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INHIBITION OF tRNA AMINOACYLATION BY ETHIDIUM DIMER AND SEVERAL OTHER BIFUNCTIONAL INTERCALATORS WITH OR WITHOUT ANTITUMOR ACTIVITY

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Abstract—The effect of various bifunctional intercalators with or without antitumor activity on the tRNA aminoacylation step was examined. The ethidium dimer appears as the strongest inhibitor of aminoacylation of *E. coli* methionine, phenylalanine and leucine tRNA and of yeast phenylalanine tRNA. Ethidium dimer binds to tRNA through two classes of sites, I and II ($K_I > 10^9 M^{-1}$, $K_{II} \simeq 10^6 M^{-1}$) [C.R. Reinhardt, *Biophys. J.* **25**, 44242 (1979)]. Surprisingly, binding of the drug to the high affinity class of sites causes only partial inhibition of tRNA aminoacylation, while full inhibition requires saturation of the second class of sites. This inhibition is reversed by displacement of the drug by an excess of DNA. In the studied systems, the 7H pyridocarbazole dimers 1 and 2 which have antitumor activity [B.P. Roques, D. Pelaprat, J. Le Guen, G. Porcher, C. Gosse and J. B. Le Pecq, *Biochem. Pharmac.* **28**, 1811 (1979); D. Pelaprat, A. Delbarre, J. Le Guen, J. B. Le Pecq and B. P. Roques, *J. med. Chem.* **23**, 1336 (1980)] inhibit aminoacylation at much higher concentrations than that of ethidium dimer. This behavior is discussed in relation to the particular structures of these drugs.

Many antitumor drugs are known to strongly interact with DNA [1–5]. Therefore, a rational strategy for discovering new antitumor compounds may consist of designing molecules with the highest possible affinity for DNA. Such molecules can be found in the series of bifunctional intercalators: when the two moieties of these molecules are intercalated in DNA the free energies of interaction of each subunit nearly add up, and the binding affinity of such molecules for DNA can become as high as 10^{10} to 10^{11} M^{-1} in conditions approaching those prevailing *in vivo* [6, 7]. Recently several bifunctional intercalators were described which indeed possess strong antitumor properties [8, 9].

It has been observed that one bifunctional intercalator, the ethidium dimer which binds DNA with a binding constant of 2×10^8 M^{-1} (at pH 7.4 in 0.2 M Na^+) [10], was able to bind to several tRNA species with even a higher binding affinity [11, 12]. This observation raised the possibility that tRNA represented a pharmacological target of the bifunctional intercalators. It was therefore of interest to determine whether the biological functions of tRNAs were modified upon binding of these compounds. It was also important to correlate an effect on the tRNA functions with the antitumor activities of these bifunctional intercalators.

Among the various biological reactions involving tRNA, the aminoacylation reaction represents a key step in protein synthesis. This reaction is very sensitive to the integrity of the tRNA tertiary structure [13]. Therefore the inhibitory effects of several bifunctional intercalators on the aminoacylation reactions catalysed by several aminoacyl-tRNA synthetases from *E. coli* or yeast were compared.

The results of such a study are reported here.

MATERIALS AND METHODS

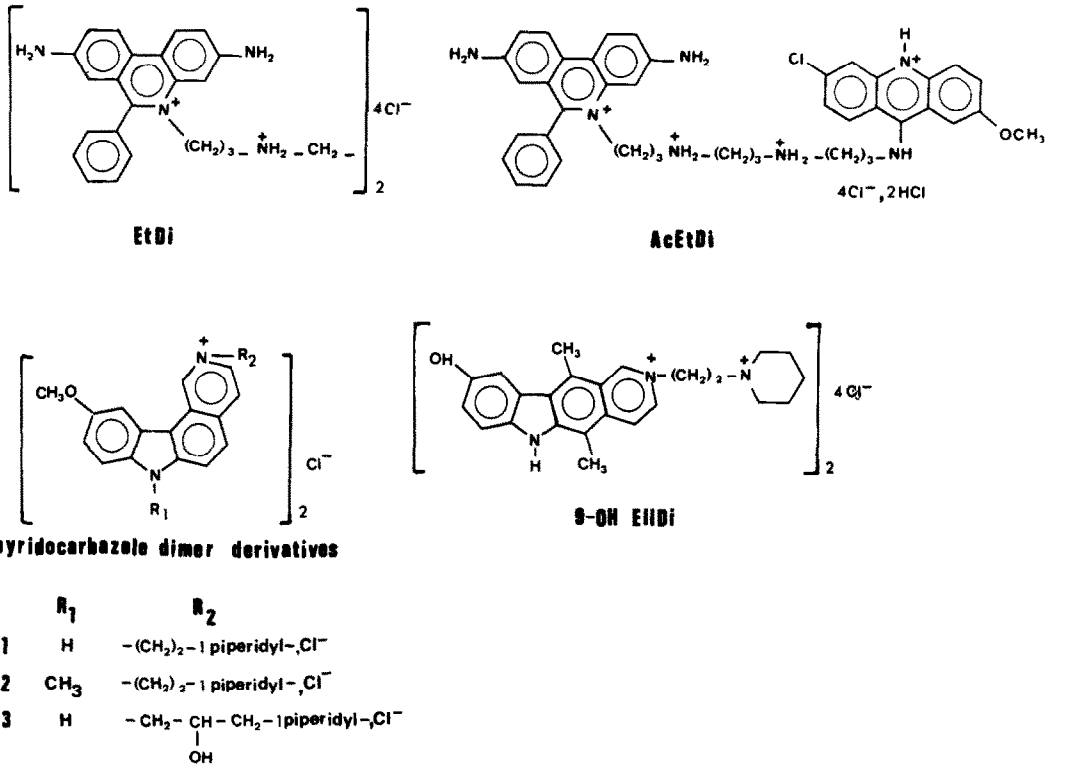
Materials

Drugs. Ethidium bromide was purchased from Sigma. The chemical structures of the compounds studied in this work are summarized in Fig. 1. The dimers were synthesized and purified as previously described [9, 14–16].

Substrates and enzymes. Pure $tRNA_f^{Met}$ from the EM 20031 *E. coli* strain (1.4 ± 0.1 nmol of methionine acceptance/A_{260} unit of tRNA) was prepared and stored in the dark as described [17]. Yeast $tRNA^{Phe}$, *E. coli* $tRNA^{Phe}$ and *E. coli* $tRNA^{Leu\,2}$ were purchased from Boehringer.

The amino acid acceptor capacities/A_{260} unit of these tRNAs were found equal to 930, 740 and 980 pmol for *E. coli* $tRNA^{Phe}$, $tRNA^{Leu\,2}$ and yeast $tRNA^{Phe}$, respectively.

Homogeneous native methionyl-tRNA synthetase, phenylalanyl-tRNA synthetase and leucyl-tRNA synthetase from *E. coli* strain EM 20031 were obtained and stored as described [18, 19].



	R_1	R_2
Di 1	H	$-(CH_2)_2-$1 piperidyl$-,Cl^-$
Di 2	CH_3	$-(CH_2)_2-$1 piperidyl$-,Cl^-$
Di 3	H	$-CH_2-CH(OH)-CH_2-$1piperidyl$-,Cl^-$

Fig. 1. Structure of the different studied compounds.

Phenylalanyl-tRNA synthetase from yeast [20] was a gift of Dr F. Fasiolo.

Enzymes and tRNA concentrations were measured according to their molar extinction coefficients at 280 and 260 nm, respectively [21].

Other chemicals. Uniformly [^{14}C]-labeled L-amino acids were purchased from the Commissariat à l'Energie Atomique (Saclay, France). Calf thymus DNA from Sigma was sonicated according to [6] and [22]. The sedimentation coefficient of sonicated DNA measured by analytical ultracentrifugation was 6–7*S* at 20°. All other chemicals were of the best commercially available grade.

Methods

Aminoacylation assay. Aminoacylation was performed in a final volume of 0.1 ml containing 100 mM Tris–HCl buffer, pH 7.6, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM ATP, 7 mM $MgCl_2$, 150 mM KCl, 50 μg/ml bovine serum albumin 50 μM [^{14}C]-labeled L-amino acid (50 Ci/mole), various concentrations of tRNA (1–3 μM), various concentrations of drugs and limiting amounts of enzymes.

Drugs from freshly prepared concentrated solutions in 50 mM sodium acetate buffer, pH 5, were added to the mixture containing tRNA. After 5 min incubation at 25° of the drugs in the presence of tRNAs, the aminoacylation reactions were initiated by addition of a pure aminoacyl-tRNA synthetase in 100 mM Tris–HCl, pH 7.6, 0.1 mM EDTA, 10 mM 2-mercaptoethanol containing 0.2 mg/ml bovine serum albumin. Rates of aminoacylation were measured at 25° for 10 min in the presence of catalytic amounts of enzymes under conditions of initial reaction velocities. The reaction was quenched by the addition of 5 ml of cold 5% trichloracetic acid containing 20 mg/ml of unlabeled aminoacids. The suspension was filtered through Whatman glass filter disc (GF/C) and the radioactivity of the washed precipitate determined in a liquid scintillation spectrometer in Bray's solution [23].

Displacement of ethidium dimer from its complex with $tRNA_f^{Met}$ *by excess DNA.* Ethidium dimer (50 μM) and *E. coli* $tRNA_f^{Met}$ (2.5 μM) in 40 μl 100 mM Tris–HCl, pH 7.6, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 125 mM KCl, 2.5 mM ATP, 17.5 mM $MgCl_2$, 125 μM [^{14}C]L-methionine (50 Ci/mole) were incubated for 5 min at 25°. After this time, 40 μl of a solution of DNA (2 mM in nucleotides) in 100 mM Tris–HCl, pH 7.6, 0.1 mM EDTA, 10 mM 2-mercaptoethanol were added. At various times (see Fig. 4) of the incubation of DNA in the presence of the tRNA–drug complex at 25°, aminoacylation was initiated by the addition of 20 μl of a 2.25 nM methionyl-tRNA synthetase solution in 100 mM Tris–HCl, pH 7.6, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, containing 0.8 mg/ml of bovine serum albumin. Initial rates of aminoacylation were measured for 2 min at 25°. A control experiment was performed in parallel, in the absence of ethidium dimer.

RESULTS

1. *Comparative inhibition of tRNA aminoacylation by ethidium monomer and dimer*

In the various systems studied (*E. coli* $tRNA_f^{Met}$,

Table 1. Total concentrations of drugs (μM) causing 50% and 90% inhibition of the tRNA aminoacylation reactions

Compound	*E. coli* $tRNA_f^{Met}$ 50%	*E. coli* $tRNA_f^{Met}$ 90%	*E. coli* $tRNA^{Phe}$ 50%	*E. coli* $tRNA^{Phe}$ 90%	$tRNA^{Phe}$ Yeast 50%	$tRNA^{Phe}$ Yeast 90%
Ethidium dimer	14–16	42–46	4–6	7–9	30–33	42–48
Ethidium bromide	38–43	>150	55–60	>150	75–78	>150
Acridine ethidium dimer	50–55	>150				
7H pyridocarbazole Di1	42–50	>150	18–22	42–48	40–45	80–85
7H pyridocarbazole Di2	>60	>150	17–22	37–42	>100	>150
7H pyridocarbazole Di3	>60	>150				
9-OH ellipticine	48–52	>120				

* tRNA concentration in the assay was 3 μM, except in the case of *E. coli* $tRNA^{Phe}$ (1 μM).

$tRNA^{Phe}$, $tRNA^{Leu2}$ and yeast $tRNA^{Phe}$), the aminoacylation activities measured with the corresponding cognate homologous aminoacyl-tRNA synthetases are much more sensitive to ethidium dimer than to ethidium monomer (Table 1). The free concentration of ethidium monomer at which the tRNA aminoacylation activities are inhibited by 50% is of the order of 50 μM in all systems. This results agrees well with previous studies [24]. By contrast, ethidium dimer causes complete inhibition in all systems in conditions where the free concentration of the drug is too small to be measured. Independent of the tRNA concentration in the assay, full inhibition of the enzymatic activity is obtained at a definite ratio of drug to tRNA for a given system (Fig. 2). This ratio varies between 8 and 15 depending on the system. On the other hand, several inhibition curves (Fig. 2) are biphasic. Two opposite cases are encountered. (1) Yeast $tRNA^{Phe}$, where the binding to tRNA of the first 2–3 ethidium dimers causes a relatively minor inhibition. (2) *E. coli* $tRNA_f^{Met}$ and $tRNA^{Leu2}$, where the binding of the first two ethidium

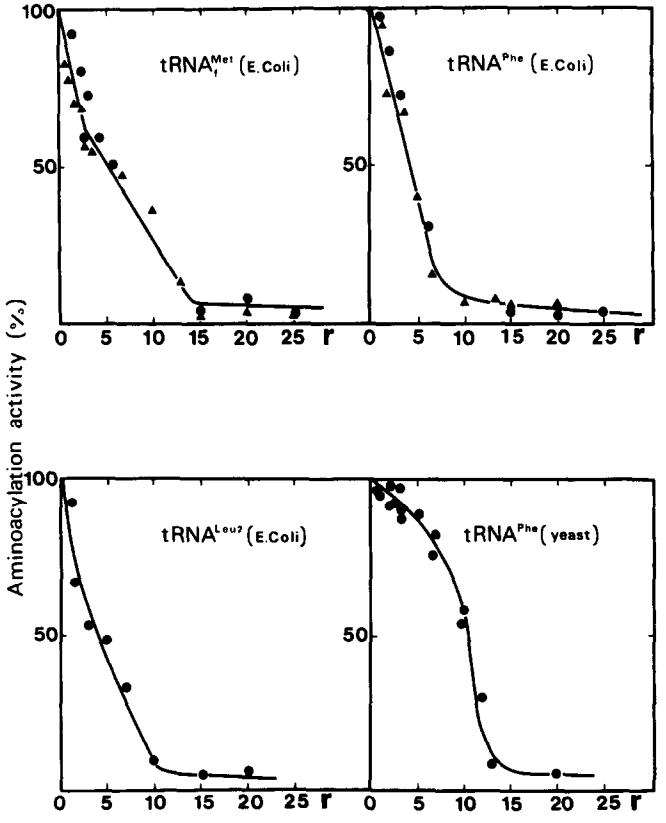


Fig. 2. Comparative inhibitory effect of ethidium dimer on the aminoacylation of *E. coli* $tRNA_f^{Met}$, $tRNA^{Phe}$, $tRNA^{Leu2}$ and yeast $tRNA^{Phe}$. The enzymatic activity in presence of ethidium dimer (expressed in percentage of the activity without dimer) is plotted as a function of r, the ratio of the number of ethidium dimer molecules per tRNA molecules in the assay. The tRNA concentration in the assay was 1 μM except in the cases of *E. coli* $tRNA_f^{Met}$ and $tRNA^{Phe}$ where the concentrations were 3 μM (●——●) or 1 μM (▲——▲), respectively. Ethidium dimer concentration was varied from 0 to 25 times the tRNA concentration in the assay.

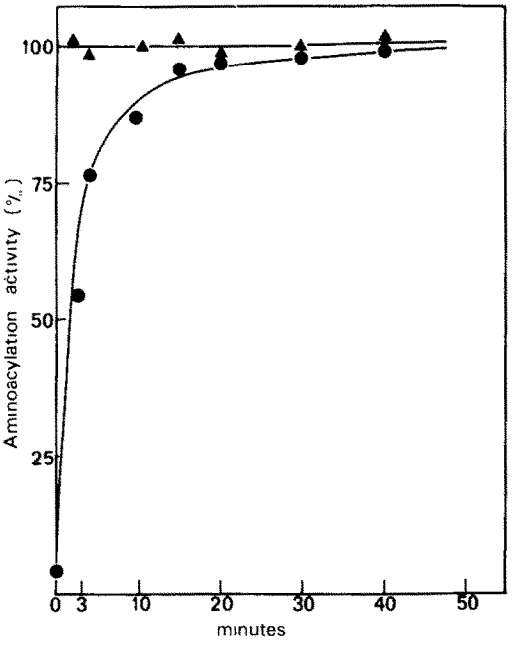


Fig. 3. Recovery of the aminoacylation activity after displacement of ethidium dimer from its tRNA complex by an excess of DNA. Two series of measurements were made: (1) The aminoacylation mixture is incubated with ethidium dimer (●——●). The reaction was performed as described in the Experimental Section. (2) Control experiment without ethidium dimer (▲——▲).

dimers causes 30% inhibition and the binding of 9–13 additional molecules is necessary to cause complete inhibition.

In the case of the *E. coli* $tRNA_f^{Met}$ system, it was observed that the inhibition promoted by ethidium dimer could be reversed by the addition of an excess of DNA with respect to tRNA (Fig. 3). From this experiment, a half-time of exchange of the order of 3 min is calculated. An accurate estimation of this time is limited by the fact that 2 min were required to measure the initial rate of the aminoacylation reaction. Nevertheless, this value is consistent with the binding process of ethidium dimer to tRNA, as will be discussed later.

Furthermore, fluorescence measurements (not shown) indicated that a $tRNA_f^{Met}$–ethidium dimer complex (two dimers per tRNA) was not dissociated by the addition of an excess of the cognate methionyl-tRNA synthetase.

2. Effects of various bifunctional intercalators, with or without an antitumor activity, on the reactions of tRNA aminoacylation

Surprisingly, none of the studied bifunctional intercalators (see Fig. 1) is able to inhibit the various aminoacylation reactions to the same extent as the ethidium dimer (Table 1).

A decreased inhibition could be accounted for by either one of the following situations:
(1) the compound interacts weakly with tRNA;
(2) the compound interacts strongly with tRNA but the interaction with the aminoacyl-tRNA synthetase and/or the aminoacylation reaction are less affected than in the presence of ethidium dimer.

In the case of ethidium acridine heterodimer, clearly the latter alternative holds. This compound is known [12] to bind to several tRNAs with an affinity comparable to that of ethidium dimer; however, nearly 50 μM of this drug is required to inhibit $tRNA_f^{Met}$-aminoacylation by more than 50%.

For the other compounds (ellipticine dimer and the 7H pyridocarbazole dimers, see Fig. 1) the interpretation is more difficult. However, fluorescence measurements (data not shown) indicate that these compounds are unable to displace ethidium dimer as well as ethidium monomer from their complexes with yeast $tRNA^{Phe}$, although they can displace ethidium dimer and monomer from their complexes with poly A. poly U. This suggests that these drugs either bind weakly to tRNA or recognize different site(s) on the nucleic acid.

DISCUSSION

Ethidium dimer was shown to have a strong affinity for DNA as well as for tRNA [10, 11]. The present study demonstrates that the binding of ethidium dimer to tRNAs is accompanied by a strong inhibition of their aminoacylation reactions. The inhibition of the enzymatic process is a consequence of the binding of the dye to tRNA and not to the enzyme: indeed, the inhibition is a function of the dimer/tRNA ratio, whatever the tRNA concentration in the assay (Fig. 2).

Ethidium dimer binds to yeast $tRNA^{Phe}$ as well as to unfractionated yeast tRNA, on two kinds of sites [11]. One or two dimers are bound per tRNA molecule with a very high affinity ($>10^9\ M^{-1}$), 4–5 dimers are bound to a second class of sites with a much lower affinity ($\simeq 10^6\ M^{-1}$). Similar results were obtained with *E. coli* $tRNA_f^{Met}$ (results not shown). The present study shows that aminoacyl-tRNA synthetases are able to recognize and aminoacylate tRNA molecules on which the two strong sites are occupied by ethidium dimer. This is especially clear in the case of the yeast $tRNA^{Phe}$ system (Fig. 2).

On the other hand, it is clear that the aminoacylating enzyme, which has a binding affinity of the order of 10^6–$10^7\ M^{-1}$ for its cognate tRNA [17, 25] has not displaced the ethidium dimer from its tRNA complex, the stability of which is at least two order of magnitude higher. The stoichiometries of ethidium dimer binding to tRNA determined by fluorescence titration [12] and by inhibition of the aminoacylation reaction are thus in agreement. Moreover, the existence of two classes of binding sites is confirmed by the present study: complete inhibition of the enzymatic process is only obtained when the second class of sites are fully occupied. The inhibition could then result from the occupancy by the drug of tRNA regions recognized by the enzyme and/or from the induction of a modified tertiary structure of tRNA.

The binding of ethidium dimer does not produce an irreversible denaturation of tRNA. The inhibition can be suppressed when the dye is displaced from its complex with tRNA by the addition of an excess

of DNA. After DNA addition, full recovery of the aminoacylation activity is obtained within a few minutes. This time could either reflect the rate of dissociation of the ethidium dimer–tRNA complex and/or the recovery of a tRNA native structure following the dissociation of the drug.

Ethidium monomer binds a single site on tRNA with a high affinity ($\simeq 10^6$ M^{-1}) [26]. Occupation of its preferred site by ethidium monomer clearly does not cause any alteration of the aminoacylation reaction. At higher concentration, the low inhibitory activity of ethidium monomer is probably related to its binding to a secondary class of sites of low affinity such as those characterized by Sturgill [26] (2–3 secondary sites per tRNA with a binding constant of 4×10^4 M^{-1}). This situation parallels that encountered with ethidium dimer.

It can be pointed out that from a pharmacological point of view the determinant factor in the inhibition of an enzymatic process *in vivo* is the free circulating concentration of the inhibitor in equilibrium with its receptor. The free concentration of ethidium dimer at equilibrium with tRNA when half of the secondary sites responsible for the inhibition are occupied, is estimated to be of the order of 1 μM (binding affinity 10^6 M^{-1}). The corresponding free concentration of ethidium monomer ensuring 50% inhibition is of the order of 50 μM (Table 1). Therefore it may be concluded that ethidium dimer is 50-fold more efficient in the inhibition of aminoacylation than ethidium monomer. This factor is markedly lower than expected from a comparison of the affinitiès of these compounds towards their primary binding site(s) on tRNA. (K ethidium dimer/K ethidium monomer $\simeq$ 1000.) This arises from the fact that the inhibition process mainly depends on the occupancy of the secondary binding sites. Since the binding affinity of ethidium dimer for DNA is 2×10^8 M^{-1}, DNA will be fully saturated before the secondary sites on tRNA are occupied. It is therefore expected that biological effects of ethidium dimer will be rather related to its binding to DNA. In agreement with this conclusion it was observed that in an *E. coli* strain sensitive to ethidium monomer, DNA synthesis was much more affected than protein synthesis by ethidium [27].

It is very striking that the other studied bifunctional intercalators with or without antitumor properties are much less efficient inhibitors of the aminoacylation reactions than the ethidium dimer.

Their inhibitory power remains in most cases of the order of that observed with ethidium monomer.

The rigidity of the interchain of these drugs [3, 11] possibly limits their binding on tRNA to these primary sites which appear unimportant in terms of biological activity. This particular behavior indicates in fact a discriminating ability of these drugs towards the tRNA tertiary structure greater than that of ethidium dimer. In this context it cannot be excluded that RNAs such a messenger, ribosomal, viral, heteronuclear . . . could represent a target of mono- and bis-intercalators. Contrary to what is observed with tRNA, the binding of these drugs to specific sites of high affinity on these RNAs might lead to inhibition of specific enzymatic processes (RNA splicing, maturation, degradation...). Such a possibility is supported by the observation that several intercalating molecules inhibit selectively the processing of 45*S* ribosomal RNA precursors in various eucaryotic cells [5, 28].

This underlines the possible complexity of the mechanism of action of such drugs, and the difficulty of correlating the action of a drug on a specific enzymatic system with a complex pharmacological property like an antitumor activity.

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