Biochemical Pharmacology, Vol. 26, pp. 269–273. Pergamon Press, 1977. Printed in Great Britain.

DIACRIDINES, BIFUNCTIONAL INTERCALATORS—IV.

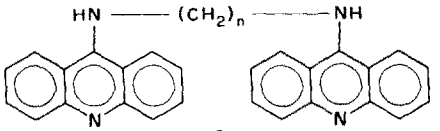
EFFECT ON NUCLEOLAR RNA SYNTHESIS

ROSARIO M. FICO and E. S. CANELLAKIS
Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510, U.S.A.

(*Received* 11 *November* 1975; *accepted* 19 *April* 1976)

Abstract—The inhibition of nucleolar RNA synthesis in HeLa cells by acridines as well as by a series of 9-NH_2 diacridines, connected through their 9-NH_2 groups by hydrocarbon chains from $(CH_2)_2$ to $(CH_2)_{12}$, and by the polyamine chains spermidine and spermine, has been investigated. Nucleolar RNA synthesis in HeLa cells is most sensitive to the longer chain diacridines, indicating that double intercalation may be essential for their action. These data are in agreement with the action *in vitro* of these compounds. However, if the degree of inhibition of nucleolar rRNA synthesis is related to the nuclear uptake, the single intercalators appear to be more effective inhibitors.

We have previously reported on the synthesis of diacridines [1] and described their action as double intercalators with nucleic acids [1], as well as some of their biological properties [2–4]. These compounds have the general structure I, where the hydrocarbon



I

chain varies in length from $(CH_2)_2$ to $(CH_2)_{12}$. In this paper, we wish to present a detailed report of the effect of this series of diacridines on the inhibition of the synthesis of nucleolar precursor rRNA in HeLa cells. Two polyamine analogues, where the connecting diamine chain is replaced by the naturally occurring polyamines, spermine and spermidine, have also been studied, as well as the parent compounds 9-amino acridine and proflavine, and their relationships to the hydrocarbon analogues were explored.

During the course of this work, it was necessary to measure the uptake and intracellular distribution of these drugs. Some are accumulated to a surprisingly high extent within the cell. Correlation of the uptake and inhibition data has allowed us to delineate the most effective compounds.

MATERIALS AND METHODS

Exponentially growing HeLa S_3 cells, a gift from Dr. Lon Hodge, were maintained in suspension culture as previously described [5], using Joklik modified minimum essential medium, 10% fetal calf serum and 1% L-glutamine (200 mM), all products of Grand Island Biochemical Co. For these experiments, cell concentrations ranged from 4 to 6 × 10^5 cells/ml.

The drugs used in this study were: ethyldiamine diacridine (C_2), butyldiamine diacridine (C_4), hexyldiamine diacridine (C_6), octyldiamine diacridine (C_8), dodecyldiamine diacridine (C_{12}), tetradecadiamine diacridine (C_{14}), sexadecadiamine diacridine (C_{16}), spermidine diacridine and spermine diacridine. To distinguish the effects of drug treatment on RNA synthesis, control cells were incubated with [8-^{14}C]-adenosine (51.2 mCi/m-mole, 0.2 μCi/ml, New England Nuclear Corp.), while drug-treated cells were incubated with [2,8-^{3}H]adenosine (32.4 mCi/m-mole, 4.0 μCi/ml, New England Nuclear Corp.). Cells were incubated for 1 hr in the absence or presence of the drug, after which time the labeled precursor was added to the respective flasks and the incubation continued for an additional hr. Incorporation was terminated by addition of the cells to frozen isotonic saline. At this time, equal numbers of control and drug-treated cells were mixed before proceeding to the next step.

Purified nuclei were prepared as follows. Washed cells were lysed by suspension in 140 mM NaCl, 10 mM Tris–HCl, pH 8.4, and 1 mM magnesium acetate containing 0.5% NP-40 (Nonidet detergent, Shell Oil, England). After a second NP-40 wash, the nuclei were treated with 1% Tween 40 and 0.5% deoxycholate in RSB buffer (10 mM NaCl; 10 mM Tris–HCl, pH 7.4; and 1.5 mM magnesium acetate) as described by Penman [6].

Nucleoplasmic and nucleolar fractions were isolated after nuclear lysis with high salt buffer containing DNAase as previously described [7] except that the lysate was centrifuged through a cushion of 25% sucrose in high salt buffer for 15 min, 0° at 28,000 g_{av} in a Spinco SW-40 rotor. The supernatant containing the nucleoplasmic fraction was carefully removed and the RNA precipitated by the addition of 2 vol. of 95% cold ethanol. This solution was kept at least 1 hr at −20° before collecting the precipitate at 1000 *g*. The precipitate was resuspended in 5 ml SDS buffer (0.5% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris–HCl, pH 7.4, and 1 mM EDTA). The pellet from the original centrifugation comprises the nucleolar fraction and was dissolved in 5 ml SDS buffer by heating at 37° for 30 min. The RNA was extracted from these two fractions by the following method developed in collaboration with Dr. S. Y. Lee, Brown University. An equal volume of phenol–H_2O (70:30)

was added to the samples, which were then heated at 56° for 5 min. An equal volume of $CHCl_3$–isoamyl alcohol (99:1) was added and the samples were reheated at 56° for 5 min. Samples were then cooled to 0°, and the pH was adjusted to 9 with 1 ml of 1 M Tris–HCl, pH 9, and kept at 0° for 15 min. The two phases were separated by centrifugation (1000 *g*, 4 min) and the lower organic layer was removed. The aqueous layer plus the interphase was extracted twice more with one half the original volume of the phenol and $CHCl_3$ solutions. After each extraction, the organic layer was re-extracted with the same 2.0 ml, 10 mM Tris–HCl, pH 9, solution. The aqueous phase and the wash solution were combined and the RNA was precipitated by adding 2 vol. of 95% ethanol at −20° and leaving the mixture at −20° for at least 1 hr. sRNA (0.6 mg, Nutritional Biochemicals Co.) was added as carrier. The precipitate was collected by centrifugation at 1000 *g* for 15 min and redissolved in a small volume of H_2O. RNA was analyzed by centrifugation in a 12–30% sucrose gradient containing 100 mM NaCl, 10 mM Tris–HCl, pH 7.4, and 10 mM EDTA, for 11 hr at 17° in a Spinco SW-40 rotor at 69,500 g_{av}. Individual fractions were collected by piercing the bottom of the tube and assayed on 3 MM filter discs (Whatmann) as trichloroacetic acid-precipitable counts.

In order to determine the uptake of the drugs into the cells, the above procedure was modified as follows: equal numbers of cells were incubated for 30 min at 37° with 1×10^{-5} M drug, then washed and lysed as described above. A single NP-40 lysate was put aside as the cytoplasmic fraction. The nuclei were further purified as above with detergent and the detergent wash was saved as an intermediate fraction; the remaining purified nuclear pellet was lysed as above and designated the nuclear fraction. All three samples were brought to 1–3% SDS, and spectrophotometric scans in the visible region were made on a Beckman DB-G spectrophotometer. Drug concentrations were measured at the peak wavelengths. In order to compare internal concentrations, the cytoplasmic, intermediate and nuclear fractions were taken to represent, respectively, 40, 20 and 40 per cent of the cell volume, as best approximated from EM photomicrographs.

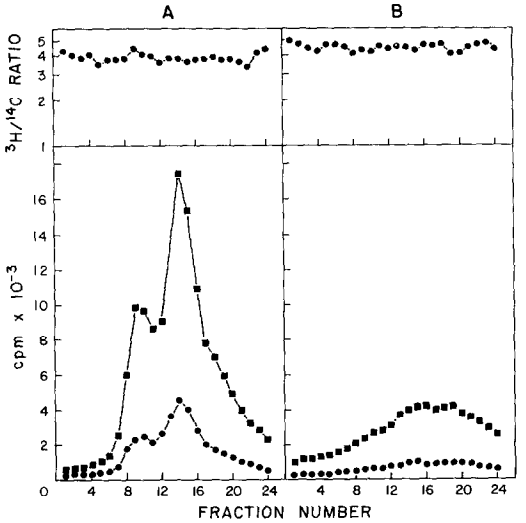


Fig. 1. Sucrose gradient analysis of control nucleolar and nucleoplasmic RNA. HeLa cells, in the absence of drug, were incubated for 1 hr with either [^{14}C]adenosine or [^{3}H]adenosine. Nucleolar and nucleoplasmic RNA were isolated and analyzed on 12–30% sucrose gradients as described in Materials and Methods. (A) Control nucleolar RNA: ●——●, ^{14}C; and ■——■, ^{3}H. Upper curve: ●——●, $^3H/^{14}C$ ratio. (B) Control nucleoplasmic RNA: ●——●, ^{14}C; and ■——■, ^{3}H. Upper curve: ●——●, $^3H/^{14}C$ ratio.

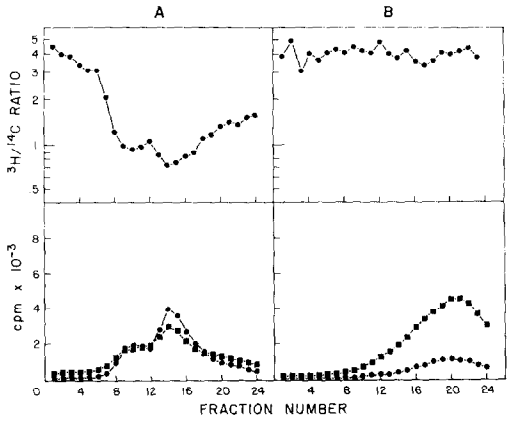


Fig. 2. Sucrose gradient analysis of drug-treated nucleolar and nucleoplasmic RNA. Same as in Fig. 1 except that cells were preincubated in the presence of 1×10^{-6} M dodecylamine diacridine. (A) Nucleolar RNA: ●——●, ^{14}C control; and ■——■, ^{3}H drug-treated. Upper curve: ●——●, $^3H/^{14}C$ ratio. (B) Nucleoplasmic RNA: ●——●, ^{14}C control; and ■——■, ^{3}H drug-treated. Upper curve: ●——●, $^3H/^{14}C$ ratio.

RESULTS

Figure 1 is typical of the distribution of radioactivity in the nucleolar and nucleoplasmic RNA obtained from a control experiment and is in agreement with other published results [7]. The nucleoplasmic fraction B consists of a heterogeneous mixture of RNA ranging in size from 60S to 5S with a broad peak from 10S to 30S. The nucleolar fraction A is composed of principally two rRNA precursor molecules, nominally defined as 45S and 32S [7]. Absence of the mature rRNA species, 28S and 18S, attests to the relative purity of this nuclear fraction [7]. Linearity of the $^3H/^{14}C$ ratio, upper curve, points out the internal consistency of our procedure, since random losses of RNA would give an erratic line. Variations in the $^3H/^{14}C$ ratio from control values indicate a change in the relative amount of RNA in that particular fraction. Since the [^{14}C]RNA (control) remains constant, any changes must be in the drug-treated RNA.

Figure 2 is an example of a drug-treated sample, the drug being dodecyldiamine diacridine, C_{12}. The nucleoplasmic fraction B parallels the control throughout the gradient, and examination of the $^3H/^{14}C$ ratios indicates only slight inhibition. These results are similar to those obtained with the parent compound, 9-amino acridine, as well as with actinomycin D, in that these compounds all inhibit the synthesis of nucleoplasmic RNA to a much smaller

Table 1. Inhibition of nucleolar RNA synthesis by diacridines*

Drug	I_{50} concn ($\times 10^{-6}$ M)
Octyldiamine diacridine (C_8)	0.04
Spermidine diacridine (C_8 analogue)	0.09
Hexyldiamine diacridine (C_6)	0.18
Dodecyldiamine diacridine (C_{12})	0.32
Proflavine	0.34
Butyldiamine diacridine (C_4)	0.64
9-Amino acridine	0.76
Spermine diacridine (C_{12} analogue)	1.50
Ethyldiamine diacridine (C_2)	5.10

* RNA was analyzed as in Figs. 1 and 2. The change in the $^3H/^{14}C$ ratios in the 45S + 32S region of the gradient was measured and used for calculating the I_{50}. Control ^3H incorporation in the 45S–32S region was 10,000–18,000 cpm (see Fig. 1). For comparison, actinomycin D (2.4×10^{-9} M) gives 40 per cent inhibition under these conditions.

extent than the synthesis of nucleolar RNA. In this study, inhibition of nucleoplasmic RNA never exceeded 25 per cent and was usually much less.

45S and 32S RNA are the precursor rRNA molecules known to be exclusively synthesized in the nucleolus [7]. As seen in Fig. 2A, RNA synthesis in the nucleolar fraction is significantly inhibited. We have taken the change in the ratios in the 45S–32S region of the gradient as an index of inhibition of nucleolar RNA synthesis. The concentration giving 50 per cent inhibition (I_{50}) for the individual compounds has been established and is given in Table 1 in decreasing order.

As seen in this table, inhibition of nucleolar RNA synthesis increases with chain length, reaching a maximum at octyldiamine diacridine, C_8, and decreasing thereafter. These longer chain compounds are 2- to 20-fold better inhibitors than the parent compound, 9-amino acridine. The polyamine analogues also follow this trend in that spermidine diacridine, the C_8 analogue, is more effective than spermine diacridine, the C_{12} analogue. However, addition of the nitrogen to the chain reduces their effectiveness as inhibitors of nucleolar RNA synthesis when compared to their respective hydrocarbon analogues.

As these experiments progressed, it became apparent that some of these compounds accumulated within the cell significantly more than others. In order to assess the effect this had on the inhibition data, drug uptake studies were undertaken. Table 2 summarizes these results. There is a very dramatic accumulation of these drugs in the cell, especially for the longer chain compounds; the C_8 analogue is concentrated by the cell more than 750-fold over its concentration in the medium. Although this uptake is probably governed by the increase in the hydrophobic nature of the connecting chains, the lower uptake for compounds with chain lengths greater than C_8 suggests that other factors are also involved. As expected, addition of ionizable nitrogens to the chains, in the spermidine and spermine analogues, greatly reduces the uptake.

The longer chain compounds are more effectively taken up, paralleling a similar trend indicated in the inhibition studies. However, when the distribution of these compounds within the cytoplasmic, nuclear wash and nuclear fractions is determined, the results indicate a change in the pattern of uptake (Table 3).

A wide variation exists in the extent of drug uptake among the various cell fractions. The ratio of the drug

Table 2. Total intracellular concentration of diacridines*

Drug	Intracellular concn ($\times 10^{-5}$ M)
Octyldiamine diacridine (C_8)	750
Hexyldiamine diacridine (C_6)	436
Tetradecadiamine diacridine (C_{14})	397
Dodecyldiamine diacridine (C_{12})	392
Sexadecadiamine diacridine (C_{16})	325
Spermidine diacridine	102
Ethyldiamine diacridine (C_2)	92
Butyldiamine diacridine (C_4)	90
Proflavine	85
Spermine diacridine	53
9-Amino acridine	41

* Cells were incubated for 30 min with 1×10^{-5} M drug. The cytoplasmic, nuclear wash and nuclear fractions were isolated and the drug concentration in each fraction was measured as described in Materials and Methods. The sum of the drug in each of these fractions, after correction for cell volume dilution, gives the total intracellular concentration. For these calculations the value 0.004 ml/10^6 cells was used.

Table 3. Relative intracellular distribution of diacridines*

Drug	Cytoplasmic fraction ($\times 10^{-5}$ M)	Nuclear wash fraction ($\times 10^{-5}$ M)	Nuclear fraction ($\times 10^{-5}$ M)	Nuclear Cytoplasmic
C_8	4.1	17.6	29.6	7.2
C_6	1.5	10.6	17.9	11.9
C_{14}	5.8	9.8	11.8	2.0
C_{16}	5.2	7.0	9.7	1.9
C_{12}	6.3	13.8	9.0	1.4
Spermidine diacridine	2.2	1.4	2.9	1.3
Spermine diacridine	1.2	0.8	1.4	1.2
Proflavine	2.7	2.0	1.1	0.4
C_4	2.9	3.4	0.5	0.2
9-Amino acridine	1.4	1.2	0.3	0.2
C_2	3.9	2.4	0.1	0.03

* Same as in Table 2 except that the concentration in each extract is listed separately. Values represent the concentration in the extracts (diluted to the same volume) and not the actual intracellular concentration. Cytoplasmic, nuclear wash and nuclear fractions represent, respectively, 40, 20 and 40 per cent of the cell volume and have been corrected for this.

concentration in the nucleus to that of the drug concentration in the cytoplasm shows that the short chain compounds, C_2 and C_4, as well as the monomers, 9-amino acridine and proflavine, are preferentially accumulated in the cytoplasm. In contrast, the longer chain compounds, which can act as double intercalators, are more highly concentrated in the nucleus. This may be an expression of the higher affinity the double intercalators have for sites within the nucleus. Interestingly enough, C_6 and C_8, the two diacridines which are best concentrated in the nucleus, are also the most effective chemotherapeutic agents against the P-388 mouse leukemia [1].

In order to arrive at a measure of the effectiveness of these drugs as inhibitors of rRNA synthesis, in relation to their uptake, we have performed the following calculations. The per cent inhibition can be defined as $\%\ \text{inh} = k \cdot \text{concn} \cdot E$, where k is an uptake parameter (k = internal concn/external concn), and E is the intrinsic effectiveness of the drug. At 50 per cent inhibition, $k_1 \cdot \text{concn}_1 \cdot E_1 = k_2 \cdot \text{concn}_2 \cdot E_2$. Using the data in Tables 1 and 3, we have calculated the relative nuclear effectiveness of these compounds as compared to C_8. These values, in decreasing order, are given in Table 4. The results indicate that the short-chain single intercalating compounds and monomers are most effective in inhibiting nucleolar rRNA synthesis when the inhibition is normalized for the uptake of these compounds in the nucleus (i.e. 9-amino acridine is 5-fold better than C_8). Within the diacridine series, increases in chain length lead to a reduction in the relative inhibitory activity for rRNA synthesis. Spermidine diacridine appears to be an exception since it shows activity similar to the monomers, although as the C_8 analogue it can act as a double intercalator.

DISCUSSION

These studies offer a unique opportunity to detail the effects of a homologous series of compounds which should presumably have the same or similar sites of action. The basic intercalating structure, the acridine ring, remains the same, while the variables are the length and the composition of the connecting chain.

The length of the connecting chain determines the ability of these diacridines to act as single or double intercalators. Space-filling models indicate that a connecting chain of four carbons, i.e. C_4 or butyldiamine diacridine, is at the limit of single intercalators. Double intercalation could presumably occur from C_6 and on [2].

When comparing the acridines to actinomycin D, we find that despite their differential uptake by the

Table 4. Relative inhibitory effectiveness*

Drug	Relative effectiveness compared to C_8
9-Amino acridine	5.2
Spermidine diacridine	4.4
Butyldiamine diacridine	3.7
Proflavine	3.2
Ethyldiamine diacridine	2.3
Octyldiamine diacridine	1.0
Spermine diacridine	0.6
Dodecyldiamine diacridine	0.4
Hexyldiamine diacridine	0.4

* As described in the text, E_1/E_2, the relative nuclear effectiveness, as compared to C_8, has been calculated using the relationship, $k_1 \cdot \text{concn}_1 \cdot E_1 = k_2 \cdot \text{concn}_2 \cdot E_2$. Values are listed in decreasing order.

nucleus, all the acridines behave like actinomycin D, in that they show minimal inhibition of nucleoplasmic RNA synthesis. However, in the T7 DNA-dependent RNA polymerase system, which initiates and transcribes the seven late cistrons of T7 DNA, they only inhibit chain initiation and have very little effect on chain elongation.* In addition, they bind both to G-C as well as to A-T regions in the DNA [1, 8]. In contrast, actinomycin D does not bind to A-T regions [9], nor does it affect chain initiation in the T7 RNA polymerase system, but only chain elongation [10–12]. This similarity in the site of inhibition of nucleolar RNA synthesis of acridines and actinomycin D, despite the differences in their mechanism of action *in vitro*, should be considered in any interpretation of their mechanism of action on nucleolar RNA synthesis.

The inhibitory effect of intercalators is dependent on their binding to DNA, and it has been shown that double intercalators bind more strongly to DNA than single intercalators [1, 8]. In addition, studies *in vitro* have shown that, among these compounds, the double intercalators have a stronger inhibitory effect on RNA synthesis than the single intercalators when assayed either with T7 DNA-dependent RNA polymerase [2] or with the *Azotobacter vinelandii* RNA polymerase [4]. Furthermore, our experimental data show that with increasing chain length, there is a dramatic increase in the uptake of the double intercalators and that lower concentrations of the C_6 and C_8 diacridines are required in the medium to inhibit rRNA synthesis. Consequently, it was anticipated that those compounds which can double intercalate would show a greater inhibitory effect on RNA synthesis *in vivo*. However, when this inhibition is normalized for the intranuclear concentration of these compounds, the shorter chain, single intercalators are the most effective inhibitors with a decrease in relative activity as the connecting chain lengthens. A possible explanation is that because of the nucleolar environment, the shorter chain compounds have a higher specificity for the site of rRNA synthesis; alternatively, within the nucleus, there may be a variety of sites for acridines to bind. Due to their stronger binding to such sites, the double intercalators may be selectively excluded from the nucleolus.

It should also be noted that we have assumed that inhibition of nucleolar RNA synthesis is only dependent on the nuclear concentration of the drug. The relatively high cytoplasmic to nuclear ratios of the most effective compounds at least suggest that some cytoplasmic controls may exist and may be responsible for the activity of the short-chain compounds.

A better understanding of how these compounds work should lead to the development of more selective drugs. This takes on added importance when it is realized that only the C_6 and C_8 analogues have any appreciable chemotherapeutic effect [1].

Acknowledgements—This research was supported in part by Grant No. 1 R01-CA-15317 from USPH Service and in part by a USPH Service Research Career award to E. S. C., No. 5 K06-GM-03070. The authors wish to thank Ms. M. A. Hare for her excellent assistance.

REFERENCES

1. E. S. Canellakis, Y. H. Shaw, W. E. Hanners and R. A. Schwartz, *Biochim. biophys. Acta* **418,** 277 (1976).
2. E. S. Canellakis, R. M. Fico, A. H. Sarris and Y. H. Shaw, *Biochem. Pharmac.* **25,** 231 (1976).
3. E. S. Canellakis and R. A. Bellantone, *Biochim. biophys. Acta* **418,** 290 (1976).
4. E. S. Canellakis, V. Bono, R. A. Bellantone, J. S. Krakow, R. M. Fico and R. A. Schulz, *Biochim. biophys. Acta* **418,** 300 (1976).
5. H. Eagle, *Science, N.Y.* **130,** 432 (1959).
6. S. Penman, *J. molec. Biol.* **17,** 117 (1966).
7. S. Penman, I. Smith, E. Holtzman and H. Greenberg, *Natn. Cancer Inst. Monogr.* No. 23, 489 (1966).
8. J. B. LePecq, M. LeBret, J. Barbet and B. Roques, *Proc. natn. Acad. Sci. U.S.A.* **72,** 2915 (1975).
9. E. Reich, *Science, N.Y.* **143,** 684 (1964).
10. J. Richardson, *J. molec. Biol.* **21,** 115 (1966).
11. R. Hyman and N. Davidson, *J. molec. Biol.* **50,** 421 (1970).
12. A. Sentenac, E. J. Simon and P. Fromageot, *Biochim. biophys. Acta* **161,** 299 (1968).

*A. Sarris and E. S. Canellakis, manuscript in preparation.