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FEBS Letters 341 (1994) 128–130

FEBS 13782

FEBS LETTERS

Inhibitor analysis of calf thymus DNA polymerases α, δ and ε

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Received 28 January 1994

Abstract

Quantitative effects of inhibitors of the replicative DNA polymerases (pol) α, δ and ε from calf thymus are reported under similar assay conditions. Carbonyldiphosphonate was a competitive inhibitor of pols δ and ε, with 4- to 6-fold selectivity compared to pol α. Aphidicolin inhibited pols α and δ with 6- to 10-fold selectivity compared to pol ε. The 'butylphenyl' nucleotides, BuPdGTP and BuAdATP, inhibited pol α with at least 1000-fold selectivity compared to pols δ and ε. The use of these inhibitors under similar assay conditions permits the discrimination of the three enzymes.

Key words: Inhibitor; DNA polymerase; Selectivity

1. Introduction

The suggestion that multiple DNA polymerases (pol) catalyze eukaryotic DNA replication (reviewed in [1]) has been supported by the use of selective inhibitors. Monoclonal antibodies and selective small molecule inhibitors of DNA polymerase α revealed a novel enzyme, designated DNA polymerase δ, that could be separated from DNA polymerase α, but that contained an intrinsic 3′ to 5′ exonuclease activity [2,3]. Among the properties of this enzyme, its activity on homopolymer templates was found to be stimulated by proliferating cell nuclear antigen (PCNA), supporting a role for the enzyme in nuclear DNA replication [4,5]. However, 3′ to 5′ exonuclease-containing pols *insensitive* to PCNA were isolated from several sources (summarized in [6]), and genetic and sequencing studies have revealed this to be a third enzyme, designated pol ε [7], also proposed to be involved in DNA replication and, possibly, repair (reviewed in [8]).

The ability to identify isolated DNA polymerases and their role(s) in DNA replication in whole cells, nuclei or nuclear preparations requires, among other properties, careful definition of the inhibitor sensitivities of the enzymes. The diterpene aphidicolin has been reported to inhibit pols α, δ and ε with similar potencies [9]. The synthetic nucleotides BuPdGTP and BuAdATP (the 'butylphenyl' nucleotides), however, are highly selective for inhibition of pol α compared to their effects on pols δ and ε ([9], reviewed in [10]). In a search for a selective inhibitor of pol δ, at a time when pol ε was still unrecognized, we first identified a pyrophosphate analog, difluoromethylene-diphosphonate, as a weak but selective inhibitor of calf thymus pol δ [11]. That discovery led to the finding that a related compound, carbonyldiphosphonate (COMDP), was a potent inhibitor of pol δ, with about tenfold selectivity relative to its effect on calf thymus pol α [12]. However, the enzyme preparation used to identify COMDP was a PCNA-insensitive form of pol δ [13], an enzyme now properly identified as pol ε [6]. Although there have been several reports in which COMDP has been suggested to inhibit pol δ selectively, no systematic study of the relative potencies of this and the other small molecule inhibitors for the three enzymes has been reported. Given that all three enzymes have been isolated from calf thymus [6], we now report a direct comparison of inhibitor sensitivities of pols α, δ and ε from that tissue, and suggest limitations in the use of inhibitors to distinguish the enzymes.

2. Materials and methods

Aphidicolin was a gift from the Natural Products Chemistry Branch, national cancer institute. Other inhibitors were synthesized as described: carbonyldiphosphonate (COMDP), tetrasodium salt, according to [12], N^2-(*p-n*-butylphenyl)-2′-de oxyguanosine 5′-triphosphate (BuPdGTP) according to [14], and 2-(*p-n*-butylanilino)-2′-deoxyadenosine 5′-triphosphate (BuAdATP) according to [15]. Calf thymus pol α was purified on an immunoaffinity matrix as described [16]. Calf thymus pol ε was the FPLC-purified fraction [13], and pol δ was separated from pol ε on hydroxyapatite [6]. PCNA was purified accord-

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SSDI 0014-5793(94)00167-T

ing to [17]. Nucleotides and oligonucleotides were from Pharmacia, and all chemicals were reagent grade.

Enzymes were assayed in 25 μl reaction volumes containing 75 mM HEPES · KOH (pH 7.5), 20% glycerol, 1.25 mM dithiothreitol, 10 mM $MgCl_2$, 250 μg/ml bovine serum albumin, 0.1 mM poly dA:oligo dT (base ratio 10:1) and 5 μM [^{3}H]dTTP (1250 cpm/pmol). One mM GMP was present in assays of pol δ and pol ε to inhibit 3′ to 5′ exonuclease activity. PCNA (50 ng) was present in each pol δ assay. Reactions were initiated by the addition of enzyme: 0.1 unit of pol α, 0.08 unit of pol δ, or 0.05 unit of pol ε. The reactions were incubated for 30 min (pol α) or 60 min (pol δ, pol ε) at 37°C, and stopped by addition of 10 mM EDTA and 2 μg of heat-denatured DNA. Polynucleotides were precipitated by the addition of 1 ml of 10% trichloroacetic acid, filtered through GFA circles (Whatman), and washed with 10 ml of 1 N HCl containing 100 mM sodium pyrophosphate, and finally with ethanol. Dried filters were counted in 1 ml of Optifluor. Control activities corresponded to 28.4, 12 and 20.8 pmol dTMP incorporated per assay tube for pols α, δ and ε, respectively.

Inhibitors were tested by addition of stock solutions in water (BuPdGTP, BuAdATP), HEPES·KOH buffer (COMDP), or dimethylsulfoxide (aphidicolin); control assays contained the same volume of inhibitor diluent. The percent inhibition of enzyme activity at 4–5 concentrations of inhibitor was plotted as a function of log concentration to obtain the IC_{50} values. Results from duplicate experiments were in the range of ± 30%. K_i values for COMDP are the average of $K_i = I/[(K_m'/K_m)-1]$, where K_m' is the apparent K_m of dTTP in the presence of COMDP at concentration I. K_m values were obtained with the computer program *Enzyme Kinetics* (D.G. Gilbert, Indiana University).

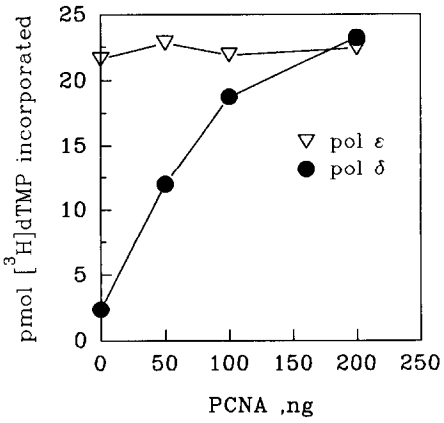

Fig. 1. Effect of PCNA on calf thymus pols δ and ε. Enzymes were assayed with poly dA:oligo dT (10:1) and 5 μM [^{3}H]dTTP as described in section 2.

3. Results and discussion

In order to compare inhibitor sensitivities under similar assay conditions, all enzymes were assayed by incorporation of [^{3}H]dTTP into poly dA:oligo dT (base ratio 10:1). This template:primer is one of only few that sustain activity of all three enzymes, although pol δ is only active in this system in the presence of PCNA [6]. A template:primer suitable for mechanistic studies of BuPdGTP, i.e. poly dC:oligo dG, poorly sustained pol δ activity even in the presence of PCNA (data not shown). To assure the identity of DNA polymerases δ and ε the response of each to PCNA was tested first. The results of Fig. 1 show that the activity of pol δ was stimulated fifteenfold by 200 ng PCNA/assay, but pol ε was unaffected. Thus, in all subsequent experiments inhibition of pol δ was tested in the presence of 50 ng PCNA/assay.

Typical percent inhibition/log concentration curves for the three enzymes are displayed in Fig. 2, and the IC_{50} values are summarized in Table 1. The enzymes were assayed in HEPES buffer because COMDP is inactivated in the customary Tris buffer [12]. COMDP inhibited pol δ and pol ε with similar potencies, demonstrating that the compound is an equivalent inhibitor of both enzymes. However, COMDP had only 4- to 6-fold selectivity for these enzymes relative to its effect on pol α (Fig. 2B), compared to the tenfold selectivity previously reported for pol ε [12]. This result may be due, in part, to the different mechanisms of inhibition of the enzymes. COMDP was found to be a non-competitive inhibitor of pol α, but a competitive inhibitor of pol ε [12]. Indeed, analysis of the inhibitory effect of COMDP in assays with poly dA:oligo dT at several dTTP concentrations revealed competitive kinetics for both pol δ and pol ε (data not shown). For pol δ the K_i was 4.0 μM, and the K_m for dTTP was 3.1 μM. For pol ε the K_i was 4.9 μM, and the K_m for dTTP was 5.7 μM. The latter result contrasts with the competitive K_i value of 1.8 μM previously reported for inhibition of pol ε in the same assay system [12].

The nucleotides BuPdGTP (Fig. 2A) and BuAdATP clearly inhibited pol α selectively, with IC_{50} values at least three orders of magnitude lower than those for pol δ and pol ε (Table 1). The typical *nanomolar* inhibitory potency of the nucleotides against pol α was not observed, because the assay involved a template, poly dA, which is not complementary to the base of either compound [18]. However, the sub-*micromolar* potencies observed are consistent with the template-independent, non-substrate mechanism by which these butylphenyl nucleotides inhibit pol α in this assay system [16].

Aphidicolin inhibited both pol α and pol δ with similar potencies, but was a weaker inhibitor of pol ε (Table 1;

Table 1
Inhibitors of calf thymus DNA polymerases[1]

Inhibitor	IC_{50} (μM)		
	pol α	pol δ[2]	pol ε
COMDP	26.2	4.4	6.7
Aphidicolin	0.5	0.9	5.8
BuPdGTP	0.026	100	87
BuAdATP	0.18	138	184

[1] Enzymes were assayed with poly dA:oligo dT (10:1) and 5 μM [^{3}H]dTTP as described in section 2.
[2] + 50 ng PCNA per assay.

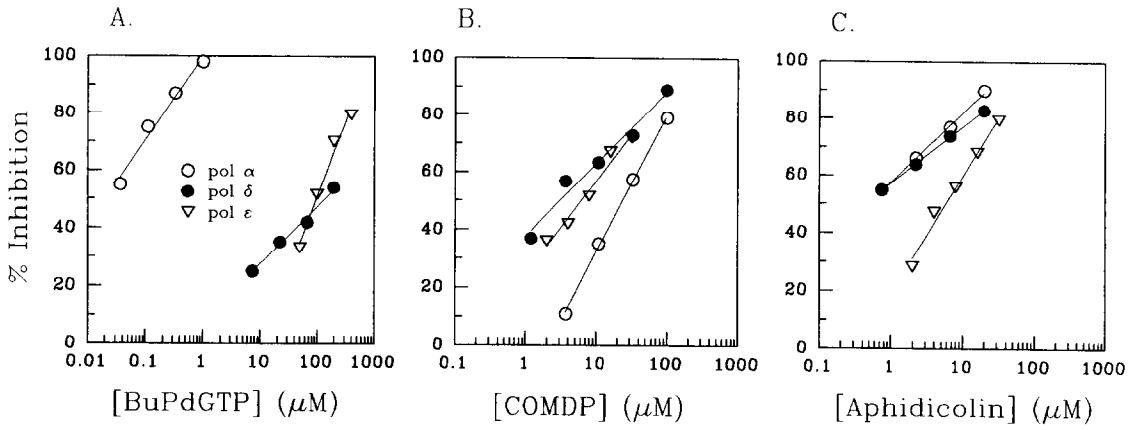

Fig. 2. Percent inhibition/log inhibitor concentration curves for calf thymus pol inhibitors: BuPdGTP (panel A), COMDP (panel B) and aphidicolin (panel C). Inhibitor assays were done as described in section 2. ○, pol α; ●, pol δ; ▽, pol ε.

Fig. 2B). Aphidicolin has been reported to inhibit pol α competitively with each dNTP [19]. Thus, the relative potencies in Table 1 suggest that aphidicolin can distinguish pol ε from both pol α and pol δ under these assay conditions.

In summary, pols α, δ and ε may be distinguished by selective inhibitors when assayed under similar assay conditions. Pol α can clearly be distinguished from pols δ and ε, first by the much greater sensitivity of the former enzyme to BuPdGTP and BuAdATP, and second by the marginally greater sensitivity of the latter enzymes to COMDP. The results of this work suggest that pol ε may be distinguished from pol δ (and pol α) by the lesser sensitivity of pol ε to aphidicolin.

The IC_{50} results for COMDP and aphidicolin (Table) are consistent with reported effects of single concentrations of the inhibitors on the calf thymus [6] and HeLa [20] DNA polymerases. However, the marginal selectivity of both COMDP and aphidicolin for the respective enzymes indicates that inhibition results may only be reliable at concentrations that partially inhibit the more sensitive enzyme. The inhibition curves in Fig. 2B show that 5 μM COMDP will inhibit both pol δ and pol ε by ~50%, but pol α will be almost unaffected. Similarly, aphidicolin at 1 μM will inhibit both pol α and pol δ by >50%, but pol ε will be almost unaffected (Fig. 2C). The most selective inhibitors, BuPdGTP (Fig. 2A) and BuAdATP, at 1–10 μM, will completely inhibit pol α, but pol δ and pol ε will be unaffected. The limitations in ability to distinguish pol δ and pol ε stimulate our continued search for selective inhibitors of these enzymes.

Acknowledgements: The authors are grateful to Joseph Gambino for technical assistance and to NATO for a collaborative research grant. This work was supported by grants GM21747 from the National Institutes of Health (to G.E.W.), 31.2859.90 from the Swiss National Science Foundation (to U.H.), and from the Italian ISS-AIDS Project (to F.F.).

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