Biochemical Pharmacology, Vol. 25, pp. 231–236. Pergamon Press, 1976. Printed in Great Britain.

DIACRIDINES - DOUBLE INTERCALATORS AS CHEMOTHERAPEUTIC AGENTS

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(Received 23 October 1975; accepted November 3, 1975)

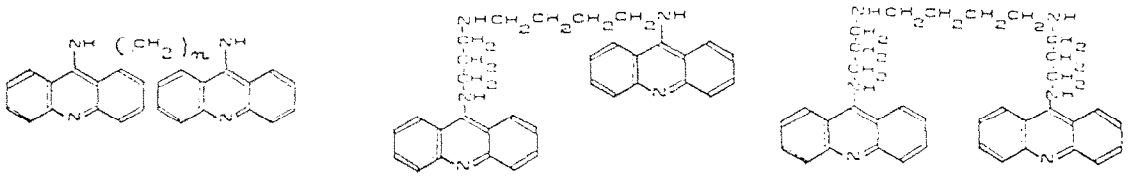
In an effort to develop compounds of chemotherapeutic efficacy which would interfere with the growth of malignant cells, this laboratory has taken advantage of the natural intercalative abilities of acridines (1) and enhanced them by forming diacridines connected by a chain of varying length and composition. The basic rationale has been that if a molecule is composed of two acridine rings, the intercalation of one ring into DNA will automatically bring the second acridine ring into close proximity to the DNA thus permitting it to intercalate in neighboring positions. The expectation is that such a diacridine would act as a 'staple' within the DNA molecule; the DNA could only be free of the diacridine if both acridine rings were to de-intercalate at the same time. Such DNA 'staples' would intercalate with the DNA molecule at positions whose limits would be defined by the length of the chain connecting the two acridine rings.

The nature of the connecting chain is important because it would define not only the ease or steric hindrance to bis-intercalation but also the partition coefficient between lipid and water. A positively charged connecting chain, not only defines the permeability of the cell to the diacridine but, by ionic interaction with adjacent phosphate diester groups of DNA, may confer rigidity to the overall structure and therefore limit the flexibility of interaction of the distal acridine rings with the DNA.

Substituents on the acridine rings play a particularly important role because they would define the possibilities of hydrogen bonding or other interactions with the overlapping DNA base pairs. In addition, substituents on the acridine rings could prevent intercalation, or once intercalated, they could also delay de-intercalation. Consequently, the nature of the ring substituents defines not only the specificity of binding but also the strength of binding.

These various theoretical considerations have lead to the development and the synthesis of a series of more than fifty diacridines of various ring structures with a variety of connecting chains. The simplest general formulation is (I) which consists of two unsubstituted acridine rings connected at the 9-position by a homologous series of a,w, polyamines where n = 2,3,4,5,6,7,8,10,12,14,16,18. A variety of diacridines has also been synthesized with

spermidine and spermine as the connecting chain as shown in (II) and (III) of Fig. 1. These two polyamines were chosen because of the long standing interests of this laboratory (2,3) as well as their reported preferential and specific interaction with A-T sites in DNA (4,5).



I II III

Fig. 1. Basic Structure of Diacridines. I Containing hydrocarbon connecting chain; II Spermidine connecting chain; III Spermine connecting chain.

Many ring substituents have been introduced. The most obvious was the quinacrine ring which has become widely known as the basic ring structure of atebrine and other effective antimalarials. In addition, a variety of 2, 3, and 4 substituents has been introduced and the chemotherapeutic efficacy of the resultant compounds evaluated. These have ranged from methyl, ethyl, propyl to methoxy, ethoxy, propoxy as well as chloro- and other derivatives. The synthesis of this series of compounds will be reported at a later date (6).

The DNA melting curves were obtained using a Gilford 2400-S spectrophotometer, programmed for a temperature rise of 0.5°/min. Calf thymus DNA was a product of Worthington Co. T7 RNA polymerase was prepared and assayed according to Niles et al. (7).

Inhibition in vivo of nucleolar RNA synthesis was measured in exponentially growing HeLa S_3 cells maintained in suspension culture as previously described (8), using Joklik modified MEM, 10% FCS and 2 mM L-glutamine (GIBCO). Control cells were introduced with 8-^{14}C-adenosine (51.2 mCi/mM; 0.2 Ci/ml) for 1 hr. After preincubation with drug for 1 hr, drug-treated cells were incubated for 1 hr with 2,8-^{3}H-adenosine (32.4 mCi/mM; 4 Ci/ml). Incorporation was terminated by addition of the cells to frozen isotonic saline; equal numbers of control and drug-treated cells were mixed and the nuclei isolated as detailed below.

Cells were lysed and nuclei isolated by two washes in 0.5% NP-40 solution (140 mM NaCl; 10 mM Tris-HCl, pH 8.4, 1 mM MgAcetate). Nuclei were detergent treated and the nucleolar fraction was isolated as previously described (9). RNA was extracted with phenol (70%) : $CHCl_3$ (1% isoamyl alcohol) (50:50) at pH 9 by a modification of the procedure of Lee et al. (10). RNA was collected by ethanol precipitation (95%, 2 vol., -20° 1 hr) and analyzed on 12%-30% sucrose gradients (100 mM NaCl; 10 mM Tris-HCl, pH 7.4, 10 mM EDTA) in a Spinco SW-40 rotor, 11 hr, 23,500 rev/min, 17°. Fractions were collected by tube puncture and measured as TCA precipitable counts on 3 mm filter discs. For the 45S RNA processing experiments, the labeling protocol was modified to fit that described by Reichman et al. (11).

For the drug uptake studies, cells were incubated in drug-containing media for 30 min. After washing, they were lysed and the nuclei isolated and lysed as described above. Each of these fractions was saved, made 1-3% sodium dodecyl sulfate and the absorbance measured at the peak wavelength for each compound. Internal cell concentration was calculated using the value 0.004 ml/10^6 cells.

The definition of the assays to be used was of particular importance because they would define the effectiveness of the compounds and the direction of the subsequent syntheses. For a physical-chemical probe we used the melting of DNA at various salt concentrations as

Table I

Comparison of 9-amino acridine (9-AA) and of Octyldiamine diacridine (C-8)

Assay	Conditions	Results	
1. Effect on Tm of DNA			
	Drug: DNA-P	Tm in 0.1 SCC	Tm in 1.0 SCC*
9-AA	1 : 10	17°	0°
C-8	1 : 10	45°	12°
2. Cellular Uptake of Drugs			
	Drug Conc. in Medium (M)	Intracellular Conc. (M)	Nucleus:Cytopl.
9-AA	1×10^{-5}	1.06×10^{-3}	0.2
C-8	1×10^{-5}	2.4×10^{-2}	1.9
3. Inhibition of Synthesis of 45S RNA			
	Drug Conc. in Medium (M)	Inhibition Per Cent	
9-AA	5.0×10^{-6}	92	
C-8	0.5×10^{-6}	86	
4. Effect on Processing of 45S RNA			
	Drug Conc. in Medium (M)	Effect	
9-AA	1×10^{-5}	Promotes Destructive Processing	
C-8	1×10^{-5}	Inhibits Processing-45S RNA Intact	
5. Inhibition of Transcription of T7 DNA by T7 RNA Polymerase			
	Drug Conc. in Medium (M)	Inhibition Per Cent	
9-AA	7.0×10^{-5}	50	
C-8	1.0×10^{-5}	50	

*Standard Saline Citrate: 0.145M NaCl - 0.0145M Na_3 Citrate.

an indication of the strength of binding. Also the melting of complementary DNA and RNA synthetic homopolymers was used as an indication of the specificity of intercalation. In addition, electric dichroism has been used to distinguish double or single intercalation. The biological assays involved tests on CDF1 mice, bearing P-388 leukemias and evaluating prolongation of their life. However, since these compounds have been found to inhibit RNA synthesis primarily, and since they interact not only with DNA but also with RNA, it became apparent that the determination of the inhibition of transcription, as well as of the processing of RNA, should be important sites for assay.

The results presented above indicate that octyldiamine diacridine (C-8), which has a long enough connecting chain to permit double intercalation, raises the Tm of DNA in 0.1 SSC by 45° while the parent compound raises it only 17°. At higher salt concentrations, 1.0 SSC, 9-amino acridine (9-AA) has no effect on the Tm of DNA, while C-8 raises it by 12°. The salt dependence of intercalation of the acridines has been noted previously and intercalation of acridines with DNA is usually studied in high salt dilutions (12); however, it is interesting to note that in isotonic salt the diacridines do affect the Tm of DNA. The remarkable extent to which the diacridines are taken up by cells is indicated by the attainment of an intracellular concentration of over 2×10^{-2} M C-8 after 30 minutes exposure of HeLa cells to 1×10^{-5} M C-8 in the medium; in addition, there appears to be a preferential concentration of C-8 in the nucleus, while 9-AA remains in the cytoplasm. This intranuclear concentration of C-8 can be calculated to be approximately 10^{-1} M. The significance of this can be appreciated from assay 5 of Table I which indicates that 1×10^{-5} M C-8 is adequate for 50% inhibition of an in vitro DNA dependent RNA polymerase reaction. This assay as well as assay 3 shows that C-8 is approximately 7 to 10 fold more effective in inhibiting RNA synthesis.

Besides the quantitative differences which are noted, there are also qualitative differences. 9-Amino acridine promotes the degradation of 45S RNA while C-8 inhibits its further processing. The implication is that C-8 in binding to 45S RNA modifies the sites where nucleolytic activity would normally occur and makes them resistant to enzymatic scission and to their subsequent conversion to 32S RNA. In contrast, 9-amino acridine appears to interact either with the 45S or the 32S RNA in a manner that promotes the extensive degradation of the RNA, so that only a small proportion of the anticipated nucleolar RNA can be found. In addition, C-8 is an effective chemotherapeutic agent when tested against the P-388 leukemic cells providing appreciable prolongation of the life of CDF1 mice when compared to 9-AA (80% vs. 15%).

Our more extensive studies, with a larger number of these compounds, indicate the individual fingerprints that each compound leaves on the cell, whether this is on the uptake of the compound, the inhibition of processing of RNA, the mechanism of intercalation with DNA,

or the inhibition of methylation of RNA. In considering these compounds as chemotherapeutic agents we should take into account that atebrine, which is concentrated to a great extent in the liver, lungs and epithelial cells has been used effectively for many years with no reported adverse effects.

Although this work was independently conceived of and formulated it should be recognized that a series of di-chloroquines similar to the series of diacridines has been described (13) and that recently a series of diacridines conceptually similar to these developed by our laboratory has been reported (14).

In closing this preliminary report it should be stated that this work was greatly aided both in its chemistry as well as in its biological chemistry by the excellent treatise of Albert (15).

ACKNOWLEDGMENTS

This research was supported in part by Contract NO1-CM-12339 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare; in part by Grant No. 1 RO1-CA-15317 from USPH Service and in part by a USPH Service Research Career award to E.S.C., No. 5 KO6-GM-03070.

The authors wish to thank Ms. M.A. Hare for her excellent assistance.

REFERENCES

1. L.S. Lehrman, J. Mol. Biol. 3, 18 (1961).
2. S.J. Friedman, K.V. Halpern and E.S. Canellakis, Biochim. Biophys. Acta 261, 181 (1972).
3. W.T. Beck, R.A. Bellantone and E.S. Canellakis, Nature 241, 275 (1973).
4. A.M. Liquori, L. Constantino, V. Crescenzi, V. Elia, E. Giglio, R. Puliti, M. Desantis-Savino and V. Vitagliano, J. Mol. Biol. 24, 113 (1967).
5. M. Suwalsky, W. Traub, U. Shmueli and J.A. Surirana, J. Mol. Biol. 42, 363 (1969).
6. E.S. Canellakis, Y.H. Shaw, W.E. Hanners and R.A. Schwartz, Biochim. Biophys. Acta, in press.
7. E.G. Niles, S.W. Conlon and W.C. Summers, Biochem. 13, 3904 (1974).
8. H. Eagle, Science 130, 432 (1959).
9. S. Penman, C. Vesco and M. Penman, J. Mol. Biol. 34, 49 (1968).
10. S.Y. Lee, J. Mendecki and G. Brawerman, Proc. Nat. Acad. Sci. USA 68, 1331 (1971).
11. M. Reichman, D. Karlan and S. Penman, Biochim. Biophys. Acta 299, 173 (1973).

12. W. Kersten, H. Kersten and W. Szybalski, Biochem. 5, 236 (1966).

13. V.E. Marquez, J.W. Cranston, R.W. Ruddon and J.H. Burckhalder, J. Med. Chem. 17, 856 (1974).

14. J.B. Lepecq, M. Lebret, J. Barbet and B. Roques, Proc. Natl. Acad. Sci. USA 72, 2915 (1975).

15. A. Albert, THE ACRIDINES, 1966, Publishers: Edward Arnold Ltd., London.