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QUARTERLY

Inhibitory effects of pentamidine analogues on protein biosynthesis *in vitro*★

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Pentamidine despite its rather high toxicity, is currently in clinical use. For development of new drugs of this type it is important to know the mechanism of their action. Two new amidines (I and II) and 4′,6-diamidino-2-phenylindole (DAPI) were found in preliminary experiments to inhibit protein synthesis *in vitro* in the cell-free rat liver system. The three compounds differed in the precise mode of action. The inhibitory effect of I on the activity of the eukaryotic elongation factor eEF-2 and ribosomes seems to suggest that the binding site of eEF-2 on the ribosome was blocked by this compound. eEF-2 has been identified as the primary target of II and eEF-1 as the primary target of DAPI in the system studied.

The amidines related to pentamidine show diverse pharmacological activities [1–3]. In particular, pentamidine is currently in widespread clinical use for treatment of *Pneumocystis carinii* pneumonia (PCP) in patients with acquired immunodeficiency syndrome [4, 5]. The precise mode of action of pentamidine is unclear and its major macromolecular targets have not been identified unequivocally, but there is considerable evidence for its direct interaction with the pathogenic genome [6, 7]. Biophysical [8, 9] and footprinting studies [10] have shown that the pentamidine molecule binds to AT-rich regions of duplex DNA. Molecular modelling has suggested [11, 12] that, like with drugs such as netropsin and DAPI [13, 14], it interacts *via* the minor groove of DNA.

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; eEF, eukaryotic elongation factor; PCP, *Pneumocytis carinii* pneumonia; TMS, tetramethylsilane.

The mode of antimicrobial action of these dicationic molecules has been ascribed to their selective binding to the minor groove of DNA at AT-rich sites and their ability to selectively interfere with the normal functioning of the pathogen topoisomerases [5]. The lack of quantitative correlation between DNA binding by those molecules and their antimicrobial activity against all the organisms studied, can be explained by DNA binding being only the first step in a multistep process. The precise mechanism of the inhibitory action of the aromatic amidines is currently unknown. It seems likely that a drug–DNA complex leads to inhibition, however, it is not known whether this is a consequence of competition for specific binding sites or is a result of changes in DNA conformation occurring on drug binding.

Perhaps the most serious concern in clinical application of the aromatic amidines is their toxicity. The selectivity of these compounds is limited due to the eukaryotic nature of fungi and therefore to the great degree of similarity between the fungal and mammalian protein synthesis machineries [15]. Limited oral bioavailability and both acute and chronic toxicity have slowed down the studies on development of this class of compounds. Pentamidine has seen continued use for the treatment of PCP despite an extensive list of adverse reactions that include nephrotoxicity, hepatotoxicity, hypotension and sterile abscesses at the injection site [4]. Serious consideration should therefore be given to evaluation of the toxic potential of these compounds as part of their development for clinical use.

As part of a continued investigation to determine the mechanism of the mode of action of pentamidine analogues, new compounds **I**, **II** and the well-known agent against trypanosomes, 4′,6-diamidino-2-phenylindole (DAPI) (Fig.1) were examined for their inhibitory effects on protein synthesis in rat liver cell-free system.

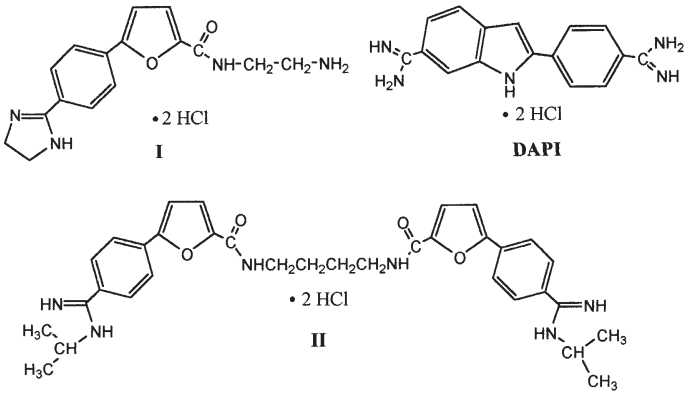

Figure 1. Structures of the pentamidine analogues.

MATERIALS AND METHODS

Materials. For the present study, two new pentamidine analogues, referred as compounds **I** and **II,** were synthesized in our laboratory. Preparation of compound **I** and **II** was satisfactorily achieved by standard chemical transformations according to the reaction sequence shown in Figs. 1 and 2. Purity of the compounds was verified by NMR and elemental analysis.

^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) spectra were recorded on a Bruker AC 200 F spectrometer, using TMS (tetramethylsilane) as an internal standard. Chemical shifts are expressed in σ value (p.p.m.) and coupling constants are given in J (Hertz). Multiplicity

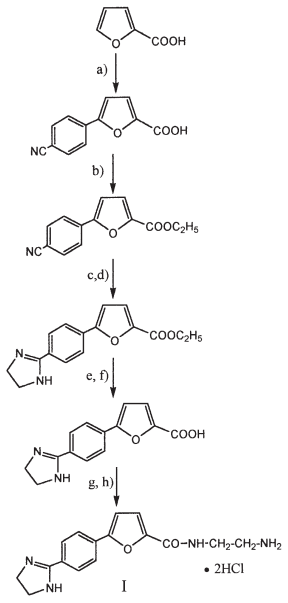

Figure 2. Synthesis of pentamidine analogue I.

Reagents and conditions: a, $H_2NC_6H_4CN$, $NaNO_2$, HCl, $CuCl_2$; b, EtOH, DCI, THF; c, EtOH, HCl; d, $H_2NCH_2NH_2$, EtOH; e, LiOH, THF/H_2O; f, HCl; g, $H_2NCH_2CH_2NH_2$, DCI, DMF; h, HCl.

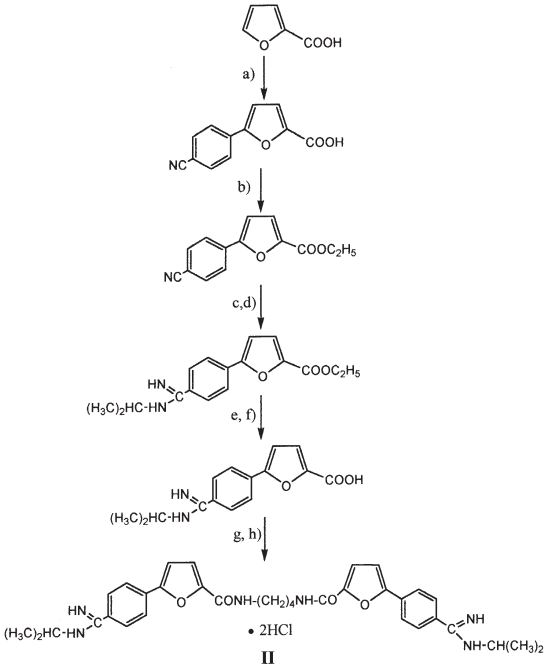

Figure 3. Synthesis of pentamidine analogue II.

Reagents and conditions: a, $H_2NC_6H_4CN$, $NaNO_2$, HCl, $CuCl_2$; b, EtOH, DCI, THF; c, EtOH, HCl, HCl; d, $CH(CH_3)_2NH_2$, EtOH; e, LiOH, THF/H_2O; f, HCl; g, $H_2N(CH_2)_4NH_2$, DCI, DMF; h, HCl.

of resonance peaks is indicated as singlet (s), doublet (d), triplet (t), quartet (q), broad (br), or multiplet (m).

DAPI was obtained from Sigma (Steinheim, Germany).

N-(2-aminoethyl)-5-[4-(4,5-dihydro-1H-imidazol-2-yl)phenyl]-2-furancarboxamide dihydrochloride (I):

m.p. (ethanol) 240–242°C;

^{1}H NMR (Me_2SO-d_6) 10.77 (br, 1H), 8.91 (t, 1H), 8.21 (d, 4H), 8.4 (d, 4H), 7.39 (d, 2H), 7.26 (d, 2H), 4.02 (s, 4H), 3.51 (t, 2H), 2.91 (t, 2H). ^{13}C NMR (Me_2SO-d_6) 164.0, 157.6, 152.5, 148.2, 134.3, 129.3, 124.5, 121.1, 115.6, 111.7, 44.3, 43.7, 38.5.

Anal. calc. for $C_{16}H_{18}N_4O_2 \cdot 2HCl \cdot H_2O$ (389): C, 49.36; H, 5.65; N, 14.39; found: C, 49.51; H, 5.69; N, 14.51.

1,4-Bis[2-[5-(4-[(N-isopropyl)amidinophenyl)furyl]-2-carboxamido]]butane dihydrochloride (II):

m.p. (ethanol) 232– 234°C;

^{1}H NMR (Me_2SO-d_6) 9.70 (br, 2H), 9.56 (br, 2H), 9.25 (br, 2H), 8.95 (t, 2H), 8.12 (d, 4H), 7.94 (d, 4H), 7.33 (d, 2H), 7.20 (d, 2H), 4.14 (m, 2H), 3.33 (m, 4H), 1.81 (t, 2H), 1.58 (t, 4H), 1.30 (d, 12H). ^{13}C NMR (Me_2SO-d_6) 161.1, 157.3, 152.5, 148.3, 134.0, 128.8, 127.6, 124.3, 115.4, 110.3, 45.1, 38.2, 26.8, 21.2.

Anal. calc. for $C_{34}H_{40}N_6O_4 \cdot 2HCl \cdot 3H_2O$ (723): C, 56.43; H, 6.64; N, 11.62; found: C, 56.31; H, 6.69; N, 12.51.

***Protein synthesis* in vitro.** The cell-free system, ribosomes, [^{14}C]leucyl-tRNA and the elongation factors from livers of male Wistar rats (200–250 g body mass) were prepared according to the methods previously described [16–18]. Protein synthesis in the cell-free system was examined by measuring the level of incorporation of [^{14}C]leucine into trichloroacetic acid-precipitable material [16].

Effect of compounds I, II and DAPI on the activity of ribosomes. A ribosome suspension, 0.1 ml (about 2 mg), was incubated

Table 1. The effect of compounds I, II and DAPI on [^{14}C]leucine incorporation into proteins in a cell-free system of rat liver

Pentamidine analogues	Compound I				Compound II				DAPI			
μg/ml	0	12.5	25	50	0	12.5	25	50	0	12.5	25	50
[^{14}C]Leucine incorporated (c.p.m.)	618	446	339	22	618	370	154	10	618	396	192	0
Inhibitory effect (%)	0.0	27.8	45.1	96.4	0.0	40.1	75.1	98.4	0.0	36.0	69.0	100

* Results are the means of three independent experiments performed in duplicate. Refer to Methods for test procedures.

at 37°C for 30 min with 200 μg **I, II** or DAPI. At the same time, 0.1 ml of ribosome suspension and 1 ml of 0.05 M Tris/HCl buffer, pH 8.0, as blank, were incubated. Both samples were layered on a sucrose gradient consisting of two layers: of 3.5 ml 1 M sucrose and 3.5 ml 0.5 M sucrose, then centrifuged for 2.5 h at 105 000 ***g***. The ribosome pellet was washed and resuspended in 0.2 ml 0.05 M Tris/HCl buffer, pH 7.4, containing 0.35 M sucrose and 0.004 M $MgCl_2$. The activity of ribosomes was examined by incorporation of [^{14}C]leucine into protein [16].

Effect of compounds I, II and DAPI on the activity of the elongation factors. The elongation factors eEF-1 or eEF-2 were preincubated at 37°C for 20 min, with 10, 20 and 40 μg of **I, II** and DAPI, respectively. Activity of eEF-2 was assayed in the total mixture for the elongation reaction as [^{14}C]leucine incorporation into protein as described previously [13].

RESULTS AND DISCUSSION

Compounds **I**, **II** and DAPI were tested for their ability to inhibit protein synthesis in rat liver cell-free system. The new compound **II** belongs to the category of elongated minor groove binders. Such molecules are expected to exhibit greater selectivity [14] as, on binding to DNA, they occupy 6–8 base pairs and not, like DAPI, 3–4 base pairs; unfortunately, most of them are strongly toxic *in vivo* [19]. The compounds **I**, **II** and DAPI at the concentration of 50 μg/ml caused total inhibition of [^{14}C]leucine incorporation into proteins (Table 1). Generally, there is a good correlation between inhibition of whole-cell growth and cell-free protein synthesis, and any differences may be indicative of differences in the uptake or in the intracellular stability of these compounds.

In order to gain more knowledge about the precise mechanism by which these com-

Table 2. Effect of compounds I, II and DAPI on the activity of ribosomes*

Ribosome	Without inhibitory solution	Inhibitory effect of					
		I		**II**		DAPI	
(μg/ml)	c.p.m.	c.p.m.	(%)	c.p.m.	(%)	c.p.m.	(%)
200	235	0	100	101	57	92	61
300	363	0	100	189	48	182	50
400	504	0	100	348	31	343	32
500	772	154	80	695	10	556	28

*Compounds **I**, **II** or DAPI (200 μg) were dissolved in 0.05 M Tris/HCl buffer at concentration of 100 mg/ml. Refer to Methods for test procedures. Results are the means of three independent experiments performed in duplicate.

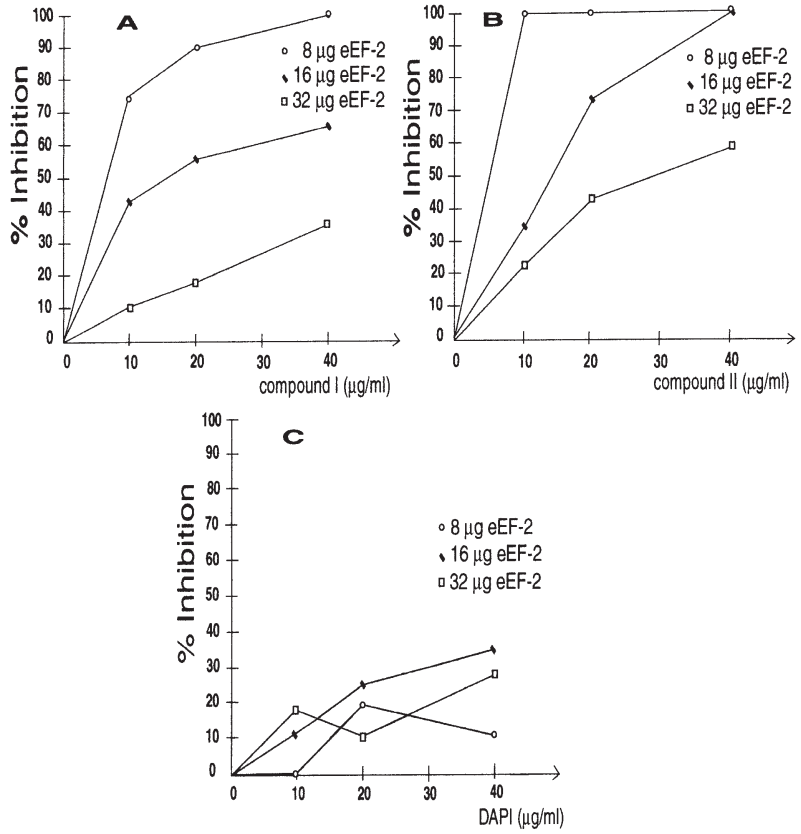

Figure 4. The effect of compounds I, II and DAPI on eEF-2 activity in the total elongation system.

*eEF-2 activity was tested by [^{14}C]leucine incorporation into proteins in the total elongation system, with increasing amount of eEF-2, but stable amounts of eEF-1. Refer to Methods for test procedures.

pounds inhibit the protein synthesis elongation cycle, the cell-free system from rats was split into ribosomes and the protein synthesis elongation factors (eEF-1 and eEF-2). They were then used to identify whether the primary site of action of the compounds studied was the ribosome or a soluble factor.

The activity of ribosome was much more affected by compound **I** than by compound **II** (Table 2). The inhibition by compound **II** and DAPI was not so significant, especially at higher concentrations of ribosomes (400 and 500 mg/ml). Although this result is not conclusive, it suggests that protein synthesis is inhibited by compound **I** in a different manner than by **II** and DAPI. This leads us to the conclusion that the ribosome is not the primary target of **II** and DAPI, and thus they cannot be treated as inhibitors of peptide bond formation. Moreover, since the aminoacyl-tRNA synthetase activity is not affected by those compounds (not shown) we can infer that one of the two elongation factors might be the target of **II** and DAPI.

The effects of compounds **I**, **II** and DAPI on the elongation factors eEF-1 and eEF-2 activities were verified in the following experiments. **I** and **II** inhibited eEF-2 activity (Figs. 4A and 4B). The molecules of **I** and **II** appeared to affect marginally the eEF-1 activity (Figs. 5A and 5B). In contrast, DAPI showed the opposite pattern, with a markedly lower potency with respect to the elongation factor eEF-2 and 100% inhibition of the activity of eEF-1 at the concentration of 40 μg/ml (Fig. 5C).

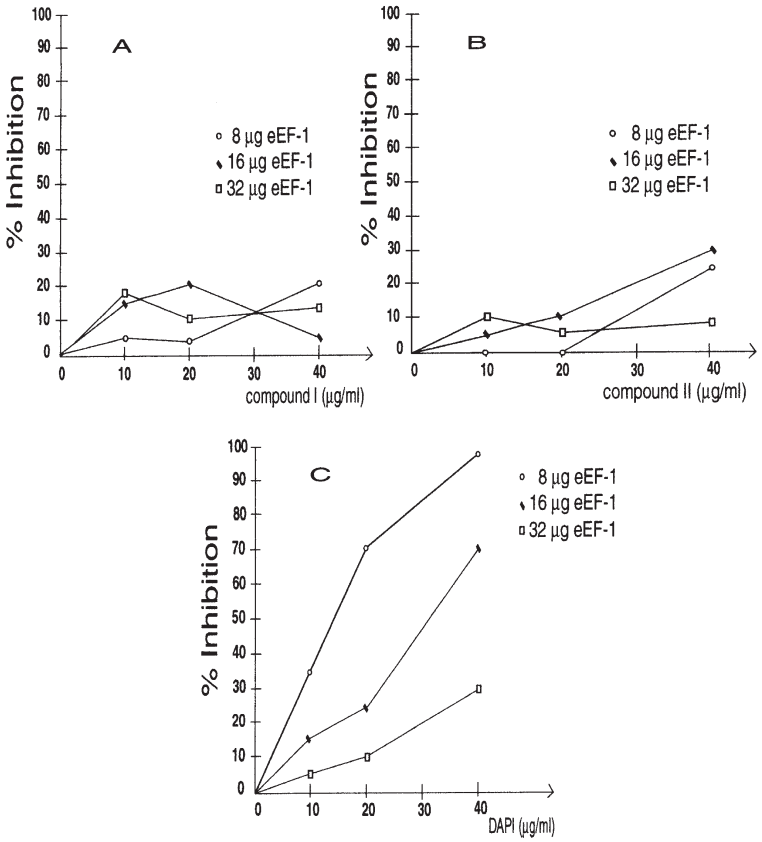

Figure 5. The effect of compounds I, II or DAPI on eEF-1 activity in the total elongation system.

*eEF-2 activity was tested by [^{14}C]leucine incorporation into proteins in the total elongation system, with increasing amount of eEF-1, but stable amounts of eEF-2. Refer to Methods for test procedures.

Our preliminary experiments have shown that even small amounts of compounds **I**, **II** and DAPI inhibit markedly [^{14}C]leucine incorporation into proteins in the cell-free rat liver system. Since all three contain the benzamidine group as the main part of the molecule, it was reasonable to assume that they all aim at the same target. However, substitutions may give rise to differences in the interactions with specific residues at the binding site of the targeted proteins. This, combined with differences in the target structure among the tested components of the rat liver cell-free system, might explain the diversity of the results obtained. It may be concluded that **I**, **II** and DAPI inhibit protein biosynthesis *in vitro* in the cell-free rat liver system, but they differ in the precise mode of action.

The most marked direct inhibitory effect of **I** on the ribosome and eEF-2 activity seems to suggest that the binding site of eEF-2 on the ribosome is blocked by this compound. The interaction between **I** and its target is greatly favored by the presence of ribosomes. It is well known that interaction with ribosomes promotes conformational changes on the two elongation factors and that these changes lead to the appearance of a latent enzyme activity, i.e., GTPase for EF-1α and EF-2 and ATPase [20, 21].

Our results indicate that the inhibition of protein synthesis by **II** and DAPI is dependent on the nonribosomal fraction more than on the ribosomes. Preliminary studies performed to elucidate the mode of action of **II** and DAPI have shown that the putative target

of these compounds are the protein synthesis elongation factors. The factor eEF-2 has been identified as the primary target of compound **II**, and the factor eEF-1, as the primary target of DAPI. Further studies are required to confirm directly our conclusions. EF-2, as well as its prokaryotic counterpart, EF-G, promotes translocation, i.e., displacement of nascent peptidyl-tRNA from the A site to the P site and movement of the ribosome along the mRNA; this is accompanied by a conformational change in the ribosome from the pretranslocational to the posttranslocational state [22]. According to recent findings [23], GTP hydrolysis catalyzed by an elongation factor might provide the energy needed for the process. EF-2 is a highly conserved protein (85% homology and 66% identity between human EF-2 and *Saccharomyces cerevisiae* EF-2) [24]. It has been shown both for EF-G and for EF-2 from different sources that this protein is able to display different conformations depending on whether it is alone or interacting with GTP, GDP, or a ribosome in either the pre- or the posttranslocational state [25, 26]. This conformational flexibility determines its biological properties and may explain how such a conserved protein can be the target of **II**. There is a clear need for further studies on the structure of DAPI, **I** and **II** complexes with the ribosome and the protein synthesis elongation factors.

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