Biochemical Pharmacology, Vol. 26, pp. 275–278. Pergamon Press, 1977. Printed in Great Britain.

DIACRIDINES, BIFUNCTIONAL INTERCALATORS—V. INHIBITION OF PROCESSING OF PRECURSOR rRNA AND INHIBITION OF METHYLATION OF *ESCHERICHIA COLI* tRNA

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 effect of a series of mono- and diacridines on the processing of precursor rRNA in HeLa cells and on the methylation of tRNA from *Escherichia coli* has been investigated. Some of these compounds are potent inhibitors of processing. In addition, at least one compound causes misprocessing of the precursor rRNA to incorrect molecular weight sizes, while another promotes the "non-conservative" processing of the rRNA. The methylation of tRNA is inhibited, to various degrees, by all of these compounds.

In the preceding paper [1], we described the effects of diacridine double intercalators on the synthesis of nucleolar RNA in HeLa cells. Presumably this effect arises due to intercalation with DNA. However, other work from this laboratory has emphasized that these diacridines can also intercalate with model synthetic ribonucleic acids of various sequences [2]. In addition, it has been reported that intercalators can interfere with the processing of precursor 45S ribosomal RNA [3]. Based on these data, it seemed reasonable to assume that diacridines may differentially intercalate with RNA and thereby interfere with various steps in the maturation of RNA. In order to verify this hypothesis, we have evaluated the effects of mono- and diacridines on two distinct steps in RNA maturation: (1) the processing of precursor 45S ribosomal RNA to 32S RNA in HeLa cells, and (2) the methylation of tRNA from *Escherichia coli* by rat liver methylases.

Some of the diacridines are potent inhibitors of the processing of 45S RNA. Furthermore, these diacridines can be distinguished by their discrete additional effects on 45S RNA processing. Under appropriate conditions, some compounds will inhibit processing and at the same time preserve the integrity of the 45S precursor rRNA molecule. At least one compound causes misprocessing of 45S RNA to a heterogeneous mixture of various high molecular weight sizes. In addition, we show that 9-amino acridine, the parent compound, will promote "destructive" processing of 45S precursor RNA, a phenomenon which so far has only been described for compounds which are incorporated into RNA [4, 5].

All of the compounds tested inhibit the methylation of tRNA to some extent. Spermine and spermidine diacridines appear to be the most effective inhibitors of this reaction.

MATERIALS AND METHODS

The structure and synthesis of the diacridines have been described [2]. Culturing of the HeLa cells and the isolation and sucrose gradient analysis of nucleolar RNA were performed as previously described [1]. Drugs used in this study were: 9-amino acridine, proflavine, ethyldiamine diacridine (C_2), butyldiamine diacridine (C_4), hexyldiamine diacridine (C_6), octyldiamine diacridine (C_8), dodecyldiamine diacridine (C_{12}), spermidine diacridine and spermine diacridine.

The processing of precursor rRNA was studied under the following conditions. HeLa cells were incubated for 15 min in the presence of either [8-^{14}C]-adenosine (51.2 mCi/m-mole, 0.4 μCi/ml) or [2,8-^{3}H]-adenosine (32.4 mCi/m-mole, 5.0 μCi/ml) (New England Nuclear Corp.). At this time, actinomycin D was added to a concentration of 0.1 μg/ml to inhibit any further rRNA synthesis [6]. The HeLa cells from the [^{14}C]adenosine samples were harvested at this point. Incubation of the [^{3}H]adenosine samples was continued for an additional 30 min; these HeLa cells were then harvested. At the time of harvesting, the [^{3}H]adenosine- and the [^{14}C]adenosine-treated cells were mixed and the nucleolar RNA was isolated as described above. In experiments where drugs were to be tested, actinomycin D and the drug were added at the same time.

For the methylation studies, rat liver methylases, dependent on exogenous putrescine, were prepared essentially as described by Pegg [7] from female 180-g Sprague–Dawley rats (Charles River Breeding Lab.). [^{3}H]Methyl-*S*-adenosyl methionine (SAM) (11.6 Ci/m-mole, 0.044 μmole/ml; New England Nuclear Corp.) was adjusted to 1 μmole/ml, 25 μCi/μmole with non-radioactive SAM (Sigma Chemical Co.) just prior to use. The reaction mixture, in a final volume of 150 μl, contained: 100 mM Tris–HCl, pH 9, 3.3 mM 2-mercaptoethanol, 20 mM putrescine–HCl, 0.2 mg/ml of *E. coli* K_{12} tRNA (Schwarz/Mann), 0.54 mg/ml of rat liver methylases and 0.13 μmole/ml of [^{3}H]SAM. All drugs were employed at a concentration of 2×10^{-4} M. The reaction mixture was incubated at 37° for 30 min and the reaction stopped by quickly cooling the samples to 4°. Portions were applied on 3 MM cellulose discs (Whatmann) and assayed as trichloroacetic acid (TCA)-precipitable counts.

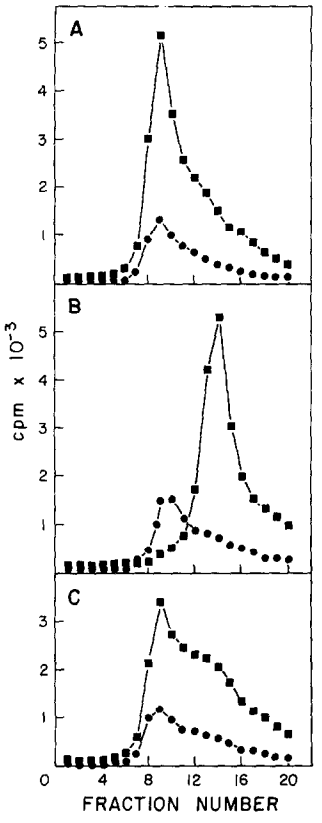

Fig. 1. Inhibition of processing of nucleolar RNA by ethyldiamine diacridine C_2. (A) HeLa cells were incubated for 15 min with [^{14}C]adenosine or [^{3}H]adenosine. (B) HeLa cells were incubated with [^{14}C]adenosine or [^{3}H]adenosine for 15 min. The [^{14}C]adenosine cells were harvested; actinomycin D was added to the [^{3}H]adenosine cells to a final concentration of 0.1 μg/ml and the incubation continued an additional 30 min, at which time the [^{14}C]adenosine and [^{3}H]adenosine cells were combined. (C) HeLa cells were incubated with [^{14}C]adenosine or [^{3}H]adenosine as in B except that at the time of addition of actinomycin D, ethyldiamine diacridine, C_2, was also added to a concentration of 4.5×10^{-5} M. Key: ^{14}C, ●——●; and ^{3}H, ■——■.

RESULTS

Effect on the processing of 45S RNA by mono- and diacridines

Inhibition of processing of 45S precursor RNA. Figure 1A shows the labeling pattern obtained after a 15-min incubation of both the [^{14}C]adenosine control samples and the [^{3}H]adenosine sample. In agreement with published results [3, 4], only the 45S precursor rRNA molecule is labeled during this time. After inhibition of the further synthesis of 45S RNA with actinomycin D, incubation of the [^{3}H]adenosine sample for an additional 30 min leads to the total conversion of the 45S precursor to the 32S stage (Fig. 1B, [3, 4]). Inhibition of this step of processing of 45S to 32S RNA can be easily monitored, since it leads to the retention of some or all of the 45S material in the same position; this is exemplified in Fig. 1C by the effect of ethyldiamine diacridine, C_2, on the processing of the 45S RNA. A survey of various acridines and diacridines gave results indicating similar inhibition or lack of inhibition.

Table 1 summarizes in a qualitative manner the results of this inhibition study. All of the diacridines tested, double and single intercalators connected with hydrocarbon chains, inhibit this processing step to some extent. On the other hand, the two polyamine analogues, i.e. diacridines connected with spermine or spermidine, do not show any inhibition of the processing of the 45S RNA. It should be kept in mind that, in order to study this particular reaction, the concentrations of drug used in the medium are high enough to completely inhibit the *de novo* synthesis of precursor 45S RNA [1]. In addition, although large differences in intracellular concentrations are achieved by these various compounds [1], reference to these results will indicate that the intracellular concentration of the polyamine analogues is at least as high as that attained by the diacridines connected by short hydrocarbon chains, which do in fact inhibit the processing of 45S RNA. Consequently, this lack of inhibition may be considered to be a property of the spermine and spermidine diacridines which differentiates them from the other diacridines studied.

In addition to this difference, other qualitative differences have been observed in the sucrose gradient analysis, which further differentiate the acridines from each other.

"Destructive" processing of 45S precursor RNA. 9-Amino acridine promotes the "destructive" processing of 45S precursor RNA. Figure 2a shows the results obtained with 9-amino acridine. Comparison with the controls in Fig. 1 reveals that the combined area under the two peaks is much less (50 per cent less) than expected, indicating a significant loss of TCA-precipitable material. Similar results, termed "destructive or non-conservative processing," have been reported for 5-azacytidine and toyocamycin [4, 5]. Both of these

Table 1. Effect of mono- and diacridines on the processing of 45S RNA

Drug	Concn ($\times 10^{-5}$ M)	Inhibition of processing*
9-Amino acridine	6.0	Yes
Proflavine	5.0	Yes
Ethyldiamine diacridine (C_2)	4.5	Yes
Butyldiamine diacridine (C_4)	4.0	Yes
Octyldiamine diacridine (C_8)	6.0	Yes
Dodecyldiamine diacridine (C_{12})	4.0	Yes
Spermine diacridine	6.2	No
Spermidine diacridine	6.0	No

* Inhibition of processing of 45S precursor RNA was qualitatively defined by the presence of a residual 45S RNA peak.

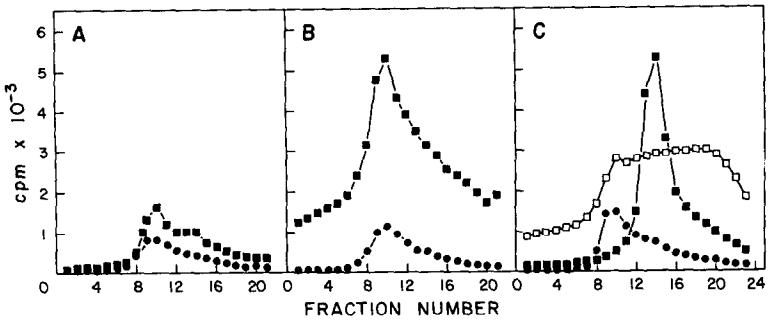

Fig. 2. Sucrose gradient analysis of nucleolar RNA during inhibition of processing. Samples were treated as described in Fig. 1 except that in panel A 9-amino acridine was added to a concentration of 6×10^{-5} M; in panel B, octyldiamine diacridine, C_8, was added to a concentration of 6×10^{-5} M. Key: ^{14}C, ●——●; and ^{3}H, ■——■. Panel C is a reproduction of Fig. 1B on which is superimposed the effect of 4×10^{-5} M dodecyldiamine diacridine, ^{3}H, □——□, to emphasize misprocessing by this compound.

drugs act by being incorporated into the RNA. The result obtained with 9-amino acridine appears to be the first example of non-conservative processing of 45S RNA caused by an unincorporated drug.

Non-degradive inhibition of processing. Octyldiamine diacridine (C_8) inhibition does not permit any extensive degradation of 45S precursor RNA. Figure 2b shows that C_8 is a very effective inhibitor of the processing of 45S precursor RNA. However, it is apparent that it also preserves the 45S RNA from any abnormal degradation, unlike 9-amino acridine or C_{12}.

Misprocessing of 45S precursor RNA. Dodecyldiamine diacridine (C_{12}) promotes the misprocessing of 45S precursor RNA. Figure 2c shows that C_{12} inhibits the processing of the 45S precursor RNA, as evidenced by the residual peak in the 45S region. In addition, the processing that does take place leads to the appearance of populations of polydisperse RNA, rather than the expected discrete 32S RNA peak.

Inhibition of the methylation of tRNA by mono- and diacridines

Methylation is a common step in the maturation of most RNA. Our results, summarized in Table 2, show that the diacridines are potent inhibitors of the methylation of *E. coli* tRNA as catalyzed by rat liver methylases. The most effective inhibitors are the polyamine diacridines in which the hydrocarbon connecting chain is replaced with spermine or spermidine. This is in sharp contrast to their lack of inhibition of the processing of 45S RNA. Among the hydrocarbon chain diacridines, C_6 appears to be the most effective.

DISCUSSION

RNA has a high level of tertiary structure which is crucial for its biological function. The diacridines have a high affinity for synthetic polyribonucleotides [2] and for purified rRNA (unpublished data). Our working hypothesis has been that these compounds bind to RNA, altering its conformation, thereby modifying the normal protein–RNA interaction upon which the biological function of RNA depends.

The finding that these compounds inhibit the processing of precursor rRNA and the methylation of tRNA supports our hypothesis. It can be envisioned that these compounds, in binding to the RNA, modify it to such an extent that the RNA can no longer function as a substrate for these enzymes. In the case of the methylase experiments, both the tRNA and the enzyme are a heterogeneous mixture and it is difficult

Table 2. Inhibition of methylation of *E. coli* tRNA by rat liver methylases*

Drug	Activity† (cpm)	Inhibition (%)
None	4180 ± 96	0
Spermine diacridine	620 ± 64	85
Spermidine diacridine	930 ± 157	78
Hexyldiamine diacridine (C_6)	1240 ± 119	70
Proflavine	2220 ± 100	47
Octyldiamine diacridine (C_8)	2890 ± 31	31
Butyldiamine diacridine (C_4)	3210 ± 98	23
Ethyldiamine diacridine (C_2)	3340 ± 146	20
9-Amino acridine	3860 ± 193	18
Dodecyldiamine diacridine (C_{12})	4430 ± 130	6‡

* Rat liver methylases were isolated and analyzed as described in Materials and Methods. All drugs were at a concentration of 2×10^{-4} M. In the absence of tRNA substrate, a background of 618 cpm was found and the data have been corrected for this.

† Mean ± S. E. M.

‡ C_{12} showed 6 per cent stimulation.

to interpret the results, except to indicate the degree of inhibition of the overall methylation reaction. Additional experiments are planned in order to investigate whether any specificity exists among these compounds for a particular reaction.

The maturation of 45S RNA is a multi-step process and some of the intermediate steps are reasonably well detailed. The effect these compounds have on processing also points out the more subtle modifications of a conformational change. It is reasonable to suggest that the binding of 9-amino acridine significantly alters the structure of precursor rRNA, thereby making it accessible to complete nucleolytic digestion. C_{12} however, apparently only modified recognition sites in such a manner that it allows erroneous but polydisperse nucleolytic products to be made. Neither of these results is observed with C_8; however, the binding of C_8 to 45S RNA appears to produce a sufficient change in the 45S precursor RNA to completely block the processing step. These results are consistent with the concept of a drug–RNA interaction leading to a conformational change. At the same time, the contrasting results observed among the mono- and diacridines imply that some differential specificity exists in their interaction with RNA. These differences in their biological activity suggest their use as tools for the further investigation of the steps involved in the maturation of RNA.

Acknowledgements—This research was supported 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. R. M. Fico and E. S. Canellakis, *Biochem. Pharmac.* **26**, 269 (1977).
2. E. S. Canellakis, Y. H. Shaw, W. E. Hanners and R. A. Schwartz, *Biochim. biophys. Acta* **418**, 277 (1976).
3. A. L. Snyder, H. E. Kann, Jr. and K. W. Kohn, *J. molec. Biol.* **58**, 555 (1971).
4. M. Reichman, D. Karlan and S. Penman, *Biochim. biophys. Acta* **299**, 173 (1973).
5. A Tavitian, S. C. Uretsky and G. Acs, *Biochim. biophys. Acta* **157**, 33 (1968).
6. S. Penman, C. Vesco and M. Penman, *J. molec. Biol.* **34**, 49 (1968).
7. A. E. Pegg, *Biochim. biophys. Acta* **232**, 630 (1971).