Inhibition of murine leukaemia virus reverse transcriptase by 2-halogenated polyadenylic acids

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Several new analogues of polyadenylic acid $[(A)_n]$, i.e. poly(2-fluoroadenylic acid) $[(f^2A)_n]$, poly(2-chloroadenylic acid) $[(cl^2A)_n]$, poly(2-bromoadenylic acid) $[(br^2A)_n]$ and poly(2-iodoadenylic acid) $[(io^2A)_n]$ have been synthesized and evaluated for their effects on the RNA-directed DNA polymerase (reverse transcriptase) activity of Moloney murine leukaemia virus. All $(A)_n$ analogues were found to be potent inhibitors of reverse transcriptase, the order of (decreasing) potency being $(f^2A)_n > (io^2A)_n > (br^2A)_n > (cl^2A)_n$. For all four $(A)_n$ analogues the inhibition of reverse transcriptase was competitive with respect to the template-primer, $(A)_n \cdot \text{oligo(dT)}$. The K_i values were $0.02 \mu\text{g/ml}$ for $(f^2A)_n$, $0.1 \mu\text{g/ml}$ for $(io^2A)_n$, $0.5 \mu\text{g/ml}$ for $(br^2A)_n$ and $8 \mu\text{g/ml}$ for $(cl^2A)_n$. With a K_i of $0.02 \mu\text{g/ml}$ (approx. $0.04 \mu\text{M}$), $(f^2A)_n$ can be considered as one of the most, if not the most, potent polynucleotide inhibitor of reverse transcriptase that has been described so far.

The RNA-directed DNA polymerase (reverse transcriptase, EC 2.7.7.49) that is associated with oncornaviruses can be considered as an appropriate target in the design of substances which would be able to inhibit oncornavirus replication, thereby preventing malignant transformation of the virus-infected cell (Chandra, 1979). A large variety of reverse transcriptase inhibitors have been developed which could, according to their mode of action, be divided into different classes, i.e. substrate analogues, template analogues, bivalent cation chelators and compounds which bind directly to the template or the enzyme (at a site other than the template-binding site) (Chandra *et al.*, 1977). Template analogues compete with the viral RNA genome for the template-binding site of the enzyme. Typical examples of such template analogues which have been reported to inhibit the reverse transcriptase associated with oncornavirions are poly(2'-O-methyladenylic acid) $[(Am)_n]$ and poly(2'-O-ethyladenylic acid) $[(Ae)_n]$ (Arya *et al.*, 1974), and, since these analogues have also been found effective in inhibiting the replication of murine leukaemia and

sarcoma viruses in both cell culture systems and animal models (mice) (Tennant *et al.*, 1972, 1973, 1974; Arya *et al.*, 1975b, 1976a), it is tempting to speculate that their inhibitory effect on virus replication is mediated by an inhibition of viral RNA-directed DNA synthesis. This assumption is further supported by the observation that the $(A)_n$ analogues are most effective when added prior to or just after virus infection (Tennant *et al.*, 1973), which suggests that an early event of the viral replicative cycle, i.e. RNA-directed DNA synthesis, is affected.

Guided by these considerations, we have recently synthesized various new $(A)_n$ analogues, including poly(2-methylthioadenylic acid) $[(ms^2A)_n]$, poly(2-ethylthioadenylic acid) $[(es^2A)_n]$, poly(2'-fluoro-2'-deoxyadenylic acid) $[(dAf)_n]$ and poly(2'-azido-2'-deoxyadenylic acid) $[(dAz)_n]$, and determined their effects on the RNA-directed DNA polymerase activity of Moloney MuLV (De Clercq *et al.*, 1979). While $(dAf)_n$ served as an efficient template for the reverse transcriptase reaction, $(ms^2A)_n$, $(es^2A)_n$ and $(dAz)_n$ strongly inhibited reverse transcriptase activity. We have now extended these observations to a new series of 2-substituted $(A)_n$ derivatives, namely poly(2-fluoroadenylic acid) $[(f^2A)_n]$, poly(2-chloroadenylic acid) $[(cl^2A)_n]$, poly(2-bromoadenylic acid) $[(br^2A)_n]$ and poly(2-iodoadenylic acid) $[(io^2A)_n]$ (Scheme I). From these 2-halogeno-substituted $(A)_n$ derivatives $(f^2A)_n$ emerged as a highly potent

Abbreviations used: abbreviations for polynucleotides follow the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [*Biochem. J.* (1970) 120, 449–454]; MuLV, murine leukaemia virus.

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Scheme 1. 2-Halogeno-substituted analogues of polyadenylic acid

R = F, poly(2-fluoroadenylic acid), (ff²A)_n;
 R = Cl, poly(2-chloroadenylic acid), (cl²A)_n;
 R = Br, poly(2-bromoadenylic acid), (br²A)_n;
 R = I, poly(2-iodoadenylic acid), (io²A)_n.

inhibitor of oncornaviral DNA polymerase. This is in marked contrast with the stimulatory effect that has previously been reported (De Clercq *et al.*, 1979; Chandra *et al.*, 1981) for its 2'-fluoro-substituted counterpart, (dAfl)_n.

Materials and methods

Polyadenylic acid analogues

The 2-halogenated AMPs were synthesized from AMP following the procedure described by Yamaji *et al.* (1978). The 2-halogenated AMPs were then converted to the 5'-diphosphates via the imidazolidate intermediates (Hoard & Ott, 1965). From these 2-halogenated ADPs the corresponding 2-halogenated (A)_n derivatives were prepared with the aid of polynucleotide phosphorylase. The exact molecular size of (ff²A)_n, (cl²A)_n, (br²A)_n and (io²A)_n was not determined. Polyacrylamide-gel electrophoresis indicated that they did not markedly differ in molecular size and that their length exceeded 200 nucleotide units.

Templates and primers

The templates (A)_n and (C)_n were obtained from P-L Biochemicals, as were the primers (dT)₁₂₋₁₈ and (dG)₁₂₋₁₈.

MuLV (Moloney)

MuLV (Moloney) was obtained from Electro-Nucleonics Laboratories (catalog no. 1024, lot no. 5004-26-58). The virus stock was derived from a Swiss mouse embryo 3T3 cell line infected with MuLV (Moloney) and contained 1.8×10^{11} virus particles (0.92 mg of protein) per ml.

DNA polymerase assay

The standard DNA polymerase assay contained in a reaction mixture of 100 μl: 25 mM-Tris/HCl

(pH 8.3), 100 mM-NaCl, 5 mM-dithiothreitol, 0.5 mM-magnesium acetate, 0.04% Triton X-100, 5.0 μCi of [methyl-³H]dTTP (specific radioactivity 30 Ci/mmol) or 5.0 μCi of [5-³H]dCTP (specific radioactivity 19 Ci/mmol) and either 5 μl (Figs. 1-4) or 0.5 μl (Fig. 5) of the MuLV stock. In addition, the reaction mixtures contained (A)_n·(dT)₁₂₋₁₈ (Figs. 1 and 3-5) or (C)_n·(dG)₁₂₋₁₈ (Fig. 2) as template-primer. The concentrations of these template-primers are indicated in the legends to the Figures. The concentrations at which the (A)_n analogues were added are also indicated in the legends to the Figures. The assay mixtures were incubated at 37°C for 60 min, unless stated otherwise, and acid-insoluble radioactivity was measured essentially as described previously (De Clercq *et al.*, 1975a).

Results

In a first set of experiments, MuLV reverse transcriptase activity was measured in the presence of (A)_n·(dT)₁₂₋₁₈ as template-primer and [methyl-³H]dTTP as radiolabelled substrate (Fig. 1). In a second set of experiments, (C)_n·(dG)₁₂₋₁₈ served as template-primer and [5-³H]dCTP as radiolabelled substrate (Fig. 2). In both experiments the (A)_n

Fig. 1. Effects of (ff²A)_n, (cl²A)_n, (br²A)_n, (io²A)_n and (A)_n on MuLV reverse transcriptase in the presence of (A)_n·oligo(dT) as template-primer

Concentration of (A)_n and oligo(dT) were 8 μM and 2 μM, respectively. Concentrations of (ff²A)_n, (cl²A)_n, (br²A)_n, (io²A)_n and (A)_n were as indicated on the abscissa.

Fig. 2. Effects of $(fl^2A)_n$, $(cl^2A)_n$, $(br^2A)_n$, $(io^2A)_n$ and $(A)_n$ on MuLV reverse transcriptase in the presence of $(C)_n \cdot oligo(dG)$ as template-primer. Concentration of $(C)_n$ and $oligo(dG)$ were $8 \mu M$ and $2 \mu M$, respectively. Concentrations of $(fl^2A)_n$, $(cl^2A)_n$, $(br^2A)_n$, $(io^2A)_n$ and $(A)_n$ were as indicated on the abscissa.

analogues were added to the reaction mixtures at a final concentration of either 0.1, 1, 10 or $100 \mu g/ml$. The four $(A)_n$ analogues exhibited a concentration-dependent inhibition of DNA synthesis, the relative order of (decreasing) potency being $(fl^2A)_n > (io^2A)_n > (br^2A)_n > (cl^2A)_n$ at all concentrations tested (Figs. 1 and 2). The inhibitory effects of the $(A)_n$ analogues on the $(A)_n \cdot (dT)_{12-18}$ - and $(C)_n \cdot (dG)_{12-18}$ -directed reverse transcriptase reactions were quite similar.

K_i values were estimated for all four $(A)_n$ analogues from Dixon ($1/v$ versus $[I]$) plots of the kinetic data obtained with $(A)_n \cdot (dT)_{12-18}$ as template-primer (Fig. 3). In these assays $(A)_n$ was used at its K_m concentration [$2.5 \mu M$, as determined previously (De Clercq *et al.*, 1979)]. The K_i values were $0.02 \mu g/ml$ for $(fl^2A)_n$, $0.1 \mu g/ml$ for $(io^2A)_n$, $0.5 \mu g/ml$ for $(br^2A)_n$ and $8 \mu g/ml$ for $(cl^2A)_n$.

Lineweaver-Burk plots were constructed for the kinetics of inhibition of the $(A)_n \cdot (dT)_{12-18}$ -directed reverse transcriptase reaction by the four $(A)_n$ analogues (Fig. 4). For all four 2-halogenated $(A)_n$ derivatives the inhibition of reverse transcriptase was simply competitive with regard to the template-primer $(A)_n \cdot (dT)_{12-18}$.

In the aforementioned experiments, $(fl^2A)_n$, $(cl^2A)_n$, $(br^2A)_n$ and $(io^2A)_n$ were added to the reaction mixtures at time zero. In another set of

Fig. 3. Dixon plots for kinetics of inhibition of $(A)_n \cdot oligo(dT)$ -directed MuLV reverse transcriptase by $(fl^2A)_n$, $(cl^2A)_n$, $(br^2A)_n$ and $(io^2A)_n$. Incubation time was 15 min and v is expressed as $10^{-5} \times$ radioactivity incorporated (c.p.m.). Concentrations of $(A)_n$ and $oligo(dT)$ were $2.5 \mu M$ and $0.625 \mu M$, respectively. Concentrations of $(fl^2A)_n$, $(cl^2A)_n$, $(br^2A)_n$ and $(io^2A)_n$ were as indicated on the abscissa ($[I]$).

experiments, programmed with $(A)_n \cdot (dT)_{12-18}$ as template-primer, the $(A)_n$ analogues were added at various times (either 0, 10, 20 or 30 min) after the reaction had started; $(fl^2A)_n$ and $(io^2A)_n$ were added at $10 \mu g/ml$, whereas $(cl^2A)_n$ and $(br^2A)_n$ were added at $100 \mu g/ml$. When present from time zero, $(fl^2A)_n$ and $(io^2A)_n$ suppressed DNA synthesis completely, $(br^2A)_n$ almost completely and $(cl^2A)_n$ only partially (Fig. 5). If the $(A)_n$ analogues were added at 10 or 20 min, they prevented further incorporation of [$methyl$ - 3H]dTMP, and if they were added at 30 min [for $(fl^2A)_n$ and $(io^2A)_n$ also if added at 20 min] they caused a diminution in the total amount of [$methyl$ - 3H]dTMP incorporated. The latter decrease obviously points to partial degradation of the DNA that had already been synthesized by the time the $(A)_n$ analogues were added.

Discussion

Our studies have indicated that the 2-halogenated derivatives of $(A)_n$ are potent inhibitors of MuLV

Fig. 4. Lineweaver-Burk plots for kinetics of inhibition of $(A)_n \cdot \text{oligo(dT)}$ -directed MuLV reverse transcriptase by $(fl^2A)_n$, $(cl^2A)_n$, $(br^2A)_n$ and $(io^2A)_n$.

Incubation time was 15 min and v is expressed as $10^{-4} \times$ radioactivity (c.p.m.). Concentrations of $(A)_n \cdot \text{oligo(dT)}$ were as indicated on the abscissa $[(A)_n]$ expressed in μM , oligo(dT) at 1/4 the concentration of $(A)_n$. Concentrations of $(fl^2A)_n$ were: 0.1 $\mu\text{g/ml}$ ($\bullet$), 0.05 $\mu\text{g/ml}$ (Δ), 0.025 $\mu\text{g/ml}$ ($\blacktriangle$) and 0 $\mu\text{g/ml}$ ($\circ$). Concentrations of $(cl^2A)_n$ were: 10 $\mu\text{g/ml}$ ($\bullet$), 5 $\mu\text{g/ml}$ (Δ), 2.5 $\mu\text{g/ml}$ ($\blacktriangle$) and 0 $\mu\text{g/ml}$ ($\circ$). Concentrations of $(br^2A)_n$ were: 1 $\mu\text{g/ml}$ ($\bullet$), 0.5 $\mu\text{g/ml}$ (Δ), 0.25 $\mu\text{g/ml}$ ($\blacktriangle$) and 0 $\mu\text{g/ml}$ ($\circ$). Concentrations of $(io^2A)_n$ were: 0.1 $\mu\text{g/ml}$ ($\bullet$), 0.05 $\mu\text{g/ml}$ (Δ), 0.025 $\mu\text{g/ml}$ ($\blacktriangle$) and 0 $\mu\text{g/ml}$ ($\circ$).

reverse transcriptase. The compounds did not show a preference with regard to the template-primer since they inhibited the $(A)_n \cdot (\text{dT})_{12-18}$ - and $(C)_n \cdot (\text{dG})_{12-18}$ -directed reverse transcriptase reactions to a similar extent. Both $(A)_n \cdot (\text{dT})_{12-18}$ and $(C)_n \cdot (\text{dG})_{12-18}$ can be considered as appropriate template-primers for the reverse transcriptase reaction, the former being the more efficient and the latter the more specific (Gerard, 1975; Gerard *et al.*, 1974; Mikke *et al.*, 1976).

With a K_i value of 0.02 $\mu\text{g/ml}$ (approx. 0.04 μM) $(fl^2A)_n$ can be considered as one of the most, if not

the most, potent polynucleotide inhibitor of reverse transcriptase that has been found so far. K_i values calculated for other polynucleotide inhibitors of reverse transcriptase, when assayed under similar conditions, were: 0.15 $\mu\text{g/ml}$ for $(ms^2A)_n$ (De Clercq *et al.*, 1979), 2.4 $\mu\text{g/ml}$ for $(dAz)_n$ (De Clercq *et al.*, 1979), 0.5–1 μM for $(I)_n$ (Arya *et al.*, 1974, 1975a), 1.8 μM for $(G)_n$ (Arya *et al.*, 1976b), 0.35 μM for $(X)_n$ (Arya *et al.*, 1976b), 12 μM for $(Im)_n$ (Arya *et al.*, 1975a), 15 μM for $(Ae)_n$ (Arya *et al.*, 1974) and 25 μM for $(Am)_n$ (Arya *et al.*, 1974). All these K_i values were determined with $(A)_n \cdot (\text{dT})_{12-18}$ as

Fig. 5. Effects of $(fl^2A)_n$, $(cl^2A)_n$, $(br^2A)_n$ and $(io^2A)_n$ on $(A)_n$ ·oligo(dT)-directed MuLV reverse transcriptase, when added at different times (as indicated by the arrows) during incubation of the assay mixtures

Incubation times were as indicated on the abscissa. Concentrations of the inhibitors were $10\mu\text{g/ml}$ for $(fl^2A)_n$ and $(io^2A)_n$ and $100\mu\text{g/ml}$ for $(cl^2A)_n$ and $(br^2A)_n$. Concentrations of $(A)_n$ and oligo(dT) were $8\mu\text{M}$ and $2\mu\text{M}$ respectively.

template-primer and detergent-disrupted virions as source of the reverse transcriptase. As suggested by previous findings (Arya *et al.*, 1976; Erickson & Grosch, 1974), the K_i for $(fl^2A)_n$ [and the other $(A)_n$ analogues] may have been lower if the assays were carried out with a purified enzyme preparation instead of disrupted virions. From an inspection of Figs. 1 and 2 and the corresponding Figures in previous publications (De Clercq *et al.*, 1975b, 1976, 1977), $(fl^2A)_n$ would also appear far more potent than other $(A)_n$ analogues for which the K_i values have not been determined, i.e. poly(2-aza-adenylic acid), poly(3-deaza-adenylic acid) and poly(7-deaza-adenylic acid).

For those $(A)_n$ analogues for which K_i values were measured under identical experimental conditions (De Clercq *et al.*, 1979; this report), the following order of decreasing potency as reverse transcriptase inhibitor could be deduced: $(fl^2A)_n > (io^2A)_n \geq (ms^2A)_n > (br^2A)_n > (dAz)_n > (cl^2A)_n$. Because of its strong inhibitory effects on reverse transcriptase activity, $(fl^2A)_n$ should be further explored for its potential to inhibit the replication of oncornaviruses, and concomitant cell transformation, in cell culture and animal systems. It also remains to be established to what extent the inhibitory effects of $(fl^2A)_n$ and the other 2-halogenated $(A)_n$ derivatives are restricted to the

reverse transcriptase of oncornaviruses or extend to other DNA polymerases, i.e. DNA polymerases α , β , and γ .

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