-Epoxypropoxy)actinomycin D to DNA

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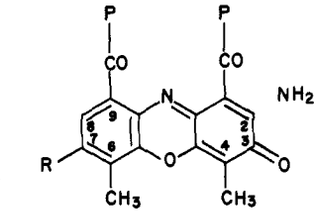
We have examined the ability of 7-(2,3-epoxypropoxy)actinomycin D (EPA) to bind covalently to DNA and to 2′-deoxyribonucleoside 5′-monophosphates in a simple system in vitro. We have observed initially that EPA binds to DNA and deoxymono- and deoxydinucleotides with intercalative or stacking interactions that are characteristic of actinomycin D (AMD). When EPA is incubated (37 °C) for a prolonged period (pH 7.4, 6 h) in contact with either DNA or deoxyribonucleotides, it forms covalent adducts. Deoxyguanosine is always the preferred site of reaction by EPA. After enzymatic digestion of EPA–DNA adduct, three deoxyguanosine (EPA–dG) adducts, one major and two minor, were isolated. These adducts are separable from one another and from other deoxyribonucleoside adducts, e.g., EPA–dA and EPA–dC by reverse-phase HPLC. The authentic EPA–dG, EPA–dA, and EPA–dC adducts were synthesized by a chemical reaction of the epoxide in EPA with the deoxyribonucleotides followed by enzymatic dephosphorylation of the products. From the EPA–DNA adduct the EPA–dG adducts accounted for ≃2.2% of EPA employed; the remainder of EPA was completely *hydrolyzed* to an epoxide ring opened diol derivative, DHPA. DHPA binds to DNA by intercalation only and it does not form covalent adducts. Another model analogue of EPA (EPAMDEA) has the same epoxide-substituted chromophore but lacks the peptide lactone functions; it fails to associate with DNA and consequently it shows no covalent binding of its epoxide with DNA. Formation of a noncovalent intercalation complex between EPA and DNA appears to be a prerequisite for the covalent reaction. Presumably because of these dual interactions, EPA demonstrates superior antitumor activities both in human leukemic cells (CCRF-CEM) in vitro and P388 and L1210 cells in mice. The DNA base specific alkylating activity of EPA, which is derived from a combination of the actinomycin D (AMD) structure and the new epoxide function in the molecule of EPA, attributes to EPA a potentially novel pharmacological behavior that is not inherent of AMD.

In an earlier paper we reported the synthesis of two carbon-7-substituted analogues of actinomycin D (AMD, **1b**). One of these analogues, 7-(2,3-epoxypropoxy)actinomycin D (EPA, **3b**) carries a potentially alkylating epoxide function on the three-carbon substituent chain at C-7. The other analogue, 7-(2,3-dihydroxypropoxy)actinomycin D (DHPA, **4b**), is an epoxide ring opened diol of EPA. Two peptide-free analogues of EPA and DHPA, EPAMDEA (**3a**) and DPAMDEA (**4a**), were also reported; in these models the peptide lactone (P) moieties of EPA and DHPA were replaced by simple diethylamino groups, which resulted in the complete loss of DNA binding properties and DNA binding related biological activities. EPA and DPHA carry not only the P groups of AMD but additional side chains at C-7 that do not interfere in their AMD-like binding affinities for DNA. They are extremely cytotoxic to tumor cells in culture and were found to be effective antitumor agents against P388 leukemia in CDF_1 mice.[1]



a, P = $N(C_2H_5)_2$
b, P = Thr-D-Val-Pro-Sar-MeVal-O

1, R = H
2, R = OH
3, R = $OCH_2CH(O)CH_2$
4, R = $OCH_2CH(OH)CH_2OH$

AMD: 1b
7-OH-AMD: 2b
EPA: 3b
EPAMDEA: 3a
DHPA: 4b
DPAMDEA: 4a

Table I. Absorption of EPA and DHPA in the Presence and Absence of Nucleotides[a]

nucleotides	nucleotide concn, mM phosphate	absorption max, nm EPA	DHPA
free drug		465	465
5′-dAMP	1	470	470
5′-dGMP	1	478	478
3′-dGMP	1	476	477
3′-dCMP	1	472	471
pdA-dT	2	473	472
pdC-dG	2	478	477
pdG-dC	2	480	480

[a] Concentration of EPA and DHPA, 5.0×10^{-5} M in 20 mM Tris-HCl (pH 7.4).

The rationale for the synthesis of an epoxide-substituted AMD analogue (EPA) was based on the clinical history of AMD, which is that AMD is effective only in a few tumors[2,3] and that the tumor lines that do not respond to AMD are generally cross resistant to other intercalating agents like adriamycin but not to alkylating agents, e.g., cytoxan and L-PAM.[4] EPA was therefore designed to act as a DNA site specific intercalator like AMD and further to act as a DNA alkylating agent which is unlike AMD. In addition, the epoxy function could conceivably bind to other macromolecules in the cell apparatus, which could widen the scope of its biological activity. In the present paper we report the result of its binding to DNA in vitro, which leads to covalent bond formation with deoxyguanosine base in DNA.

DNA Base and Base Pair Sequence Specific Binding Studies. The absorption maxima of EPA and DHPA on binding to DNA in buffer suffer simultaneous bathochromic and hypochromic shifts, from λ_{max} 465 nm (ϵ 15 000) to λ_{max} 480 nm (ϵ 8000);[1] the difference spectra between DNA-bound drugs and the free drugs show a negative maximum at 444 nm ($-\Delta\epsilon$ = 10 000) and a weaker positive maximum at 485 nm ($+\Delta\epsilon$ = 7000). Since DNA has no absorbance in this region, the extent of DNA

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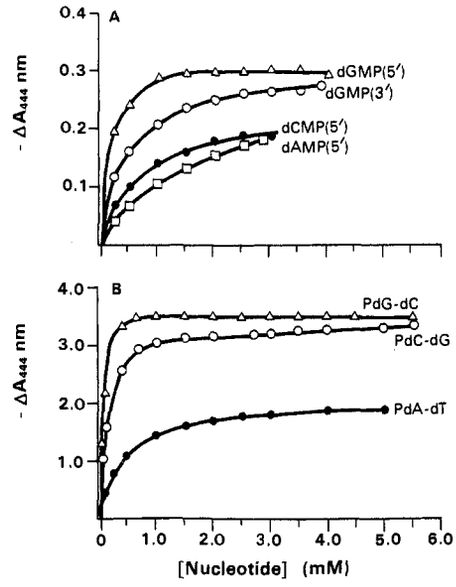

Figure 1. The change in absorbance of EPA at 444 nm as a function of added nucleotide concentration. The concentration of EPA was 5.0×10^{-5} M at the start of each titration. All measurements were made in 5 mM phosphate buffer pH 7.0 and 20 °C.

binding can be estimated from $-\Delta A_{444}$ values, which show an approximate linear relationship with the formation of intercalated drug–DNA complexes.[5] The time required for stabilization of this equilibrium binding is about 30 min when [drug]/[DNA] ratios are high; at limiting [drug]/[DNA] ratio of 1:10 the time required is 1 h. Deoxyribonucleotides and dinucleotides demonstrate similar changes showing reproducible $-\Delta A_{444}$; however, the positive changes at higher wavelengths depend on the nature of the nucleotide substrates employed (Table I).

The visible spectral changes provide a useful means of monitoring nucleotide complex formation. The change in absorbance ($-\Delta A_{444}$) of a solution of EPA and DHPA (5.0×10^{-5} M) in the presence of the nucleotide was plotted against the concentration of the nucleotide used (Figure 1A and B). EPA shows large absorbance changes in complexing with guanine-containing nucleotides. The shape of pdG–dC titration curve, when compared to the shape of the other dinucleotide titration curve, indicates that pdG–dC binds to the analogue more strongly than the other dinucleotides. The absorption maximum shifts to 480 nm on addition of excess pdG–dC ($\simeq$1 mM). An analysis of visible absorption binding curves shows that EPA binds to pdG–dC in a cooperative manner, while pdC–dG shows pseudosigmoidal and pdA–dT shows hyperbolic titration curves. The approximate Hill constants for the binding of pdG–dC to EPA is 1.9 and for DHPA is 1.8 (figure not shown), which suggest cooperative binding situations. In comparison, AMD exhibits a Hill constant of 2.0 for complex formation with pdG–dC, which is indicative of a highly cooperative complex formation.[6,7] DHPA behaves like EPA in binding to mono- and dinucleotides.

UV-Visible Absorbance Changes on Binding Covalently to DNA and Nucleotides by EPA. In addition to the absorbance A_{465}, EPA shows an additional absorbance A_{260} that is equivalent to its A_{465}. The experimentally observed absorbance value of A_{260} is found to be relatively unperturbed on binding to a base in DNA; this is true whether the drug is bound intercalatively or covalently. (The [drug]/[DNA–base] was estimated by ^{3}H and ^{14}C.) In the absence of all intercalatively bound species, EPA–DNA adduct shows absorption maxima at 465 and 260 nm. In EPA–DNA complexes, the observed A_{260} is the sum of individual contributions from free EPA and DNA. In the intercalated state, the A_{480} is 0.55 of its value at A_{465} in free form; therefore, A_{260} of the intercalated complex is adjusted by multiplying A_{480} by a factor 1.88 to correct for the contribution of the drug, and the difference of this value from the observed A_{260} in the complex is due to DNA only. Lack of any shift in the spectra of EPA adducts either with DNA or deoxyribonucleosides relative to spectrum of drug signifies that the drug chromophore must be well outside chromophoric regions of the substrates; it strongly suggests that simultaneous intercalative and covalent binding by the same drug molecule to a DNA base is not possible.

Table II. Frequency of Covalent and Noncovalent Intercalative Modes of Binding of EPA to Nucleotides on DNA Helix[a]

	[DNA base]/[drug]: method of assay			
	A_{260}/CPM		A_{260}/A_x[b]	
	A[c]	B	A	B
EPA (**3b**)	32 ± 1	480 ± 10	35 ± 4	525 ± 55
DHPA (**4b**)	33 ± 2	ND	36 ± 6	ND
EPADEA (**3a**)			ND	ND
AMD (**1b**)	26 ± 2	ND[d]	29 ± 5	ND

[a] Purified calf thymus DNA in 20 mM Tris-HCl (pH 7.4) buffer at 37 °C. [b] A_x for EPA and DHPA are A_{480} and for AMD is A_{465} in the intercalated complex; A_{465} for the adducts. [c] A, fractions of DNA–drug complexes that are precipated with ethanol; the ratio is based on the total amount of drug that is bound to DNA covalently and intercalatively. B, fractions of DNA–drug adducts that are bound by covalent bonds. [d] ND, none or not detected.

It is important to point out that the experimentally observed A_{260} values in the intercalatively bound complexes may not represent the actual situation. Because of proximity, there must be some electronic interactions between the chromophores of the substrates and ligands. We have previously noted that as a result of identical interactions with a fluorescent analogue, 7-amino-AMD, the nucleic acids uniformly enhanced the fluorescence of the analogue at the regions of 595 (uncorrected) and 644 nm (corrected).[8] The deoxyribonucleosides and deoxyribonucleotides also behaved like the nucleic acids except dG, which quenched the fluorescence. Clearly some electronic interactions were taking place between the chromophores, and this is exhibited by the shift in the visible absorbance of high wavelength region for both EPA and DHPA. Shifts are also observed in the CD spectra both in high and low (260 and 280 nm) wavelength regions.[1] Absorbance at 260 nm may be influenced likewise but in a much smaller degree, and the quenching in dye chromophore at 260 nm be compensated by the enhancement in the DNA chromophores; the net result is that there is no observable change in A_{260} values. This is only a hypothesis and needs to be examined by DNA-phosphorescence experiments.

Time Course for the Reaction of EPA with DNA and Its Bases. The time dependence of the reaction of EPA with DNA shows that EPA is virtually stable for 2 h in the presence of DNA and for >4 h with its nucleotide bases. EPA is hydrolyzed to only 55% DHPA in 17 h at 37 °C in the absence of DNA. Reaction of EPA with DNA

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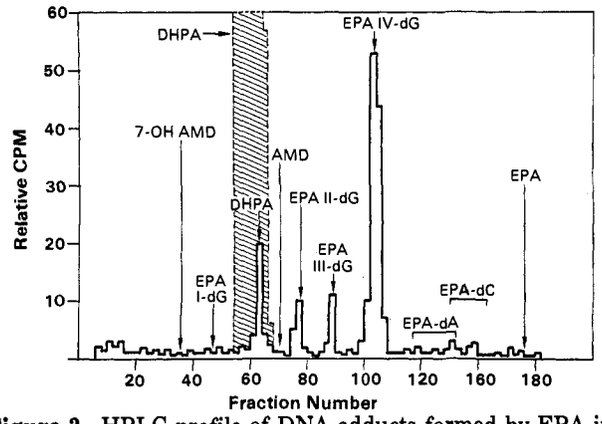

Figure 2. HPLC profile of DNA adducts formed by EPA incubated with DNA for 6 h. DNA modified by EPA or [^{3}H]EPA was isolated and enzymatically digested. The modified deoxynucleosides, separated by Sephadex LH-20 chromatography, were cochromatographed on HPLC with authentic standards. Fractions (0.5 min × 2) were collected and after dilution their specific activities were determined. Peaks in HPLC were monitored at A_{465} and at A_{254}. The shaded peak represents A_{465} tracing of the hydrolysis product DHPA in the absence of deoxynucleosides.

is complete in 6 h; 2.2% of total EPA forms EPA–DNA adduct, and the rest is hydrolyzed to DHPA, 28% of which intercalates to DNA and the remainder stays in the free form. The status is unchanged at 44 h. DHPA and AMD are unaffected at 44 h, in both unbound and bound states. EPAMDEA neither binds nor changes. At [drug]/[DNA] = 1:10, EPA and DHPA intercalates to every 32–36 bases and AMD binds to every 26 bases in DNA. The bindings were estimates by A_{260}/CPM and $A_{260}/A_{nm(max)}$ and the results agree well (Table II). Deoxyguanosine is always the preferred site for reaction by EPA in both DNA and nucleotides. The extent of adduct formation by EPA is 35–40% with dGMP, ~20% with dAMP, and ~25% with dCMP in a 44-h reaction. The rest is converted to DHPA. The chemical reaction and the physical association rates of EPA with nucleotides follow the same order of preference, i.e., 5′-dGMP > 5′-dCMP ≃ 5′-dAMP. However, compared to its rate of intercalation, the rate of adduct formation by EPA is much slower, which may reflect a relative rigidity in the structure of the DNA–EPA complex, which retards its reaction with nucleophiles in DNA but not with the medium surrounding it.

Isolation of Covalent EPA Conjugates. After reaction with EPA, EPA–DNA adduct was purified from unbound and intercalatively bound drugs and hydrolyzed to mononucleoside adducts. The procedure to purify EPA–DNA adduct include (i) precipitation of EPA–DNA adduct with ethanol to separate it from unbound drug, (ii) removal of intercalatively bound drug from the adduct by stepwise dialysis in high salt and urea solution, (iii) complete enzymatic hydrolysis of the dialyzed addut to deoxyribonucleoside adducts, and (iv) separation of these into purified components by Sephadex chromatography and HPLC. In addition, an estimate of the unbound and intercalatively bound drugs were made from the EtOAc extracts of the aqueous supernatants after separation of DNA fraction in step (i) and the diffusates from dialysis of the adduct in step (ii). The method of assay included absorption spectrometry and scintillation spectrometry in conjunction with HPLC (sensitivity of assays are <100 and ≃1 pmol, respectively).

EPA–nucleosides were analyzed by reverse-phase HPLC using appropriate markers (Figure 2). The first peak to elute (62 min) cochromatographed with DHPA; the material in the peak constituted about 0.5% of the total EPA employed and ~15% of all the peaks eluted. This peak was followed by three peaks, one major and two minor. All three peaks coeluted with standard EPA–dG adducts prepared by reaction of EPA with 5′-dGMP and dephosphorylation of the reaction products (Experimental Section); four EPA–dG standards were prepared by this method (see below). In the HPLC chromatogram (Figure 2), the peak positions are designated as EPA I-dG (44 min), EPA II-dG (77 min), EPA III-dG (88 min), and EPA IV-dG (104 min), in the order of their retention times. Only three of these components were present in the enzymatic hydrolyzates of EPA–DNA adduct; the fraction corresponding to EPA I-dG was absent in this digest. The EPA IV-dG adduct was the major component and represented about 70–75% of the total adducts. The major component was acetylated and was fund to coelute with the corresponding acetylated[9] standard (150 min), confirming its identity. Two minor peaks, EPA II-dG and EPA III-dG were present in about equal proportions and these fractions together constituted 24–30% of all the three adducts formed. All peaks compared well with the respective standards by cochromatography and the peak augmentation technique. Little radioactivity was detected in the regions where deoxyadenosine, deoxycytidine adducts, and free EPA (176 min) elute. The large shaded peak (fractions 54–68) in the chromatogram represents an aliquot of DHPA from the intercalatively bound fractions and is superimposed to show that even relatively large fractions of DHPA can be separated efficiently from the adducts. We have also found that our procedure can separate DHPA (and EPA) from mixtures with deoxyribonucleosides; the deoxynucleosides are more polar and elute at or close to the solvent fronts.

Synthesis of Standard Samples of EPA–Deoxyribonucleoside Adducts. These 1:1 adducts were made by a direct reaction of the epoxide group in EPA with the deoxyribonucleoside 5′-phosphates followed by enzymatic removal of the phosphate groups in the products by digestion with alkaline phosphatase. Chromatography on a Sephadex LH-20 column purified the adducts from the enzyme, salt, and the free drug; the details are given in the Experimental Section. All these adducts consist of one drug molecule bound to one DNA base, but the exact structure and conformation are not known at this time. Of these the deoxyguanosine adducts were found to be a mixture of four adducts, which were resolved into pure components by HPLC. They served as useful markers for the HPLC peaks of the products isolated in the above in vitro DNA experiments. These and the other EPA–dC and EPA–dA adducts isolated in these experiments will be used as the standards and markers in our future in vivo experiments.

Discussion

In the present studies EPA is found to form covalent adducts with DNA (6 h) with the sole preference for the deoxyguanosine base. EPA is also found to react with several monomeric deoxyribonucleotides (14 h). The reaction is most facile with DNA; with the nucleotides the order of this reaction is 5′-dGMP >> 5′-dAMP ≃ 5′-dCMP (37 °C). At temperatures of 37 and 20 °C, EPA also intercalates into DNA, like AMD and DHPA. EPA is relatively stable in aqueous buffer, but in the presence of DNA it is activated to form DNA adducts or its hydrolytic product, DHPA. The reaction is influenced by

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the affinity of the drug for a specific sequence in DNA and the ease of its covalent reaction with the nucleophiles in or around DNA in the intercalated state. At the primary stage, the rate of EPA reaction may depend mainly on the stereochemistry of the drug–DNA complex and also on the process of sequestering of the drug on the DNA surface, which increases the probability of its reaction with nucleophiles. It is relevant to add that the formation of intercalated EPA–DNA complex may go through several stages, e.g., rupturing of hydrogen bonds between DNA base pairs in localized segments of DNA, entry of the drug chromophore at the ruptured sites, and re-formation of the ruptured bonds.[11,12] During these stages several sites on DNA should be available for reaction with the epoxide group in EPA. Since only a small amount of the adduct is isolated in these reactions, it indicates that the epoxide group is away from the sites of intercalation or that there is a relatively rigid stereochemical restraint on the reaction by this function in intercalated EPA. This might make the drug available only at those specific sites (or sequences) in the nuclei where the drug action can be expressed at an optimum level. Our tentative estimate is that only one molecule of EPA per 100 000 base pairs in the cell nuclei can bring about 50% inhibition of growth of CCRF-CEM cells in vitro.[13]

In EPA–DNA adduct only one molecule of the drug is bound to every 500 bases, whereas in the intercalated complex the ratio is one EPA to 32 bases; these ratios for AMD and DHPA in their intercalated forms are about the same as for EPA (Table II). The cytotoxicity values (ID_{50}, CCRF-CEM)[1] are 9 ng/mL for EPA, 85 ng/mL for DHPA, and 60 ng/mL for AMD. Assuming that the other factors, e.g., cellular transport of the agents are similar and that the analogues act primarily via interaction with DNA, the superior activity of EPA should derive from its unique covalent binding with DNA.

The formation of deoxyguanosine adducts was studied by complete enzymatic hydrolysis of the EPA–DNA adduct to nucleoside adducts. Although some separation of the EPA–nucleoside adducts can be achieved by Sephadex LH-20 chromatography, our HPLC analysis gave much higher degree of resolution. HPLC analysis revealed three products, all of which were identified as deoxyguanosine adducts with the help of markers, but the exact chemical nature and the conformation of the adducts are not known at this time. In related studies using benzo[*a*]pyrene[18] and aflotoxin[19] in which the biologically active forms are also epoxide ring added species, the major adducts are those that link their chromophores to either the exocyclic 2-amino group[20] or the 7-nitrogen[19] in the guanine ring of deoxyguanosine. In addition, the nucleoside adducts can be present in the diastereoisomeric forms that can be resolved by HPLC.[16] On the basis of the consideration of racemic starting EPA and the number of possible sites for covalent bond formation, the production of multiple deoxyguanosine adducts here can be rationalized.

In the HPLC chromatogram the presence of the isolated free DHPA (the unshaded peak in Figure 2) constitutes about 0.5% of EPA employed. Although the amount is small compared to total EPA, it is significant in comparison to the total amount of the adducts formed, which is about 2.2% of total EPA. This amount of unconjugated DHPA should not survive as a contaminant after the rigorous treatment made for removal of all noncovalently bound drug. It could represent some form of covalent adduct that was dissociated during conversion of DNA adduct to nucleosides adducts and their isolation. One possibility is that it could originate from some O-alkylated adduct.[16] In some cellular systems, transient demethylation of O-methylated adducts has been observed,[16] but to the best of our knowledge there is not reported instance of this process for in vitro DNA systems; however, a similar observation of a free hydrolytic product from benzo[*a*]pyrenediol epoxide after reaction with in vitro DNA has been reported.[21] Very recently, two ribonucleoside adducts of N^2-methyl-9-hydroxyellipticine substituted at the 2′-O position of ribose have been reported.[22]

From the absence of the peak of 7-hydroxy-AMD (**2b**) in our chromatogram (Figure 2), the possibility of the de-O-alkylation of the EPA can be ruled out.

Conclusion

The adducts formed by reaction of EPA with calf-thymus DNA are characterized as deoxyguanosine adducts. Further studies including ^{13}C NMR and circular dichroic spectral investigations are necessary to establish the exact structures and conformations of these adducts. The present studies using in vitro DNA provide the basis for our future studies with cellular DNA. Evidence of for-

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(13) We take an opportunity to make an approximate estimate of the specificity of binding EPA to CCRF-CEM (CEM) cell nuclei: The average cell volume of a CEM cell is 574×10^{-15} L. The volume of 5×10^5 cells is 2.8×10^{-4} mL (half a million cells were used per milliliter of medium in CEM ID_{50} experiments). ID_{50} value of EPA in CEM cells is 9 ng/mL, i.e., 6.4 nM. DNA content in 0.5×10^6 cells is ~ 2.5 μg[14] $\simeq$ 75 nmol (P); therefore, the calculated amount of base pairs in 0.5×10^6 cells $\simeq$ 37.5 nmol. Octanol–water partition coefficient of EPA is 96.0 and in cell to medium partition is 97.1 (2 h), which means that after 2 h of incubation the cells have 323-fold higher concentration than in the medium. Half a million cells pick up only 9.56% of the drug present in the medium; i.e., drug concentration at ID_{50} inside cells (in 1 mL of experimental medium) is 0.590 pmol. Approximately 65% of this is distributed in nuclear compartment (the rest is in the cytosol).[15] Therefore 0.383 pmol of EPA is distributed over 37.5 nmol of base pairs in cell nuclei. On the basis of this estimate, only one molecule of EPA is bound to about 98 000 base pairs (which effects 50% inhibition of growth of CEM cells; for 100% inhibition, the concentration needed is 180 ng/mL, by extrapolation).
We also found that after 4 h of incubation in drug-free medium, 60% of intracellular EPA taken up (after 2 h of incubation in drug medium) was still inside CEM cells, while only 20% of AMD is found to be retained at the same time.[15]
Retention of AMD is practically nil in AMD-resistant P388/ADR cells; only 2–5% of AMD is retained (4 h) and compared to this over 40% EPA is retained; it may be a major reason why EPA is a more effective agent in P388/ADR leukemia.[15]
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mation EPA–DNA adducts with cellular DNA, both in vitro and in vivo, and comparison with the ones generated in these studies and the future studies will contribute to the understanding of biochemical pharmacological behavior of EPA in in vivo systems. We are hopeful that the products formed with the deoxyribonucleosides will provide additional markers that can be used for comparison with those found in the in vivo investigations.

Experimental Section

Actinomycin D (batch no. NCS 3053, lot L55461-0-10) was obtained from the National Cancer Institute. The nucleotides and dinucleotides were purchased from Collaborative Research, Watertown, MA, and the ^{14}C-labeled nucleotides from New England Nuclear, Boston, MA.

[^{3}H]EPA (50 mCi/mmol) and [^{3}H]DHPA (50 mCi/mmol) were synthesized from [^{3}H]AMD (14 Ci/mmol, Amersham). Radioactivity in [^{3}H]AMD was located exclusively in the methyl groups of both the chromophore and the peptide lactone residues of AMD and were not exchangeable with aqueous or organic solvents. The samples were >98% pure as determined by HPLC. Calf thymus DNA, protein free, type I (Sigma Chemical Co., St. Louis, MO, further purified by phenol extraction), deoxyribonuclease I (P.L. Biochemicals, Milwaukee, WI), snake venom phosphodiesterase (P.L. Biochemicals), and bacterial phosphodiesterase type III (Sigma Chemical Co.) were used.

Sephadex LH-20 (particle size 25–100 μm) was obtained from Pharmacia Fine Chemicals. Glass distilled water was used in all cases. Methanol and acetonitrile were HPLC grade; all other solvents were spectral and reagent grade. All reactions were done in the absence of light and subsequent procedures were performed in subdued light.

Thin-layer chromatography was done on silica gel plates from E.M. Laboratories, Inc. Solvents use were (A) butanol–formic acid–water (75:13:12), (B) ethyl acetate–acetone (3:1), and (C) Cifferi, the organic phase of ethyl acetate–methanol–water (20:1:20). Buffer A is 20 mM Tris-HCl, pH 7.4. Buffer B is 20 mM Tris-HCl (pH 7.8), 0.1 M NaCl–0.5 mM $MgCl_2$.

Varian MCH-10 octadecylsilane C_{18} reversed phase column (4.0 × 300 mm) fitted with a guard column (Supelco guard column) in a Varian Model 5020 gradient liquid chromatograph equipped with CD-111L chromatography data system were used for HPLC analysis. UV–visible spectra and absorbances were determined on a Gilford 250 spectrophotometer using 1- and 10-cm path length cells.

Specific activity was determined in scintillation vials with liquiscint scintillation mixture (National Diagnostic, NJ). The cocktail was allowed to stand for 16 h before measurement in a Packard Tri-Carb Model 3375 scintillation spectrometer.

Analysis of Covalent Binding with DNA. Preparation of DNA–Drug Adducts. Tritiated drugs [^{3}H]EPA, [^{3}H]DHPA, and [^{3}H]AMD (all 50 mCi/mmol) were used in these experiments. Two sets of reactions were carried out. In the first set, one of each tritiated drug (14 μM) and purified calf thymus DNA (140 μM) were mixed in 3 mL of buffer A. In the second set one of each nontritiated drug and also EPAMDEA (140 μM) was mixed with the above DNA (1.4 mM) in 30 mL of buffer A. The mixtures were incubated at 37 °C for 6 h. At the end of the reaction the mixtures were chilled and adjusted to 0.1 M NaCl, and DNA was precipitated with 2.5 times the volume of ethanol. The solutions were allowed to stand at −20 °C in the dark for 16 h. Next, the precipitates were pelleted in siliconized glass tubes (centrifugation at 10000 rpm, 45 min) and the supernatants were separated from the pellets. The supernatants were concentrated by blowing N_2 to one-third of their original volumes and were extracted with EtOAc (water saturated) until no more drugs were extractable when examined by either radioactivity or HPLC. After evaporation of EtOAc, residues were analyzed by HPLC for the amounts of unbound drugs.

DNA pellets from the above were dialyzed with use of Amicon PM-10 filters and 7.0 M NaCl solution until the diffusates were free from any drugs that were dissociated from the DNA in this process. The diffusates were lyophilized, extracted with MeOH, and analyzed by HPLC for the drugs that were bound by intercalation. Alternatively, aliquots of the DNA pellets were analyzed, before dialysis, to estimate the amounts of drugs bound both intercalatively and covalently by using either A_{260}/CPM(drug) and A_{260}/A_{480} values, after appropriate corrections were made for A_{260} from A_{480} and A_{465} values (vide infra). The DNA fractions in the dialyzates were redialyzed in buffer A and the [DNA]/[drug] ratios of covalently bound drugs, fractions B (Table II), were determined from these fractions.

The fractions that showed relatively high ratios of [drug]/[DNA] were redialyzed in a Paula's apparatus[5] using UM-10 membrane (Amicon) in 10 mM phosphate buffer containing 5 M urea; this treatment further ensured complete separation of the strands in DNA and helped to dissociate all the intercalatively bound drugs from the DNA phase. DNA's from these dialyzates were redialyzed, twice with buffer A, and the combined diffusates from all the dialysis were analyzed to estimate any residual drugs that were bound intercalatively and had escaped dissociation from DNA in the previous operations. After adjustment the final ratio of A and B (Table II) were estimated.

The DNA fractions from the last dialyzates were dissolved in minimum of buffer A and stored at 4 °C in the dark. Aliquots were analyzed for [DNA] at A_{260} and [drug] by CPM (scintillation spectrometry) and at A_{465}. The remainder of these samples were used for the preparation of the deoxyribonucleoside adducts (see the section below).

In order to examine the relative stability of EPA and DHPA, 5 mL of 140 μM solutions of each nontritiated drug containing 1.4 mM DNA was processed and analyzed as above at 2, 4, 6, 17, and 44 h. AMD was used as the standard, and drug solutions (no DNA) were used as controls. Analyses were performed only to the extent needed to determine stability and the rates of transformation, if any, in these systems. The experimental results were used to design the above DNA-binding experiments.

Enzymatic Hydrolysis of EPA–DNA Adduct. Approximately 35 A_{260} units of EPA-modified and 3.5 units of [^{3}H]-EPA-modified DNA were used in two separate experiments. Adducts in buffer B (4 mL) were digested with (i) 50 units/mL of DNase I for 5 h at 37 °C; (ii) one-tenth volume of 1.0 M Tris-HCl (pH 7.9) was added and digestion was continued for 44 h with the addition of 5 units/mL of phosphodiesterase (snake venom); (iii) the resulting deoxyribonucleotides were converted to deoxyribonucleosides by addition of 3 units/mL of alkaline phosphatase (bacterial), and incubation was continued for 3 days. To separate adducts from deoxyribonucleosides, the digests were added to a Sephadex LH-20 column (0.8 × 15 cm) which had been preequilibrated with water. The column was first washed with water to remove unmodified deoxynucleosides, enzymes, and salt and finally with 50–80% MeOH (v/v) to elute EPA–deoxyribonucleoside adducts (EPA–dG). The methanolic fractions eluted >95% of the radioactivity of EPA that was applied on the column. These methanolic eluents were concentrated under reduced pressure. Volumes were adjusted to a minimum in MeOH. For analysis, aliquots were injected on MCH-10 octadecylsilane 4.0 × 300 mm HPLC column (Varian Associates) using a concave gradient of 65–90% water–methanol at a flow rate of 1 mL/min for 180 min. The eluates were monitored at dual wavelengths 260 and 465 nm (λ_{max} for EPA, DHPA, and EPA–dG adducts; see the next section). Fractions were collected (0.5 min) and were quantitated by scintillation spectrometry. The peaks were identified by comparison with authentic material and also standard deoxyribonucleoside–EPA adducts synthesized for this purpose (next section). Identities were confirmed, in every case, by cochromatography and peak augmentation techniques. In the case of the major peak material, the identification was further extended to the acetylated products of standard vs. sample.[9] Quantitation were also accomplished, whenever possible, with the help of a HPLC-CDS-111L data system (Varian). Identity of all adducts as 1:1 covalent conjugates of EPA and nucleoside base was made initially on the basis of A_{465}/A_{260} and CPM(drug)/A_{260} ratios after adjustments and finally by comparison with standards and their ^{3}H-labeled drug/^{14}C-labeled nucleoside values (see the following section).

Reaction of 5′-dGMP, 5′-dCMP, and 5′-dAMP with EPA. Formation of EPA–Deoxyribonucleoside Adducts. [U-^{14}C]dGMP, [U-^{14}C]dAMP, and [U-^{14}C]dCMP were diluted with the corresponding nonradiolabeled materials to 50 μCi/mmol for each. Three separate experiments were carried out, one containing

nonradiolabeled drugs and nucleotides, and second containing triated EPA (100 μCi/mmol) and nonradiolabeled nucleotides, and the third containing ^{14}C-labeled nucleotides and [^{3}H]EPA; each reaction mixture had EPA (140 μM) and a nucleotide (140 mM) in buffer A. The reactions volumes were 50 mL in experiment 1, 5 mL in experiment 2, and 2 mL in experiment 3. The mixtures were incubated at 37 °C for 14 h. The samples were extracted with water-saturated EtOAc, and the aqueous fractions were chromatographed on a Sephadex LH-20 column as in the previous experiments. Unreacted nucleotides and nucleosides (occasional contaminants) were eluted 20 mM NH_4HCO_3 buffer. With use of a linear gradient of 20% MeOH–20 mM NH_4HCO_3 to 90% MeOH–20 mM NH_4HCO_3, the adducts were eluted with 50% MeOH–buffer. Treatment with alkaline phosphatase (bacterial) followed by chromatography of the digests on a Sephadex LH-20 column separated the nucleoside adducts from the enzymes and the free drug (DHPA). As before, successive elutions, first with water (to remove enzymes) and then with 20% MeOH–H_2O (v/v) and 50–80% MeOH–H_2O in a linear gradient, eluted all the drug–deoxyribonucleoside adducts. The solvents were removed under reduced pressure and the adducts were analyzed by HPLC (previous experiment in Figure 2). The specific activity ratios ($^{3}H/^{14}C$) and/or the UV absorbance ratios (A_{465}/A_{260}) were employed to calculate the molar ratios of the drug and nucleoside in the adducts. The molar extinction values of the adducts, which were determined from the specific activities, were found to be $\epsilon_{260} = (2.8 \pm 0.25) \times 10^4$ and $\epsilon_{465} = (1.6 \pm 0.2) \times 10^4$ M^{-1} cm^{-1}, respectively. These values affirm that one EPA is bound to one nucleoside molecule and that the chromophore of the drug is not stacked on the nucleoside base.

In order to examine the stability of EPA in the presence of nucleotides, solutions of EPA, with or without the nucleotides, were incubated for times as in the previous experiment. Actinomycin D was used as the standard in these reactions. In the above experiments the free drugs, EPA (when it is not completely hydrolyzed to DHPA) and DHPA, are eluted from the columns in 20% MeOH–H_2O.

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Trequinsin, a Potent New Antihypertensive Vasodilator in the Series of 2-(Arylimino)-3-alkyl-9,10-dimethoxy-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinolin-4-ones

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Series of 3-substituted-9,10-dimethoxy-3,4,6,7-tetrahydro-2*H*-pyrimido[6,1-*a*]isoquinoline-2,4-diones and 2-substituted-9,10-dimethoxy-6,7-dihydro-4*H*-pyrimido[6,1-*a*]isoquinolin-4-ones were synthesized and tested for blood pressure lowering properties in anesthetized normotensive cats and conscious spontaneously hypertensive rats. Several compounds in the 2-(arylamino)-9,10-dimethoxy-6,7-dihydro-4*H*-pyrimido[6,1-*a*]isoquinolin-4-one series display a high order of activity. The most active compounds are the alkyl derivatives of the 2-mesitylamino/2-mesitylimino tautomeric forms. The 2-(mesitylimino)-3-methyl analogue trequinsin is a potent antihypertensive agent and displays a hemodynamic profile characteristic of an arteriolar dilator. It is also a potent inhibitor of both cAMP phosphodiesterase and platelet aggregation.

Vasodilators in current use for the clinical management of hypertension are of two types: (1) receptor- or enzyme-dependent agents, such as the peripheral postsynaptic α_1-adrenoceptor blocking agents prazosin and indoramin, and the angiotensin converting enzyme inhibitor captopril and (2) the direct acting agents on arteriolar smooth muscle, such as hydralazine, diazoxide, nitroprusside, and minoxidil.[1] The attribute of these antihypertensive vasodilators that distinguishes them from other classes of antihypertensive agents, such as β-adrenoceptor blocking agents, centrally acting drugs and adrenergic neuron blocking drugs, is their ability to selectively lower peripheral resistance. They thereby reverse the major hemodynamic abnormality of a markedly elevated systemic vascular resistance that characterizes human essential hypertension. Both types of vasodilators, however, produce similar, although not absolutely identical, side effects that are related to their main hemodynamic action.

The major side effects of the directly acting vasodilators hydralazine, diazoxide, and minoxidil that attenuate their antihypertensive effectiveness are the baroreceptor reflex increases in sympathetic activity that raise cardiac output and produce tachycardia, the augmentation of plasma renin activity, and the retention of sodium with plasma volume expansion.[2] A need has thereby arisen for vasoactive antihypertensives that would lack completely or

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