

Aurintricarboxylic Acid and Polynucleotides as Novel Inhibitors of Ribonucleotide Reductases

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Ribonucleoside diphosphate reductases isolated from *Escherichia coli*, baker's yeast, Ehrlich ascites tumor cells, and a unicellular green alga (*Scenedesmus obliquus*) are inhibited strongly and uniformly by the polymeric triphenylmethane dye, aurintricarboxylic acid. The molecule appears to interact simultaneously with the enzyme's various nucleotide and catalytic (iron-organic radical) sites. Oligo- and polynucleotides are also inhibitory. These reactions serve as models of the probably physiologic regulation of ribonucleotide reduction exerted by natural inhibitors. Partial characterization of an inhibitor fraction found in wheat seed embryo is described.

Introduction

Inhibitors have a prominent role in studies of the universally distributed, cell cycle-correlated ribonucleoside diphosphate reductases (EC 1.17.4.1) because these enzymes offer several structurally different targets for inactivation: The nucleotide (substrate, and effector) binding sites, the essential cysteine residues, the protein-bound iron center, and the tyrosine free radical of the catalytic protein subunit [1]. Metal-chelating and radical scavenging compounds, in particular of the N-hydroxyurea and polyhydroxybenzoic acid type have found wide application for mechanistic studies and as potential antineoplastic and antiviral agents [2–4]. Compounds of oligo- or polynucleotide nature have been found to serve as natural inhibitors of ribonucleotide reduction, *e.g.* in seeds, fungi, and other eukaryotic cells [5–8]. However, despite such general vulnerability of the ribonucleotide reductases to inhibition, the effect of a particular compound upon the enzymes isolated from different organisms may vary from strongly inhibitory to ineffective, depending on structure and accessibility of the various protein sites in each individual case. The combination, within one molecule, of several structures suitable for the interaction with ribonucleotide reductases might provide more universal and efficient inhibitors.

It occurred to us that such properties exist in the triphenylmethane dye aurintricarboxylic acid which has long been known as a potent inhibitor of protein-nucleic acid interactions, for example in ribosomal protein biosynthesis, in RNA polymerase reactions, and with ribonucleases [9–11]. Schleich *et al.* have shown by nmr spectroscopic, synthetic, and biochemical studies that the active inhibitory component of dye preparations is not the monomeric chromophore but a polymer fraction of $\bar{M}_n = 6000$, composed of approximately 12–15 monomers in various methylene-linked triphenylmethyl structures such as schematized in Fig. 1 [12]; an additional, particularly interesting feature is the material's content of a stable free radical [11]. High affinity between this form of aurintricarboxylic acid and ribonucleotide reductases is predicted as the compound could at the same time occupy nucleotide sites (in analogy to the widely recognized interaction of aromatic dyes with the "nucleotide fold" of many other enzymes), act as multidentate metal-chelating salicylic acid, and interfere with the enzymes' radical centers.

Considering the existence of multitudinous, up to six nucleotide binding sites on a ribonucleotide reductase protein like that of *Escherichia coli* [1, 13] and the natural inhibitory substances mentioned above oligo- and polynucleotides should also serve as models of protein nucleotide interaction; previous experiments with a tumor cell enzyme [14] support that idea. An inhibitory oligonucleotide

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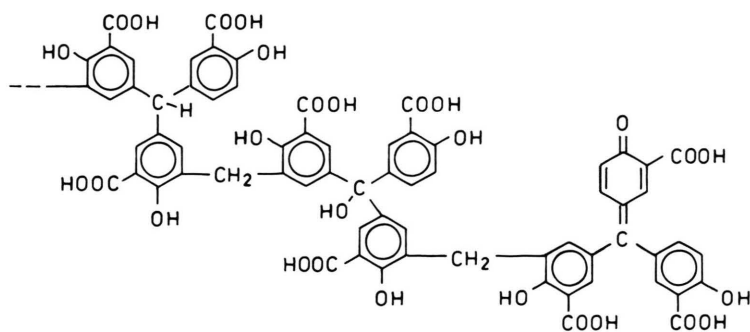


Fig. 1. Structures present in polymeric fractions of aurintricarboxylic acid, as identified by NMR and synthetic studies [12].

fraction isolated from wheat embryo [5] was included in our present study.

Materials and Methods

All chemicals, reagents, enzymes, and nucleotides, were of highest purity available and were obtained from Boehringer, Mannheim; Merck, Darmstadt; and Serva, Heidelberg. Radioactive nucleotides came from Amersham Buchler, Braunschweig. Polymeric aurintricarboxylic acid, purified by dialysis and ultrafiltration (fraction I, [12]), was a generous gift of Dr. T. Schleich, University of California, Santa Cruz, USA. Yeast tRNA^{Phe} (Boehringer) was cleaved enzymatically, or by removal of Y base and aniline treatment as described [15].

Ribonucleotide reductase from thymine-starved *E. coli* B3 cells (ATCC 23 851) was purified by the published procedure [16]; a 90% homogeneous preparation (separated subunits B1 plus B2) was available for aurintricarboxylate inhibition studies whereas 40% pure enzyme (free from nucleotide-degrading activities) served for assaying the other inhibitors. Fluorodeoxyuridine-treated cultures of *Scenedesmus obliquus* were a source of the algal enzyme as described [17, 18]; *Saccharomyces cerevisiae* ribonucleotide reductase was prepared from fluorouracil-treated batch cultures of baker's yeast [6, 19]; and mouse Ehrlich ascites cells (strain Karzel), kindly provided by Dr. M. Löffler, Physiologische Chemie, Universität Marburg, were used for enzyme preparation as previously [20, 21].

Ribonucleotide reductase activity was determined using [5-³H]cytidine diphosphate (spec. activity, 20 Ci/mmol) or [8-³H]guanosine diphosphate (spec. activity, 0.6 Ci/mmol) as substrates [16–21]. Assay condition were individually optimized to ensure linear product formation with respect to time,

mg enzyme protein, and (where applicable) substrate concentration. In brief, assays contained, in a total volume of 0.10 or 0.30 ml: 0.10–0.30 mM CDP or 0.50 mM GDP, respectively (in case of substrate saturation); 2–6 mM, or 30 mM (yeast enzyme) dithiothreitol as reductant; 3 mM ATP, or 0.1–1 mM dTTP as positive allosteric effectors; and 3–15 mM Mg²⁺ ions (except in case of *Scenedesmus* enzyme). Inhibitors were added as specified below. After incubation of the enzyme assays for 30 min at 30 °C or 37 °C (mouse tumor enzyme) all nucleotides were converted to nucleosides by treatment with 4 units of alkaline phosphatase (from calf intestine, Boehringer), and the amount of radioactive deoxyribonucleoside was analyzed by LC or HPLC on Beckman M-71 or Aminex A-7 cation exchange resins in borate buffer system [17, 19].

Ribonucleotide reductase inhibitor from wheat [5, 22]: An aqueous extract (10 mM Tris-HCl, pH 7.5) from 4 g homogenized wheat embryo was boiled for 10 min, centrifuged after chilling, and the supernatant was mixed with 5 vol. icecold ethanol. The precipitate formed after 15 min was isolated and redissolved in 10 ml Tris buffer. In the following chromatography steps, column eluates were monitored by a dual wavelength UV detector, and inhibitor fractions were recognized by their absorption ($A_{254} > A_{280}$) as well as in inhibition assays using *E. coli* enzyme. The yellow solution was passed over a column (2 × 40 cm) of Sephadex G-25 and the first, colorless elution peak was recovered. This material was freed from protein by chromatography on a column (2 × 20 cm) of DEAE cellulose (Whatman, DE 32), equilibrated in 0.14 M triethylammonium carbonate (pH 7.0) solution and eluted with a gradient (0.14 to 1.0 M) of the same salt; the inhibitor was contained in a peak eluting at 0.7 M salt concentration, and was reprecipitated with

ethanol. It was finally fractionated by reverse phase chromatography on a column (1.2×21 cm) of RPC-5-Analog (Bethesda Research Laboratories) equilibrated in the above Tris-HCl buffer plus 0.20 M KCl; strongly inhibitory fractions were obtained by stepwise elution with 0.30 M and 0.45 M KCl, respectively, and were desalted on Sephadex G-25. Yield, 40 and 50 OD₂₆₀ units, respectively. The compounds were characterized as polyribonucleotides by their phosphate content, the presence of ribose but not 2-deoxyribose, cleavage by several ribonucleases but not by DNase or proteases, and UV absorption at 258 nm (pH 7). These fractions are not yet homogeneous when analyzed on urea-containing polyacrylamide (20%) gels. Fraction II (eluted from RPC-5 at 0.3 M KCl) shows an unresolved pattern in the $M_r = 4000$ –9000 size category. Chromatography on a column (1.2×80 cm) of Sephadex G-75, equilibrated in 0.075 M Tris-HCl, pH 7.5, containing 0.75 mM EDTA and 0.20 M NaCl, and calibrated with oligonucleotides and RNA of known molecular weight indicated essentially the same size range.

For inhibition of ribonucleotide reductases, appropriate volumes of inhibitor solution were included in enzyme assays. Stock solutions of polymeric aurintricarboxylic acid ($\bar{M}_n = 6000$) in water were made by weight. Oligo- and polynucleotide solutions were quantitated by their UV spectrum, using mean residue molar extinction coefficients at 260 nm of $\epsilon = 13000$ for the adenylates (approximate chain length of poly-A: 200), and of $9000 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for wheat fraction II (chain length 10–25 nucleotides). The amount of material in case of undefined chain length is indicated in OD₂₆₀ units (equivalent to 1 ml solution of $A_{260} = 1$).

Results

The effect of polymeric aurintricarboxylic acid on the activity of four representative ribonucleoside diphosphate reductases, *viz.* the proteins isolated from *Escherichia coli*, baker's yeast (*Saccharomyces cerevisiae*), a unicellular green alga (*Scenedesmus obliquus*), and from mouse Ehrlich ascites tumor cells has been tested. The material is strongly inhibitory towards all four enzymes (Fig. 2). Concentrations required for 50% inhibition vary only fivefold: $I_{50} = 5 \times 10^{-6}$ M (*S. obliquus*); 1.2×10^{-5} M (*E. coli*, ascites cells); and 2.5×10^{-5} M (yeast

enzyme). It may be noted that these small individual variations parallel known enzyme properties. Thus, the *E. coli* and mammalian ribonucleotide reductases are closely related in many details of structure and mechanism [1] while the enzyme of yeast differs, among other things in its unusual resistance to high concentrations of the common reductase inhibitor hydroxyurea [19]; on the contrary plant enzymes are most sensitive proteins and easily inactivated [5, 17].

An attempt was made to determine the type of inhibition produced by the dye with purified bacterial (*E. coli*) or algal (*S. obliquus*) ribonucleotide reductase. The data (Fig. 3, a, b) indicate mixed inhibition with a strong decrease in V and increases in apparent K_m values. As the difficulties and limitations of radioactive ribonucleotide reductase assays, especially in presence of inhibitors, do not permit more precise kinetic experiments it cannot entirely be ruled out that the inhibition actually approaches noncompetitive behaviour; however, competitive or uncompetitive inhibition is excluded with certainty. Determination of apparent K_i values was not meaningful under these circumstances.

One structural property of aurintricarboxylic acid is its amphipathic, polyanionic character in which it would resemble a polynucleotide. Another analogy is that the ribonucleotide reductase-inhibiting nucleotide fractions found in various organisms are also effective in several heterologous enzyme systems, for example an inhibitor from wheat embryo (where the endogeneous reductase is not available in purified form) with *E. coli* ribonucleotide reductase [5]. With a view on these observations we have compared the effect of a number of synthetic and natural oligo- and polynucleotides on the bacterial enzyme (Table I). The inhibitor fraction from wheat included in this study is a mixture of guanosine-rich, singlestranded ribonucleotides of 10–25 residues chain length ($M_r = 4000$ –9000) [22]. The data summarized in Table I indicate that inhibition of enzyme activity by polynucleotides is common and is not restricted to the natural inhibitor. However, a distinct structure specificity is seen in that compounds of intermediate chain length (*i.e.*, a nonanucleotide, the wheat inhibitor fraction, and tRNA^{Phe} fragment molecules 15–39 nucleotides in length) and of mixed base composition are more effective than are

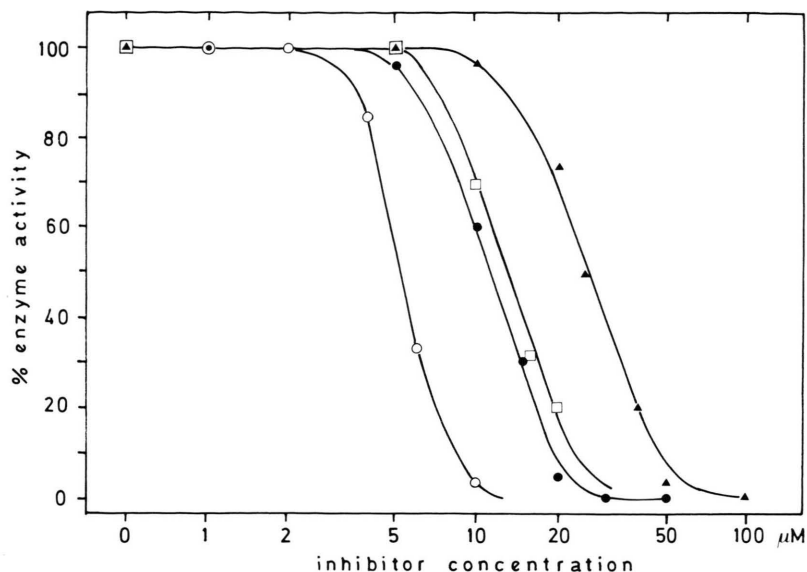


Fig. 2. Inhibition of bacterial and eukaryotic ribonucleoside diphosphate reductases by polymeric aurintricarboxylic acid ($M_r = 6000$). Substrates: CDP, or GDP of saturating concentrations. Source of enzyme: *Scenedesmus obliquus* (○); *Escherichia coli* (●); Ehrlich ascites cells (□); baker's yeast (▲).

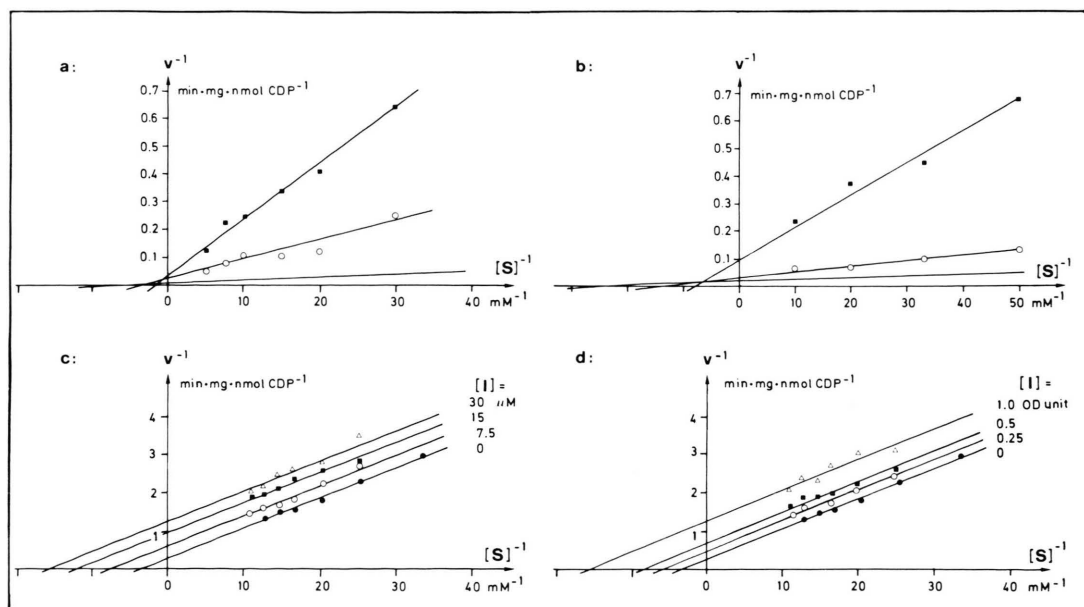


Fig. 3. Lineweaver-Burk plots for reduction of CDP by *E. coli* and *S. obliquus* ribonucleotide reductase in presence of inhibitors. a: *E. coli* enzyme without addition (—), plus 10 μM (○), or 15 μM aurintricarboxylic acid (■). b: *S. obliquus* enzyme without addition (—), plus 6 μM (○), or 10 μM aurintricarboxylic acid (■). c: *E. coli* enzyme without addition (●), in presence of 7.5 μM (○), 15 μM (■), or 30 μM (Ap)₈A (△). d: *E. coli* enzyme without addition (●), in presence of 0.25 OD₂₆₀ units (○), 0.5 OD units (■), or 1.0 OD units (△) of the wheat inhibitor fraction II. Two batches *E. coli* ribonucleotide reductase of different specific activity were used (*cf.* Materials and Methods).

Table I. Inhibition of *E. coli* ribonucleotide reductase by oligo- and polynucleotides.

Compound	% inhibition in standard assay ^a	I_{50}	K_i (app.)
ApApA	0		
(Ap) ₈ A	40	1×10^{-4} M	1×10^{-5} M
Poly-A	10	2×10^{-6} M	1.5×10^{-4} M
Poly-C	15		
Poly-G	15		
Poly-U	15		
Poly-G,U	50		
yeast tRNA ^{Phe}	0		
yeast tRNA ^{Phe} /2 (1/4) ^b	60		
wheat inhibitor fraction II	70	1.6×10^{-5} M	3×10^{-6} M

^a Equal amounts of inhibitory material (5 OD₂₆₀ units/0.3 ml) were included in standard CDP reduction assays.

^b Mixture of half molecules prepared by chemical scission, or of half and quarter molecules prepared by limited ribonuclease digestion.

the homopolyribonucleotides of several hundred residues. In contrast the trinucleoside diphosphate ApApA proved totally indifferent towards the enzyme system, as did intact yeast tRNA; the difference in inhibition before and after chemical or enzymatic fragmentation of a tRNA molecule is remarkable.

The analogy between model polynucleotides and the potent ribonucleotide reductase inhibitor from wheat is strengthened by the identical type of inhibition they produce (Fig. 3, c, d). In contrast to the effect of aurintricarboxylic acid, double-reciprocal plots of the rates of CDP reduction by *E. coli* enzyme in presence of the wheat polynucleotides, nonaadenylate, or polyadenylate all clearly indicate uncompetitive inhibition. The apparent K_i values (Table I), obtained from linear replots of $[I]$ vs. $1/V$ (not shown) of nonaadenylate and wheat inhibitor fraction II are one or two orders of magnitude lower than the apparent K_m of cytidine diphosphate ($K_m = 10^{-4}$ M).

Discussion

We have shown that polymeric aurintricarboxylic acid is a potent, apparently universal inhibitor of ribonucleoside diphosphate reductases of bacterial and eukaryotic origin. This observation contributes primarily to a better understanding of that highly complex enzyme family as a whole. Its significance

for specifically affecting a particular enzyme in cell extracts or *in vivo* may be limited as the dye interacts with a great number of nucleotide-converting enzymes; in addition to RNA polymerase or ribonuclease inhibition [10, 11] it was found, *e.g.*, that thymidylate synthase from *Scenedesmus obliquus* is also efficiently inhibited by aurintricarboxylic acid (B. Bachmann, unpublished data).

The quite uniform mode of action (Fig. 2) contrasts markedly with the effects of benzohydroxamates, polyhydroxybenzoic acid derivatives, or heterocyclic thiosemicarbazones which can differ by several orders of magnitude in their inhibition of various ribonucleotide reductases [2–4]. The leveling effect of aurintricarboxylic acid is best explained by its chemical structure (Fig. 1) in which different possibilities for interaction with protein sites are combined, *viz.* as nucleotide analog, metal chelator, radical scavenger, and general polyanion. These components may contribute to enzyme inactivation by varying degrees in each case but add up to comparable overall effects.

Inhibition of ribonucleotide reductases by oligo- and polynucleotides also appears a general phenomenon. Earlier observations by Cory [14] who described inhibition of Ehrlich tumor cell reductase by various RNA preparations (including tRNA) and by polyribonucleotides, in particular polycytidylate, are corroborated by our present results with the *E. coli* enzyme (Table I). However the purer bacterial enzyme exhibited much higher selectivity than was observed with the tumor enzyme; more systematic studies are required to establish whether this difference is due to the source of enzyme, or to contaminating RNA fragments in the less purified preparation.

Interaction of ribonucleotide reductases with all these model nucleotides supports the accumulated biochemical evidence that modified oligo- and polynucleotides, found in a mould (*Achlya sp.*), in yeast, wheat embryo, and hamster cells [5–8], do have a function in the endogenous regulation of deoxyribonucleotide synthesis and cell proliferation. Analysis of these apparently important compounds themselves has been severely hampered by the difficult structure elucidation [22, 23] and by lack of pure homologous enzyme samples. In this connection it must not be overlooked that too general and strong an affinity between polynucleotides and ribonucleotide reductases would pose specificity prob-

lems for crucial effector functions. However the data in Table I, although limited in number, suggest that sufficient specificity can reside in size, chemical composition, and probably secondary structure of a polynucleotide to differentiate physiologic, regulatory compounds from other, weakly inhibitory small oligonucleotides and RNA species present in a cell.

It must be stated that the highly unusual structures and mechanisms of the multisite, multireactant ribonucleotide reductases make it difficult, if not impossible to treat them in terms of standard enzyme kinetics. Nevertheless in a qualitative view the increasingly complex effects produced by simple substrate analogs such as arabinonucleotides (competitive inhibition [24]), by polynucleotides (uncompetitive inhibition), and by the oligomeric dye (mixed inhibition) appear to reflect the structure complexity of inhibitors. The oligo- and polymers may not directly interact with substrate ribonucleotide sites, which are highly specific [1]. Rather the

effector nucleotide sites, of somewhat lower specificity, and further cationic residues on the protein surface should be capable to bind the nucleotide inhibitors through a combination of ionic and base substituent-mediated interactions, with the consequence of decreased cooperativity [13, 24] among the substrate, allosteric effector, and catalytic sites. Additional inactivation of the iron-organic radical protein subunit of a ribonucleotide reductase could explain the strong, yet kinetically less defined inhibition by aurintricarboxylic acid. More detailed information about these processes, including the functioning of natural inhibitors has to await further structure analysis of the ribonucleotide reductase proteins.

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