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Development of Selective Inhibitors of DNA Polymerase Delta: A Thesis

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DEVELOPMENT OF SELECTIVE INHIBITORS OF DNA POLYMERASE DELTA

A Thesis Presented

By

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I dedicate this to my Best Man.

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ABSTRACT

This thesis is divided into three parts, united by the theme of the development of selective inhibitors of mammalian cell DNA polymerase delta (pol δ). The first part consists of an investigation of the cytotoxic mechanism(s) of certain 2-substituted adenine analogs, selected on the basis of their inhibitory properties towards DNA polymerase alpha (pol α) and mammalian cell DNA synthesis. The second is a direct search for inhibitors of isolated pol δ, and an investigation of inhibitory mechanisms. The third consists of measurement of the effects of a selective pol δ inhibitor on cellular DNA synthesis.

Mechanism of cytotoxicity of 2-substituted adenine analogs. The mechanism of inhibition by 2-(p-n-butylanilino)-2'-deoxyadenosine (BuAdA), and related

compounds, of Chinese hamster ovary (CHO) cell [^{3}H]thymidine ([^{3}H]TdR) incorporation, was investigated. The potency of the compound could largely be explained by its potency (IC_{50} = 23 μM) as an inhibitor of CHO cell [^{3}H]TdR uptake. BuAdA inhibited incorporation by CHO cells of [^{32}P]phosphate into DNA relatively weakly, displaying an IC_{50} value of 80 μM.

Differential inhibition of DNA polymerases alpha and delta. Known DNA polymerase inhibitors of a structurally wide range were screened for their ability to inhibit pol δ derived from calf thymus selectively with respect to pol α derived from the same tissue. Pyrophosphate (PP_i) and difluoromethanediphosphonate each inhibited pol δ weakly, but with greater potency than pol α. Based on this lead, an expanded series of PP_i analogs was screened. Carbonyldiphosphonate (COMDP) inhibited pol δ with a potency (K_i = 1.8 μM) twenty-two times greater than that displayed for pol α. Kinetic studies indicated that COMDP inhibited pol δ competitively with the dNTP specified by the template, but not competitively with the template:primer. Analogous experiments with pol α showed that the compound inhibited that enzyme uncompetitively with the dNTP, and not competitively with the template:primer. COMDP was a weak inhibitor of the 3' → 5' exonuclease activity of pol δ, displaying an IC_{50} value greater than 1 mM.

Inhibition of permeabilized cell DNA synthesis by a

selective pol δ inhibitor. The potency of COMDP as an inhibitor of permeabilized CHO cell DNA synthesis (IC_{50} = 200 μM) did not clearly indicate the participation of pol δ in cellular DNA replication.

Prospectus. The thesis concludes with a prospectus for the development of pol δ inhibitors with improved properties compared to COMDP.

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ABBREVIATIONS

BSA	bovine serum albumin
CHO	Chinese hamster ovary
dA	deoxyadenosine
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid
HMPA	hexamethylphosphoramide
Leu	leucine
$NaBH_4$	sodium borohydride
$NaPP_i$	sodium pyrophosphate
NMMPR	nitrobenzylthioinosine
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
pol α	DNA polymerase alpha
pol β	DNA polymerase beta
pol	DNA polymerase gamma
pol δ	DNA polymerase delta
ppm	parts per million
TCA	trichloroacetic acid
TdR	thymidine
TMS	trimethylsilane

Tris	tris(hydroxymethyl)aminomethane
Urd	uridine

INHIBITOR ACRONYMS

Acronym		Structure (Figure, Page)
2AA	2-anilinoadenine	III-3, 46
ACGTP	9-[(2-hydroxyethoxy)methyl]guanine triphosphate	IV-2, 81
araATP	9-β-D-arabinofuranosyl)adenine 5'-triphosphate	IV-1, 78
araCTP	9-β-D-arabinofuranosyl)cytidine 5'-triphosphate	IV-1, 78
AZTTP	(3'-azido)-2'-deoxythymidine 5'-triphosphate	IV-4, 86
BuAA	2-(p-n-butyalanilino)adenine	III-1, 41
BuAdA	2-(p-n-butyalanilino)-2'-deoxyadenosine	III-1, 41
BuAdATP	2-(p-n-butyalanilino)-2'-deoxyadenosine 5'-triphosphate	I-1, 7
BuPdG	N^2-(p-n-butylphenyl)-2'-deoxyguanosine	III-1, 41
BuPdGTP	N^2-(p-n-butylphenyl)-2'-deoxyguanosine 5'-triphosphate	I-1, 7
BuPG	N^2-(p-n-butylphenyl)guanine	III-1, 41
ClAP	2-anilino-6-chloropurine	III-3, 46
COMDP	carbonyldiphosphonate	IV-11, 108

DAP	2,6-diaminopurine.....III-6, 55
DAPdR	2-amino-2'-deoxyadenosine.....III-6, 55
DCAA	2-(3,4-dichloroanilino)adenine.....III-2, 45
DCACl	2-(3,4-dichloroanilino)-6-chloropurine.....III-2, 45
DCACldR	2-(3,4-dichloroanilino)-6-chloro-9-(2-deoxy-β-D-ribofuranosyl)purine.....III-4, 48
DCAdA	2-(3,4-dichloroanilino)-2'-deoxyadenosine.....III-4, 48
DCAdAMP	2-(3,4-dichloroanilino)-2'-deoxyadenosine 5'-phosphate.....III-5, 50
DCAdATP	2-(3,4-dichloroanilino)-2'-deoxyadenosine 5'-triphosphate.....III-5, 50
DCMP	2-(3,4-dichloroanilino)-6-methoxypurine.....III-2, 45
DCMTP	2-(3,4-dichloroanilino)-6-methylthiopurine.....III-2, 45
DCPG	N^2-(3,4-dichlorophenyl)guanine.....III-2, 45
DCTG	N^2-(3,4-Dichlorophenyl)-6-thioguanine.....III-2, 45
ddTTP	2',3'-dideoxythymidine 5'-triphosphate.....IV-4, 86
EMPdGTP	N^2-(3-ethyl-4-methylphenyl)-2'-deoxyguanosine 5'-triphosphate.....IV-2, 81
F_2MDP	difluoromethanediphosphonate.....IV-5, 87

HexdGTP	N^2-(n-hexyl)-2'-deoxyguanosine 5'-triphosphate	IV-2, 81
MDP	methanediphosphonate	IV-5, 87
PAA	phosphonoacetic acid	IV-5, 87
PFA	phosphonoformic acid	IV-5, 87
PhdGTP	N^2-(phenyl)-2'-deoxyguanosine 5'-triphosphate	IV-2, 81
PP_i	pyrophosphate	IV-5, 87

CHAPTER I

INTRODUCTION

A. Roles of the Mammalian Cell DNA Polymerases in DNA Replication

An understanding of mammalian cell DNA replication will require an understanding of the properties and roles of the enzymes which catalyze the key reactions in that process. There are four known enzymes in mammalian cells capable of catalyzing DNA polymerization, DNA polymerases alpha (pol α), beta (pol β), gamma (pol γ) and delta (pol δ) (reviewed in Fry & Loeb, 1986). The roles of the four DNA polymerases in DNA replication and repair have not been precisely determined, and that determination represents one of the fundamental problems in modern biology.

DNA polymerases beta and gamma probably do not contribute to genomic DNA replication. The roles of pol β and pol γ in mammalian cell DNA metabolism seem limited to the repair of genomic DNA and metabolism of mitochondrial DNA, respectively. Experiments employing the antibiotic aphidicolin (Brundret et al., 1972) (Figure IV-3) have provided evidence against the hypothesis that pol β, pol γ or both participate in DNA replication. The compound is a potent inhibitor of pol α (Ikegami et al., 1978) and pol δ (Lee et al., 1981; Goscin & Byrnes, 1982), and is without

effect on pol β and pol γ (Ikegami et al., 1978). DNA replication is highly sensitive to inhibition by aphidicolin (Ikegami et al., 1978; Hanaoka et al., 1979; Berger et al., 1979; Huberman et al., 1981), consistent with the hypothesis that neither of the aphidicolin resistant DNA polymerases participate in DNA replication. A second line of evidence concerns the level of activity of the various DNA polymerases as a function of the stage of the cell cycle. One would expect that the activity of a replicative DNA polymerase would increase prior to S phase. The level of pol α activity positively correlates with the level of replicative DNA synthesis throughout the cell cycle (see below), but the levels of pol β and pol γ are independent of DNA replication (Chang et al., 1973; Fry & Loeb, 1986). In addition, Zmudzka et al. (1988) showed that the cellular level of pol β mRNA also was independent of the cell cycle. Experiments using selective inhibitors of pol β have supported the idea that the mission of that enzyme is DNA repair (Miller & Chinault, 1982a; 1982b; Cleaver, 1983; Yamada et al., 1985), although the experiments are complicated by, for example, the participation of pol α in DNA repair, and the apparent ability of pol α and pol β to compensate for one another in repair synthesis when the other enzyme is selectively inhibited (Fry & Loeb, 1986). Pol γ is found in mitochondria (Bolden et al., 1977), and is

likely the only DNA polymerase in mitochondria (Bolden et al., 1977; Zimmerman et al., 1980). It is believed to be the only DNA polymerase involved in mitochondrial DNA metabolism (Fry & Loeb, 1986). Pol γ also has been identified in nuclei (Bertazzoni et al., 1977; Hübscher et al., 1977), although there is no positive evidence for a role for that enzyme in genomic DNA metabolism (Fry & Loeb, 1986).

A role for DNA polymerase alpha in DNA replication is firmly established. The most firmly established role for any of the known mammalian DNA polymerases in DNA metabolism is that of pol α in genomic DNA replication. Three major lines of evidence, summarized below, indicate that pol α is a major or the sole replicative DNA polymerase in mammalian cells. (i) The level of pol α activity in cells closely tracks the level of replicative DNA synthesis throughout the cell cycle (Fry & Loeb, 1986). Pol α activity is low in quiescent cells (Bertazzoni et al., 1976), and increases dramatically when cells are stimulated to divide (Chang et al., 1973; Bertazzoni et al., 1976) or when synchronized cells enter S phase (Spadari & Weissbach, 1974; Chiu & Baril, 1975). Wong et al. (1988) found that the level of pol α mRNA is significantly higher in proliferating cells than in quiescent cells. Wahl et al. (1988) showed that pol α mRNA levels increased just prior to the peak of DNA

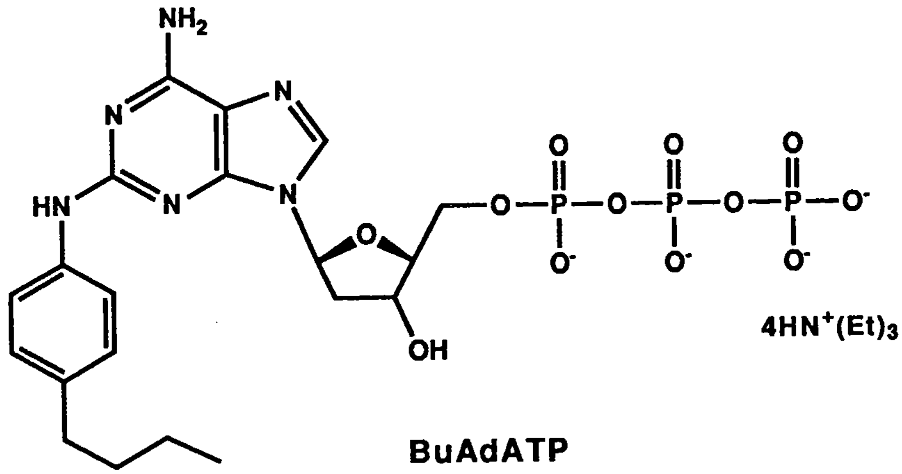
synthesis in quiescent cells stimulated to divide, suggesting that regulation of pol α gene expression is at the transcriptional level. Thömmes et al. (1986) demonstrated that the increase in pol α activity during S phase was accompanied by an increase in the synthesis of the enzyme. (ii) Inhibitors of pol α inhibit mammalian cell DNA replication (Fry & Loeb, 1986). Ikegami et al. (1978) found that aphidicolin prevented cell division in sea urchin embryos as a result of its inhibition of DNA synthesis. Pedrali-Noy et al. (1980) later showed that aphidicolin was capable of synchronizing HeLa cells by blocking their entry into S phase. Liu and Loeb (1984) isolated pol α from aphidicolin resistant mammalian cells, and found that the enzyme was aphidicolin resistant. Unfortunately, in vivo studies with aphidicolin are ambiguous with respect to the roles of pol α and pol δ in DNA synthesis, because aphidicolin inhibits the two enzymes with equal potencies (Lee et al., 1981; Goscin & Byrnes, 1982). The possibility that inhibition of DNA synthesis by aphidicolin is at least partially a result of inhibition of pol α in vivo is supported by the observations of Miller et al. (1985), who found that DNA synthesis in permeable human fibroblasts could be inhibited up to 70 to 80% by the monoclonal antibody SJK 132-20 (Tanaka et al., 1982), a reagent which neutralizes pol α but not pol δ (Byrnes, 1985). (iii) A

mouse cell line which was temperature sensitive in DNA replication yielded a temperature sensitive mutant form of pol α (Murakami et al., 1985; Eki et al., 1986; Eki et al., 1988).

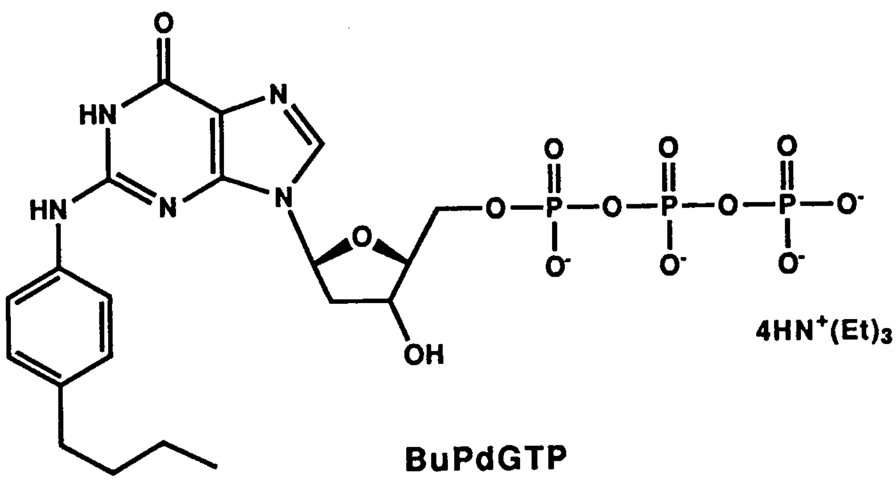
Role of DNA polymerase delta in DNA replication.

Although pol δ has been known for about thirteen years (Byrnes et al., 1976), there is no direct evidence for its involvement in DNA replication. There are no known conditional mutants of pol δ. Until recently, there were neither any selective small molecule inhibitors of pol δ (Talanian et al., submitted), nor any inhibitory monoclonal antibodies specific for pol δ (Lee et al., 1989). In the absence of pol δ-specific reagents, the function of pol δ has been inferred indirectly by assessment of the amount of replicative DNA synthesis which is resistant to inhibition by selective pol α inhibitors but sensitive to inhibition by aphidicolin. Dresler and Frattini (1986; 1988) found that N^2-(p-n-butylphenyl)-2'-deoxyguanosine 5'-triphosphate (BuPdGTP, Figure I-1) (Wright & Dudycz, 1984), a potent inhibitor of pol α (Khan et al., 1984) which inhibits pol δ only weakly (Byrnes, 1985; Lee et al., 1985), inhibited replicative DNA synthesis in permeabilized human fibroblasts with a potency several orders of magnitude less than that which it displayed for inhibition of pol α. Dresler and Frattini concluded that the unexpected resistance of DNA

synthesis to inhibition by BuPdGTP may have been a result of the participation of a BuPdGTP-insensitive enzyme, possibly pol δ, in DNA synthesis. Hammond et al. (1987) made somewhat different observations, but came to the same conclusion. They found that pol α-specific neutralizing monoclonal antibodies or BuPdGTP inhibited replicative DNA synthesis in permeable cells by roughly 50%, and that the remaining activity was resistant to those inhibitors but sensitive to inhibition by aphidicolin. The latter results were consistent with the possibility that replicative DNA synthesis was catalyzed in roughly equal proportions by pol α and pol δ. Other, less direct studies have also supported the hypothesis that pol δ participates in DNA replication. For example, Prelich et al. (1987a) have shown that the pol δ cofactor proliferating cell nuclear antigen (PCNA) (see below) is required for SV40 replication; to the extent that SV40 replication is an accurate model for genomic DNA replication, that observation implicates pol δ in genomic DNA replication. The observations of Hurwitz and coworkers (Lee et al., 1988) have, however, put the latter conclusions in doubt. They found that PCNA stimulates SV40 replication by counteracting the effect of an inhibitory factor, rather than by the direct stimulation of pol δ. Stronger evidence for the involvement of PCNA, and thus by association pol δ,



BuAdATP



BuPdGTP

Figure I-1. Structures of BuAdATP and BuPdGTP

in cellular DNA replication, is the observation of Liu et al. (1989) that inhibition of Chinese Hamster Ovary (CHO) cell PCNA biosynthesis, by means of introduction into cells of an antisense oligonucleotide complementary to PCNA mRNA, effectively blocked entry of CHO cells into S phase.

B. Properties of DNA Polymerase Delta

DNA polymerase delta: a mammalian DNA polymerase with 3' → 5' exonuclease activity. Pol δ was first isolated from rabbit erythroid hyperplastic bone marrow (Byrnes et al., 1976). The enzyme has since been isolated from a variety of sources, including calf thymus (Lee et al., 1980), CV-1 cells (Hammond et al., 1987), human placenta (Lee & Toomey, 1987) and HeLa cells (Nishida et al., 1988), suggesting that pol δ is a ubiquitous mammalian DNA polymerase. The major feature of pol δ which distinguished it most clearly from the other known mammalian DNA polymerases was its possession of a 3' → 5' exonuclease activity, which seemed to be on the same peptide as the DNA polymerase activity (Byrnes et al., 1976; Goscin & Byrnes, 1982). Most replication specific prokaryotic DNA polymerases have an associated 3' → 5' exonuclease activity which increases the replication fidelity of the DNA polymerase by a "proofreading" or "editing" mechanism (Goodman, 1988; Kunkel, 1988). With few exceptions (Chen et al., 1979; Ottiger & Hübscher, 1984;

Skarnes et al., 1986; Reyland et al., 1988), experiments indicate that pol α is devoid of a 3' → 5' exonuclease activity, and the absence of that activity is believed to be the reason for the relatively low replication fidelity of pol α *in vitro* (Loeb & Kunkel, 1982). It is believed that, if the low fidelity of pol α *in vitro* is not an artefact, other mechanisms, including "accessory" nucleases, must compensate *in vivo* for the low replication fidelity of pol α *in vitro*. In the case of pol δ, replication fidelity is increased significantly by the action of its associated 3' → 5' exonuclease activity (Byrnes et al., 1977; Kunkel et al., 1987; Sabatino & Bambara, 1988); behavior which strongly supports the hypothesis that pol δ is a replicative DNA polymerase *in vivo*.

Enzymatic Properties of DNA polymerase delta; comparison with DNA polymerase alpha. Pol α and pol δ also can be distinguished on the basis of the requirements and behavior of their DNA polymerase activities, as well as the presence of associated activities. Pol δ prefers synthetic template:primers such as poly(dA):oligo(dT) and the alternating copolymer poly(dA-dT), although the pattern of template preferences of that enzyme depends on its source and the method of its purification (So & Downey, 1988). Pol α prefers template:primers with a high primer density, although, like pol δ, the template preference of pol α is a

function of its method and source of purification (Kaguni & Lehman, 1988). Pol α and pol δ also can be distinguished by their processivity; the number of nucleotides that, on average, the enzyme adds to a growing primer chain before the enzyme dissociates from the template:primer. Pol α is generally regarded as a nonprocessive (distributive) enzyme, adding on the order of 5 to 100 nucleotides to a growing primer chain before dissociation (Fry & Loeb, 1986). In the presence of PCNA, the processivity of pol δ is at least two orders of magnitude greater than that generally reported for pol α (Downey et al., 1988; So & Downey, 1988). In the absence of PCNA, pol δ synthesizes DNA in a distributive manner (Tan et al., 1986; Prelich et al., 1987b), although processive forms of pol δ in the absence of PCNA are known (Focher et al., 1989; see below). The difference in the observed processivities of pol α and pol δ *in vitro* may not reflect their processivities *in vivo*; under the appropriate conditions, which may or may not reflect *in vivo* conditions, highly processive synthesis by both enzymes is possible (Sabatino et al., 1988). Another distinguishing characteristic is that pol δ is capable of strand-displacement synthesis (Downey et al., 1988), whereas pol α is not (Murakami et al., 1986; Downey et al., 1988). Finally, pol α contains a tightly associated DNA primase activity which catalyzes the synthesis of RNA primers,

allowing the enzyme to use single stranded DNA as a template (Fry & Loeb, 1986). With one possible exception (Crute et al., 1986), pol δ does not appear to contain a DNA primase activity.

PCNA: A stimulatory cofactor of DNA polymerase delta. PCNA is a cell cycle regulated nuclear protein whose rate of synthesis closely tracks the rate of DNA synthesis in proliferating cells throughout the cell cycle (for a review, see Celis et al., 1987). PCNA is identical with a previously described cell cycle regulated nuclear protein called cyclin (Mathews et al., 1984). PCNA is an accessory protein of pol δ (Tan et al., 1986; Bravo et al., 1987; Prelich et al., 1987b), capable of stimulating the activity of that enzyme more than two orders of magnitude (Tan et al., 1986), depending on the assay conditions. Tan et al. (1986) and Prelich et al. (1987b) have observed that PCNA stimulates pol δ by increasing its activity and its processivity. PCNA-dependent and PCNA insensitive forms of pol δ have been described (Crute et al., 1986; Focher et al., 1988c; 1989); calf thymus has been the source of both (Lee et al., 1984; Focher et al., 1989). The relationship between PCNA-sensitive and insensitive forms of pol δ is not known. There are no known preparations of pol α which are stimulated by PCNA.

Structural relationship between DNA polymerases alpha

and delta. During most of the history of pol δ, the structural relationship between it and pol α was unknown. The hypothesis that pol δ was a precursor to or breakdown product of pol α, or perhaps a posttranslationally modified form of pol α, seemed at least as likely as the hypothesis that they were different gene products. Cotterill et al. (1987) observed that separation of the polymerase subunit from the four peptide Drosophila pol α-primase complex revealed a 3' → 5' exonuclease activity; later, the same group observed (Reyland et al., 1988) that the isolated polymerase subunit is, like pol δ, far less sensitive to inhibition by BuPdGTP than the intact pol α-primase. The latter observations supported the hypothesis that pol α and pol δ were somehow structurally related. However, recent observations seem to have tilted the argument in favor of the hypothesis that pol α and pol δ are the products of different genes. First, Lee and Toomey (1987) have shown by Western blotting that a mouse heteroantiserum raised against pol δ reacted with pol δ but not with pol α, and that monoclonal antibodies raised against pol α displayed no reactivity with pol δ. The same group (Lee et al., 1989) later showed that of eight pol δ-neutralizing monoclonal antibodies, two were inhibitory to pol α, indicating that the two proteins are distinct, but have some structurally common relationship. The second and perhaps more important

observation was that of Focher et al. (1989), and Wong et al. (1989) who independently showed by tryptic peptide analysis that the DNA polymerase catalytic subunits of calf thymus pol α and pol δ were different. The gene encoding human pol α has been cloned and sequenced (Wong et al., 1988); until the pol δ gene also has been cloned and sequenced, the structural relationship between the two enzymes will remain unclear.

Differential inhibitors of DNA polymerases alpha and delta. Pol α and pol δ display similar sensitivities to aphidicolin (Lee et al., 1981; Goscin & Byrnes, 1982) and sulfhydryl-reacting compounds such as N-ethylmaleimide (Byrnes, 1984). For some time, those observations were cited as evidence for the hypothesis that pol δ was a form of pol α. Khan et al. (1984) and Lee et al. (1985) showed that BuPdGTP and its dATP analog, 2-(p-n-butyalanilino)-2'-deoxyadenosine 5'-triphosphate (BuAdATP, Figure I-1) displayed differential inhibition of pol α and pol δ, inhibiting the former enzyme with far greater potency than the latter. Byrnes (1985) also showed that the pol α-neutralizing antibody SJK 132-20 (Tanaka et al., 1982) had no effect on pol δ at concentrations which were completely inhibitory to pol α. Differential inhibition of the two enzymes reinforced the hypothesis that the two enzymes were distinct gene products. Further, selective inhibition of

pol α by BuPdGTP and monoclonal antibodies facilitated experiments designed to determine the role of pol α, independently of pol δ, in DNA metabolism. Analogous experiments designed to determine the role of pol δ were not possible, since all known differential inhibitors of the two enzymes did so by selectively inhibiting pol α.

C. Why Might There Be Two Replicative DNA Polymerases in Mammalian Cells?

Asymmetry at the replication fork. All known DNA polymerases catalyze DNA synthesis by adding dNMP monomers to a growing primer chain exclusively in the 5' → 3' direction (Kornberg, 1980). As a result, there is a fundamental asymmetry at the replication fork, wherein the "leading strand" is synthesized toward the replication fork in a continuous manner, and the "lagging strand" is synthesized away from the fork from a number of short RNA primers in a discontinuous manner. The demands on a DNA polymerase synthesizing leading and lagging strands at the replication fork are therefore likely to be very different.

Hypothesis: DNA polymerases alpha and delta catalyze lagging and leading strand synthesis, respectively. Based on the properties of pol α and pol δ, a model has been proposed in which the two enzymes act coordinately at the DNA replication fork, the former catalyzing lagging strand

synthesis, and the latter catalyzing leading strand synthesis (Focher et al., 1988a; Downey et al., 1988). Continuous synthesis on the leading strand would best be catalyzed by a highly processive enzyme such as pol δ (see above), while discontinuous synthesis might be catalyzed most rapidly by the less processive pol α. Frequent primer synthesis would be necessary during discontinuous synthesis, while continuous synthesis would require priming only rarely. Thus, the presence of a primase activity tightly associated with pol α and not with pol δ (see above) makes the former enzyme a more suitable candidate for lagging strand synthesis than the latter. Finally, the ability to conduct strand displacement synthesis, a capability of pol δ but not of pol α (see above), might be crucial to the function of a leading strand DNA polymerase, since it may be required to replicate through duplex regions of DNA, whereas a lagging strand polymerase would not. Focher et al. (1988a) have observed that the ratio of pol α to pol δ activity in calf thymus was invariably 1:1 using eight different extraction procedures and in three subcellular locations. Zhang and Lee (1987) observed that the ratio of pol α to pol δ activity in neonatal rat heart was constant during maturation and terminal differentiation of heart cells. Both sets of observations are consistent with the hypothesis that pol α and pol δ function as an asymmetric

dimer.

The asymmetric dimer hypothesis of Focher et al. (1988a) and Downey et al. (1988) is not without precedent. It was first proposed in 1980 (Sinha et al., 1980) to explain the properties of a reconstituted T4 bacteriophage DNA replication system. The hypothesis has been supported by the observations of McHenry (1982; 1988), who has described the properties of a heterodimeric form of E. coli DNA polymerase III. The two halves of the dimer differed according to the identity of their associated subunits, and had the functional properties expected of a leading and lagging strand DNA polymerase.

D. Statement of the Problem

State of the art. As described above, a role for pol α in mammalian cell DNA replication is firmly established, and a role for pol δ is not. However, considerable circumstantial evidence suggests a role for pol δ in DNA replication, and a consideration of the properties of pol δ and pol α as well as the expected requirements of leading and lagging strand DNA polymerases suggests a specific and plausible model for the cooperation of the two enzymes in DNA replication. The role of pol δ in DNA replication, as well as its structural and functional relationships with pol α, remain to be determined.

Approach. At the start of the work described here, discrepancies had been observed between the potencies of several nucleoside and nucleobase analogs as inhibitors of pol α and of mammalian cell DNA replication (see Chapter III), suggesting that inhibition of pol α was an insufficient model for inhibition of cellular DNA synthesis. There also was considerable evidence, which has since been supported, that pol α was probably not the only replicative DNA polymerase. Both sets of observations suggested that pol δ might participate in mammalian cell DNA replication.

I approached the problem of the possible role of pol δ in DNA replication by seeking selective inhibitors of that enzyme which might be used to probe its in vivo function. I began the search by investigating the cytotoxic mechanisms of several compounds which appeared to inhibit cellular DNA synthesis with unexpectedly great potencies relative to their potencies as inhibitors of pol α and, thus, may have acted on cells by potent inhibition of pol δ. When I found that the apparent potencies of these compounds as inhibitors of cellular DNA synthesis was a function of the method chosen for measuring cellular DNA synthesis, I took a different approach, seeking directly an inhibitor of pol δ which was selective for that enzyme relative to pol α. When I found such a compound, I thoroughly characterized its action as an inhibitor of pol δ, and then measured its

effects on cellular DNA synthesis, in an attempt to detect the participation of pol δ in mammalian cell DNA replication.

CHAPTER II

MATERIALS AND METHODS

A. Materials

[^{3}H]TdR, [^{3}H]Urd, [^{3}H]Leu, [^{3}H]dTTP, [^{3}H]dGTP, [^{32}P]phosphate, [^{32}P]dGTP, [^{32}P]dCTP and [^{32}P]dATP were purchased from New England Nuclear or Amersham. Poly(dA), poly(dC), oligo (dT) [(d)T_{12-18}], oligo(dG) [(d)G_{12-18}] and Sephadex G-25 were purchased from Pharmacia. Calf thymus DNA was purchased from Worthington. Except as noted, all other materials were purchased from Sigma or VWR.

B. Inhibitors

Melting points were determined on a Mel-temp apparatus and are uncorrected. Elemental analyses (C, H & N) were performed by the Microanalysis Laboratory at the University of Massachusetts, Amherst, MA; phosphorus elemental analyses were done by the method of Peterson (1978). ^{1}H NMR spectra in Me_2SO-d_6 were obtained at 250 MHz on a Bruker WM250 instrument, at 200 MHz on a Bruker AC/P200 instrument, or at 60 MHz on a Perkin Elmer R12B/TT7 Fourier Transform instrument; ^{31}P NMR spectra in D_2O were obtained at 102 MHz on a Bruker WM250 instrument. The ^{1}H NMR spectrum at 200 MHz, some 250 MHz spectra, and all ^{31}P NMR spectra were obtained by Steven Freese. Chemical shifts are reported in

ppm relative to external TMS (^1H) and external 85% H_3PO_4 (^{31}P).

N^2-(3,4-Dichlorophenyl)-6-thioguanine (DCTG). A solution of N^2-(3,4-dichlorophenyl)guanine (DCPG)[1] (924 mg, 3.18 mmol) in pyridine (35 mL) was treated with P_2S_5 (2.8 g). After 3 h at reflux, the volume of the solution was reduced about 50% by rotary evaporation. The remaining solution was poured into water (210 mL), and the resulting blue-grey precipitate was purified twice by dissolving in 1 N NaOH, treating with charcoal, and precipitating with conc. HCl. The resulting tan colored solid was dissolved in 1 N NaOH, and precipitated by adding glacial acetic acid. The solid was filtered and washed with ethanol, giving 564 mg (57%) of pale yellow DCTG: mp dec. > 220 °C; ^1H NMR: 60 MHz (Me_2SO-d_6): δ 10.62 (s, 1-NH), 9.34 (s, 2-NH), 8.32 (s, 8H), 8.13 (s, 2-φH), 7.98 (m, 5-φH, 6-φH). Anal. ($C_{11}H_7N_5Cl_2S \cdot 1.2H_2O$): Calcd.: C: 39.58, H: 2.72, N: 20.98; Found: C: 39.91, H: 2.60, N: 20.37.

2-(3,4-Dichloroanilino)-6-methylthiopurine (DCMTP). A mixture of DCTG (329 mg, 1.05 mmol) in 10 mL of 0.1 N NaOH and 10 mL ethanol was treated with iodomethane (75 μL, 1.19 mmol) in ethanol (2 mL). After 45 min, water was added, and the solution was chilled, giving a yellow precipitate. The precipitate was chromatographed on a silica gel column (3 x

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3.5 cm) with a gradient of chloroform to 8% isopropyl alcohol in chloroform. The product was crystallized from aqueous ethanol after treating with charcoal, giving 146 mg (43%) of the reddish-brown colored product DCMTP: mp 267-270 °C; ^{1}H NMR: 60 MHz (Me_2SO-d_6): δ 11.96 (br s, 9-H), 9.73 (s, 2-NH), 8.34 (s, 8-H), 8.11 (s, 2-φH), 7.65 (m, 5-φH, 6-φH), 2.69 (s, CH_3); Anal. ($C_{12}H_9N_5Cl_2S \cdot 0.75H_2O$): Calcd.: C: 42.43, H: 3.12, N: 20.62; Found: C: 42.43, H: 2.99, N: 20.26.

2-(3,4-Dichloroanilino)-6-methoxypurine (DCMP). A solution of 2-(3,4-dichloroanilino)-6-chloropurine (DCACl)[2] (299 mg, 0.95 mmol) in 10 mL of 1 N sodium methoxide in methanol was heated at reflux. After 23 h, the reaction mixture was neutralized by addition of glacial acetic acid (1 mL), and poured into 50 mL of water. The resulting precipitate was chromatographed on a silica gel column (2.5 x 2.5 cm). Elution with a gradient of chloroform to 20% isopropyl alcohol in chloroform yielded one major product, which was crystallized from methanol to give 257 mg (87%) of nearly colorless DCMP: mp > 350 °C; ^{1}H NMR: 60 MHz (Me_2SO-d_6): δ 12.61 (br s, 9-H), 9.67 (s, 2-NH), 8.30 (s, 8-H), 8.06 (s, 1-φH), 7.64 (m, 5-φH, 6-φH), 4.11 (s, CH_3). Anal. ($C_{12}H_9N_5OCl_2 \cdot 1\ 1/3MeOH$): Calcd.: C: 44.26, H: 3.71, N: 19.85; Found: C: 44.44, H: 3.53, N: 19.59.

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2-Anilinoadenine (2AA). A solution of 2-anilino-6-chloropurine (ClAP) (Focher et al., 1988b) (84.7 mg, 0.344 mmol) in methanol saturated with ammonia (5 mL) was heated in a sealed tube at 130 °C for 4.5 h. Addition of 2 mL of water precipitated a small amount of a dark solid, which was removed by filtration. The filtrate was chilled, and the solid which precipitated was crystallized from ethanol to yield 50.3 mg (65%) of 2AA as a colorless solid: mp 284-288 °C; ^{1}H NMR: 60 MHz (Me_2SO-d_6): δ 12.24 (s, 9-H), 8.69 (s, 2-NH), 7.83 (d, o-φH), 7.78 (s, 8-H), 7.21 (t, m-φH), 6.85 (m, p-φH), 6.74 (s, NH_2); Anal. ($C_{11}H_{10}N_6 \cdot 0.2H_2O$): Calcd.: C: 57.48, H: 4.56, N: 36.56; Found: C: 57.75, H: 4.51, N: 36.65.

2-(3,4-Dichloroanilino)-6-chloro-9-(2-deoxy-3,5-di-p-toluoyl-β-D-ribofuranosyl)purine (blocked DCACldR) and its 7-isomer. A mixture of 2-(3,4-dichloroanilino)-6-chloropurine (DCACl)[3] (507 mg, 1.612 mmol) and NaH (50% suspension in mineral oil, 77.4 mg, 1.6 mmol) in dry acetonitrile (50 mL) was stirred at room temperature for 30 min. 2-Deoxy-3,5-di-p-toluoyl-β-D-ribofuranosyl chloride (626.3 mg, 1.61 mmol) was added in small portions over 40 min, and stirring was continued for 30 min. The mixture was diluted with chloroform (50 mL) and filtered through Celite. The filtrate was evaporated to dryness, and was purified by

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HPLC. The HPLC was equipped with a Waters 6000A pump, a refractometer detector and a preparative scale silica column (10 μM particle size, 2.2 x 50 cm); 10% acetone in toluene was used as the mobile phase. The column was run at a flow rate of 13.5 mL min^{-1}. The 9-isomer eluted between 200 and 253 mL, and the 7-isomer eluted between 331 and 368 mL. The 9-isomer was crystallized from ethanol to give 712.5 mg (66%) : mp 184-188 °C; ^1H NMR: 250 MHz (Me_2SO-d_6): δ 10.38 (s, 2-NH), 8.55 (s, 8-H), 6.56 (t, 1'-H, J_{av} = 5.7 Hz), 5.83 (m, 4'-H), 4.53 (m, 5',5'',3'-H), 2.86 (m, 2',2''-H); Anal. ($C_{32}H_{26}N_5O_5Cl_3 \cdot 0.2$ toluene): Calcd.: C: 58.53, H: 4.06, N: 10.22; Found: C: 58.61, H: 4.19, N: 10.21. The 7-isomer was crystallized from ethyl acetate, giving 171 mg (15%) of a colorless product: mp 174-175.5 °C; ^1H NMR: 250 MHz (Me_2SO-d_6): δ 10.21 (s, 2-NH), 8.93 (s, 8-H), 6.76 (t, 1'-H, J_{av} = 4.8 Hz), 5.73 (m, 4'-H), 4.52 (m, 5',5'',3'-H), 2.84 (m, 2',2''-H). Anal. ($C_{32}H_{26}N_5O_5Cl_3$): Calcd.: C: 57.63, H: 3.93, N: 10.50; Found: C: 57.40, H: 4.06, N: 10.47.

2-(3,4-Dichloroanilino)-6-chloro-9-(2-deoxy-β-D-ribofuranosyl)purine (DCACldR). A solution of blocked DCACldR (313.5 mg, 0.47 mmol) in methanol saturated with ammonia (20 mL) was stirred in a loosely capped flask for 26 h. The solution was neutralized with glacial acetic acid and chilled. The precipitate was crystallized from aqueous ethanol to give 155 mg (77%) of DCACldR as a colorless

solid: mp 150-153 °C; ^{1}H NMR: 250 MHz (Me_2SO-d_6): δ 10.33 (s, 2-NH), 8.55 (s, 8H), 8.11 (d, 2-φH, J = 2.0 Hz), 7.72 (d, 5-φH, J = 9.0 Hz), 7.55 (m, 6-φH, J = 8.9 Hz), 6.34 (t, 1'-H, J_{av} = 6.6 Hz), 4.40 (m, 3'-H), 3.86 (m, 4'-H), 3.53 (m, 5',5''-H), 2.77, 2.40 (m's, 2'-H, 2"-H); Anal. ($C_{16}H_{14}N_5O_3Cl_3 \cdot 1/3EtOH$): Calcd.: C: 44.88, H: 3.62, N: 15.70; Found: C: 45.28, H: 3.30, N: 15.80.

2-(3,4-Dichloroanilino)-2'-deoxyadenosine (DCAdA). A solution of blocked DCACldR (303 mg, 0.454 mmol) in methanol saturated with ammonia (8 mL) was heated in a sealed tube at 130 °C for 6 h. After evaporation of the solution to dryness, the residue was crystallized from methanol to yield 115 mg (62%) of colorless DCAdA: mp 203-206 °C; ^{1}H NMR: 200 MHz (Me_2SO-d_6): δ 9.32 (s, 2-NH), 8.20 (d, 2-φH), 8.12 (s, 8H), 7.77 (dd, 6-φH, J = 2.4 Hz, J = 8.9 Hz), 7.43 (d, 5-φH, j = 8.9 Hz), 7.15 (s, NH_2), 6.26 (t, 1'-H, J_{av} = 6.8 Hz), 5.30 (br s, 3'-OH), 4.88 (br s, 5'-OH), 4.38 (m, 3'-H), 3.83 (d, 4'-H, J = 2.7 Hz), 3.53 (t, 5',5''-H, J = 4.9 Hz), 2.72, 2.28 (m's, 2',2''-H); Anal. ($C_{16}H_{16}N_6O_3Cl_2 \cdot 1/2H_2O$): Calcd.: C: 45.73, H: 4.32, N: 20.00; Found: C: 46.28, H: 3.93, N: 19.61.

2-(3,4-Dichloroanilino)-2'-deoxyadenosine 5'-phosphate (DCAdAMP). A solution of DCAdA previously dried *in vacuo* (100 mg, 0.24 mmol) in trimethyl phosphate (1.3 mL) was chilled to -4 °C and stirred for 30 min. Phosphoryl

chloride (75 μL, 0.8 mmol) was added during 2 h, and stirring was continued for 4 h. Cold water (1.3 mL) was then added, and the solution was neutralized with 1 M NaOH. The solution was applied to a DEAE-Sephadex column (bicarbonate form; 25 x 2.5 cm), and eluted with a 24 h linear gradient of 0.1 to 1.0 M triethylammonium bicarbonate at a flow rate of 160 mL h^{-1}, with the collection of 15 mL fractions. Fractions # 180 to 247, which contained the desired product, were pooled and evaporated to dryness. The residue was dissolved in water, and the solution was filtered (0.2 μm), and lyophilized to give the triethylammonium salt of DCAdAMP (79 mg, 56%). ^{31}P NMR: 102 MHz (D_2O): δ 3.90 (d, J_{PH} = 4.6 Hz). Anal. [$C_{16}H_{17}N_6O_6Cl_2P \cdot N(Et)_3$]: Calcd.: P: 5.23; Found: P: 4.88.

2-(3,4-Dichloroanilino)-2'-deoxyadenosine 5'-triphosphate (DCAdATP). A solution of DCAdAMP (35.8 mg, 0.06 mmol) in HMPA (0.5 mL) was treated at room temperature with 1,1'-carbonyldiimidazole (42.5 mg, 0.26 mmol). After stirring for 2 h, this activation step was quenched by the addition of 38 μL methanol, and stirring was continued for 1 h. A solution of tributylammonium pyrophosphate (110 mg, 0.24 mmol) in HMPA (2 mL) was added dropwise, and stirring was continued for 24 h. The reaction was quenched by the addition of 10 mL of cold water. The resulting solution was applied to a DEAE-Sephadex column (bicarbonate form; 25 x

2.5 cm), and eluted with a 24 h linear gradient of 0.2 to 1.0 M triethylammonium bicarbonate at a flow rate of 160 mL h^{-1}, with the collection of 15 mL fractions. Fractions # 142-156, which contained the desired product, were pooled and evaporated to dryness. The residue was dissolved in water, and the solution was filtered (0.2 μm), and lyophilized to give the tetrakis(triethylammonium) salt of DCAdATP (16 mg, 25%). ^{31}P NMR: 102 MHz (D_2O): δ -6.04 (d, -γ P, J = 22.6 Hz), -11.01 (m, α-P), -21.98 (t, β-P, J_{av} = 20.7 Hz). Anal. [$C_{16}H_{19}N_6O_{12}Cl_2P_3 \cdot 4N(Et)_3$]: Calcd.: P: 8.80; Found: 9.19.

The following inhibitors were synthesized by others in the lab of Dr. George Wright, as described: BuAA, BuACl, BuAOMe, BuPTG, BuASMe and BuAdA (Wright et al., 1987); BuAdATP (Khan et al., 1985); BuPG (Wright et al., 1982); BuPdG, BuPdGTP (Wright & Dudycz, 1984). The aphidicolin analogs 17-acetylaphidicolin, 17,18-diacetylaphidicolin, "16-ketoaphidicolin" (3,18-dihydroxy-17-noraphidicolan-16-one) and 3-epiaphidicolin were prepared by Dr. Lili Arabshahi, (Arabshahi et al., 1988) according to the methods of Dalziel et al. (1973). F_2MDP also was prepared by Dr. Arabshahi, by the method of Blackburn et al. (1981). EMPdGTP, PhdGTP and HexdGTP were prepared by Stephen Freese.[1] DCAA, DCACl and DCPG were prepared by Joseph

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[1] S. Freese and G. Wright, unpublished.

Gambino.[2] The following inhibitors were synthesized in the lab of Dr. Charles McKenna (Universty of Southern California), as described: BrPAA, FClPAA, FBrPAA, ClPPA, FMDP and COMDP (Talanian et al., submitted), the latter by a minor variation of the method of Quimby et al. (1967).

AraATP, araCTP, ddTTP, PP_i, PFA, PAA, DAP and DAPdR were purchased from Sigma. MDP was purchased from Aldrich. ACGTP and AZTTP were gifts from Dr. Wayne Miller (Burroughs-Wellcome Co.). FPAA, F_2PAA, ClPAA, Cl_2PAA, ClBrPAA, Br_2PAA, COPAA, FPPA, ClPPA, FBrMDP, FClMDP, ClMDP, Cl_2MDP, ClBrMDP, BrMDP and Br_2MDP were gifts from Dr. Charles McKenna (University of Southern California). Aphidicolin was a gift from the Natural Products Branch, National Cancer Institute.

C. Cell Culture and Measurement of Macromolecule Precursor Uptake and Incorporation

Growth of CHO cells. CHO SC1-9 cells were grown in suspension culture at 37 °C at densities of 4 to 12 x 10^5 cells mL^{-1}, in McCoy's 5A medium supplemented with 10% fetal bovine serum. Densities were determined by hemocytometer.

Incorporation of [^{3}H]TdR into CHO cell DNA. Incorporation of [^{3}H]TdR into CHO cell DNA was measured essentially as described (Wright et al., 1987). A suspension of exponentially growing CHO cells (4 to 8 x 10^5

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cells mL^{-1}) was distributed in 3 mL aliquots into capped plastic tubes containing drug solutions in Me_2SO or Me_2SO itself as control, and incubated at 37 °C for 3 h with shaking. [^{3}H]TdR (1.5 μCi, 20 Ci $mmol^{-1}$) was then added to each tube, and incubation was continued for 30 min. Incorporation was then quenched by the addition of 3 mL of ice-cold phosphate buffered saline (PBS) (pH 7.0), and the mixtures were centrifuged at 1000 x g for 10 min. The supernatants were aspirated, and the pellets resuspended in 2 mL of 1 N NaOH. The mixtures were then incubated at 65 °C for 1 h in order to hydrolize RNA, after which 2 mL of an ice-cold solution of 20% trichloroacetic acid (TCA) (w/v) and 20 mM sodium pyrophosphate ($NaPP_i$) was added. After chilling on ice for 15 min, the mixtures were filtered through GF/A discs (Whatman), and the discs were washed with a cold solution of 1% TCA (w/v) and 10 mM $NaPP_i$, then with ethanol, and dried. The discs were transferred to scintillation vials, 1 mL of Opti-Fluor (Packard) was added to each, and radioactivity was measured in a scintillation counter. Control incorporation of [^{3}H]TdR per tube was 7000 to 9000 cpm above background, which was typically 100 to 200 cpm.

Incorporation of [^{3}H]Urd into CHO cell RNA.

Incorporation of [^{3}H]Urd into CHO cell RNA was measured as the difference between total TCA-precipitable counts and

that which remained after base-catalyzed hydrolysis, essentially as described (Wright et al., 1987). A suspension of exponentially growing CHO cells (4 to 8 x 10^5 cells mL^{-1}) was distributed in 3 mL aliquots into capped plastic tubes containing drug solutions in Me_2SO or Me_2SO itself as control, and incubated at 37 °C for 3 h with shaking. [^{3}H]Urd (3 μCi, 47 Ci $mmol^{-1}$) was then added to each tube, and incubation was continued for 45 min. Incorporation was then quenched by the addition of 3 mL of ice-cold phosphate buffered saline. Aliquots of 1 mL of each sample were then prepared for measurement of [^{3}H]Urd incorporation into either (1) DNA, or (2) DNA and RNA, as follows: (1) The mixtures were centrifuged at 1000 x g for 10 min, and the supernatants were aspirated. The pellets were resuspended in 0.5 mL of 1 N NaOH, incubated at 65 °C for 1 h, and [^{3}H]Urd incorporation was measured as described above in the case of [^{3}H]TdR incorporation. (2) A solution of 0.5 mL of ice-cold 10% TCA (w/v) and 10 mM $NaPP_i$ was added to each tube. The tubes were then chilled on ice for 15 min, after which the mixtures were filtered through GF/A discs (Whatman), and the discs were washed and counted as described above in the case of [^{3}H]TdR incorporation. Incorporation of [^{3}H]Urd into RNA was calculated as the difference between the counts obtained by (1) and (2). Control incorporation of [^{3}H]Urd into RNA was 6000 to 9000

cpm above background, which was typically 100 to 200 cpm.

Incorporation of [^{3}H]Leu into CHO cell protein.

Incorporation of [^{3}H]Leu into CHO cell protein was measured essentially as described (Wright et al., 1987). A suspension of exponentially growing CHO cells (4 to 8 x 10^5 cells mL^{-1}) was distributed in 2 mL aliquots into capped plastic tubes containing drug solutions in Me_2SO or Me_2SO itself as control. [^{3}H]Leu (2 μCi, 50 Ci $mmol^{-1}$) was then added to each tube, and the mixtures were incubated at 37 °C for 3 h with shaking. Incorporation was then quenched by the addition of ice-cold solutions of 100 μL bovine serum albumin (BSA) (1 mg mL^{-1}) and 200 μL of 25% (w/v) TCA, and the tubes were chilled on ice for 15 min. The mixtures were centrifuged at 1000 x g for 10 min, and the supernatants were aspirated. The pellets were washed three times by resuspending in 0.5 mL of 0.1 N HCl, centrifuging and aspirating the supernatant. The pellets were resuspended in 0.5 mL of 1 N NaOH, and incubated at 60 °C for 30 min. The solutions were neutralized with conc. HCl, and transferred to scintillation vials. Aqueous counting scintillant (Packard) (4 mL) was added, and the radioactivity in each sample was measured in a scintillation counter. Control incorporation of [^{3}H]Leu was 8000 to 10,000 cpm above background, which was typically about 100 cpm.

Measurement of [^{3}H]Thymidine Uptake by CHO cells.

[^{3}H]TdR uptake by CHO cells was measured as described by Aronow and Ullman (1985), with certain modifications. Exponentially growing CHO cells (4 to 8 x 10^5 cells mL^{-1}) were centrifuged and resuspended in McCoy's 5A medium (unsupplemented) containing 10 μM aphidicolin, at a density of about 2 x 10^7 cells mL^{-1}. Drug solutions in Me_2SO or Me_2SO itself as control were added, and the suspensions were incubated at 37 °C with occasional agitation for 15 to 45 min. Separately, 1.5 mL plastic microcentrifuge tubes were prepared containing 100 μL of unsupplemented McCoy's 5A medium with [^{3}H]TdR (10 μCi mL^{-1}, 20 Ci $mmol^{-1}$) layered over 100 μL of an oil mixture [silicone oil (Aldrich 17,563-3) : light mineral oil (Fisher 0-121), 94:6]. 100 μL of the cell suspension were added to the aqueous layer of the tube, and after a 15 sec incubation, the tube was centrifuged at 15,000 x g for 25 sec, causing the cells to pellet below the oil layer. The aqueous layer was aspirated, and the walls of the tube were washed twice by addition and aspiration of 0.5 mL PBS (pH 7.0). The oil layer was aspirated, and the cell pellet was suspended in 0.1 mL of 2% (v/v) Triton X-100. 1 mL of scintillation fluid was added, and the samples were counted. Control uptake of [^{3}H]TdR was 7000 to 10,000 cpm above background.

Incorporation of [^{32}P]phosphate into CHO cell DNA.

Incorporation of [^{32}P]phosphate (orthophosphate) into CHO

cell DNA was measured essentially as described (Robichaud & Fram, 1987). A suspension of exponentially growing CHO cells at a density of about 4 to 8 x 10^5 cells mL^{-1}) was distributed in 2.5 mL aliquots into capped plastic tubes containing drug solutions in Me_2SO or Me_2SO itself as control, and the tubes were shaken at 37 °C. After 1 h a solution of [^{32}P]phosphate (12.5 μCi, 200 mCi $mmol^{-1}$) was added, and shaking was continued. After 3 h, 2.5 mL of ice-cold phosphate buffered saline (pH 7.0) was added, and the tubes were chilled on ice for 15 min. The mixture was centrifuged at 1400 x g for 10 min, and the supernatant was aspirated. Workup and counting were then performed as described above in the case of [^{3}H]TdR incorporation. Incorporation of [^{32}P]phosphate by the control samples was 8000 to 12,000 cpm above background.

[α-^{32}P]dATP incorporation in permeable CHO cells.

Permebilization of and measurement of DNA synthesis in CHO cells was performed essentially as described by Dresler (1984) in the case of human fibroblasts. CHO cells, used at a density of 4 to 8 x 10^5 cells mL^{-1}, were prelabelled by supplementing the cell culture medium with [^{3}H]TdR (5 nCi mL^{-1}, 20 Ci $mmol^{-1}$), in order to control for variations in the number of cells in each assay tube. Cells were made permeable by incubation at 0°C for 30 min in a solution of 10 mM Tris (pH 7.6), 4 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose,

and 3 mM DTT. The cells were washed twice in the same buffer to remove endogenous nucleotides. 100 µL of the permeable cell suspension (10^7 cells mL^{-1}) was mixed with 50 µL of a reaction mix containing inhibitors in Me_2SO or a solution of 10 mM HEPES-K^+, or the solvents themselves as controls, to give final concentrations of 40 mM Tris (pH 7.6), 8 mM $MgCl_2$, 5 mM ATP, 167 mM sucrose, 2 mM dithiothreitol, 0.67 mM EDTA, 50 µM each of dCTP, dGTP and dTTP and 1 µM [α-^{32}P]dATP (15 Ci $mmol^{-1}$). Samples were incubated at 37 °C for 8 min, and workup and counting were performed as described (Dresler, 1984). Control samples typically incorporated 2000 to 3000 cpm of ^{32}P, and contained 2000 to 3000 cpm of the ^{3}H prelabel.

D. Enzymes

DNA polymerase delta. PCNA-independent calf thymus DNA polymerase δ was provided by Drs. Federico Focher and Ulrich Hübscher (University of Zürich-Irchel). Two enzyme preparations were used; one, designated HAP-δ, was purified through step III, hydroxyapatite chromatography (Focher et al. 1988c). The second preparation, designated FPLC-δ, was purified through step V, FPLC-MonoS chromatography (Focher et al. 1989). FPLC-δ was not stimulated by the pol δ cofactor PCNA, nor did it contain PCNA (Focher et al., 1989). FPLC-δ contained four major peptides and was used in

the analysis of the mechanism of inhibition of pol δ by COMDP, and in assays of exonuclease activity.

DNA polymerase alpha. Calf thymus DNA polymerase α was prepared by Drs. Kimberly Foster and Russell Hammond, using the immunoaffinity chromatography method of Chang et al. (1984).

Other enzyme. Terminal deoxynucleotidyltransferase was purchased from New England Nuclear.

E. Enzyme Assays

Poly(dA):oligo(dT) as the template:primer. Assays of pol α and pol δ (either HAP-δ or FPLC-δ) using poly(dA):oligo(dT) as the template:primer contained the following in 25 µL: 75 mM HEPES-K^+ (pH 7.5), 1.25 mM DTT, 20% (v/v) glycerol, 10 mM $MgCl_2$, 250 µg mL^{-1} BSA, 0.5 µg poly(dA):oligo(dT) (base ratio 10:1), 10 µM [^{3}H]dTTP (1250 or 2000 cpm $pmole^{-1}$), and about 0.005 to 0.02 units of the enzyme to be assayed. One unit of DNA polymerase activity is defined as the amount of enzyme which catalyzes the incorporation of 1 nmole of dTMP in 60 min at 37 °C under standard assay conditions. The mixtures were incubated at 37 °C for 30 min, and the material which could be precipitated by TCA was determined as described by Neville and Brown (1972).

Activated DNA as the template:primer. Assays of pol α

using activated calf thymus DNA as the template:primer contained the following in 25 µL: 20 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, 4 mM DTT, 10 mM $MgCl_2$, 250 µg mL^{-1} BSA, 50 µM each of dATP, dCTP and dGTP, 20 µM [^{3}H]dTTP (500 cpm $pmole^{-1}$), 10 µg DNAse activated calf thymus DNA, and about 0.1 to 0.5 units of enzyme. The mixtures were incubated at 37 °C for 30 min, and the material which could be precipitated by TCA was determined as described by Neville and Brown (1972). Assays of pol δ (HAP-δ) using activated calf thymus DNA as the template:primer contained the following in 25 µL: 75 mM HEPES-K^+ (pH 7.5), 1.25 mM DTT, 20% (v/v) glycerol, 10 mM $MgCl_2$, 250 µg mL^{-1} BSA, 100 mM KCl, 1 mM GMP or AMP, 25 µM each of dATP, dCTP and dGTP, 10 µM [^{3}H]dTTP (1250 cpm $pmole^{-1}$), 3 µg DNAse activated calf thymus DNA, and about 0.002 to 0.005 units of enzyme. The mixtures were incubated at 37 °C for 90 min, and the material which could be precipitated by trichloroacetic acid was determined as described by Neville and Brown (1972).

In some experiments, the dNTP expected to be competitive with certain inhibitors was omitted, giving a "truncated" assay (Wright & Brown, 1976). In these assays, in which sensitivity of the enzyme to inhibition by compounds expected to be competitive with the omitted dNTP was maximized (Wright & Brown, 1976), enzyme activity was typically 25% that observed under "full" assay conditions,

i.e., in which all four dNTPs were present.

In experiments in which dTTP was the omitted dNTP, [α-^{32}P]dGTP (1250 cpm pmole^{-1}) at 10 μM served as the labeled substrate.

Preparation of exonuclease substrates. The 3'-terminal end of oligo(dT)$_{12-18}$ was labeled by addition of radiolabelled dNTPs catalyzed by terminal deoxynucleotidyltransferase. The following components were incubated at 37 °C for 60 min in a final volume of 100 μL: 0.2 M sodium cacodylate, pH 7.5, 5 mM $MgCl_2$, 250 μM $CoCl_2$, 1 mM β-mercaptoethanol, 100 μg mL^{-1} BSA, 100 μM oligo(dT)$_{12-18}$, 20 units of terminal deoxynucleotidyltransferase, and either 160 μM [α-^{32}P]dCTP (5 Ci mmol^{-1}) or 150 μM [^{3}H]dTTP (20 Ci mmol^{-1}). The reaction was terminated by incubation at 100 °C for 1 min, and the labeled DNA was purified by chromatography on Sephadex G-25. Given the amount of DNA used and the specific activity of the added nucleotides, I calculated that the products of the reaction were oligo(dT)$_{12-18}$•([^{32}P]dC)$_{0.33}$ and oligo(dT)$_{12-18}$•([^{3}H]dT)$_{0.3}$. The oligonucleotides were hybridized to poly(dA) [base ratio 10:1 poly(dA):primer] prior to use as exonuclease substrates.

Exonuclease assays. Assays of the 3' → 5' exonuclease activity of pol δ were identical to those of the polymerase activity except that poly(dA):oligo(dT)$_{12-18}$•([^{32}P]dC)$_{0.33}$

or poly(dA):oligo(dT)$_{12-18}$·([^{3}H]dT)$_{0.3}$ were substituted for poly(dA):oligo(dT), and [^{3}H]dTTP and GMP were omitted. Reactions using the mismatched substrate required about 0.075 (DNA polymerase) units of enzyme and incubation for 12 min. Reactions using the properly paired substrate required about 0.5 (DNA polymerase) units of enzyme and incubation for 20 min. Reactions were terminated by addition of 25 µL of 200 mM EDTA, and the samples were prepared for scintillation counting as described by LaDuca et al. (1986). The samples were spotted onto DE81 discs (Whatman), and washed three times in 50 mL of a cold solution of 0.3 M ammonium formate (pH 7.8) and 10 mM $NaPP_i$, then in ethanol, and dried. The discs were transferred to scintillation vials, 1 mL of Opti-Fluor (Packard) was added to each, and radioactivity was measured in a scintillation counter.

F. Calculation of K_i Values of DNA Polymerase Inhibitors on the Basis of Lineweaver-Burk Plot Data

K_i values for inhibition of DNA polymerases were determined as follows: The data of Lineweaver-Burk plots (velocity^{-1} *vs.* substrate concentration^{-1}) were fitted by least squares regression. The equations of the regression lines were used to calculate X- and Y-axis (velocity^{-1} and substrate concentration^{-1} axis, respectively) intercepts and line slopes, as appropriate. The slope and intercept values

were used to calculate K_i values by two methods, which depended on the pattern of inhibition (see below for the three cases of competitive, noncompetitive and uncompetitive inhibition). The K_i values reported in the text are the arithmetic means of the two calculated values.

Competitive inhibition. One K_i value was calculated by plotting the negative inverse of the X-intercepts determined as described above *vs.* inhibitor concentration. The data were fitted by least squares regression, and the negative of the X-intercept of the regression line so obtained gave a K_i value. A second K_i value was determined by plotting the slopes of the regression lines of the Lineweaver-Burk plots *vs.* inhibitor concentration; the regression line of that plot was determined, and the negative of the X-intercept of that line gave a K_i value.

Noncompetitive inhibition. One K_i value was calculated by plotting the Y-axis intercepts of the regression lines of the Lineweaver-Burk plots *vs.* inhibitor concentration. A least squares regression line was determined, and the negative of the X-intercept of that line gave the K_i value. The second K_i value was determined on the basis of the Y-intercept values of the regression lines of the Lineweaver-Burk plots as described above in the case of competitive inhibition.

Uncompetitive inhibition. One K_i value was determined

on the basis of the Y-axis intercepts of the regression lines of the Lineweaver-Burk plots as described above in the case of noncompetitive inhibition. The second K_i value was determined on the basis of the X-intercept values of the regression lines of the Lineweaver-Burk plots as described above in the case of competitive inhibition.

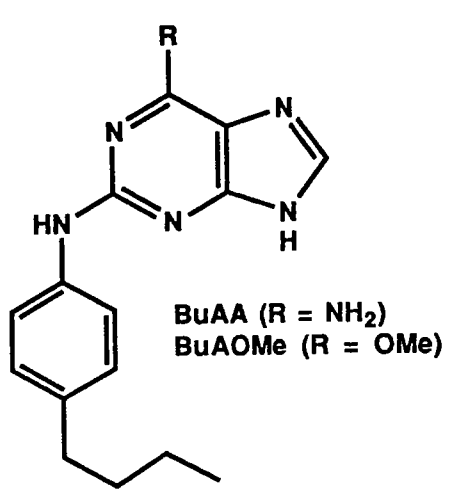
CHAPTER III

INHIBITION OF MAMMALIAN CELL DNA SYNTHESIS

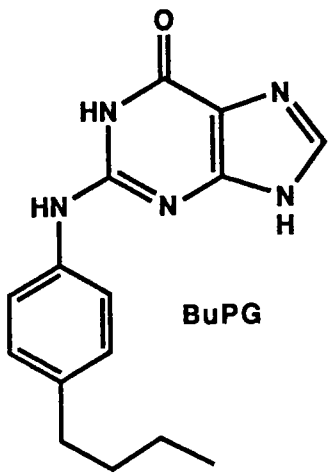
A. Introduction

Original goal of the project. The original goal of the project was to explain the sometimes poor correlation between the potencies of several nucleoside and nucleobase analogs as inhibitors of the activity of isolated pol α and as inhibitors of mammalian cell thymidine (TdR) incorporation. In particular, I was interested in elucidating the cytotoxic mechanism(s) of 2-(p-n-butylanilino)-2'-deoxyadenosine (BuAdA, Figure III-1). BuAdA inhibited HeLa cell thymidine incorporation with a potency (IC_{50} = 1 μM) (Wright et al., 1987) about an order of magnitude greater than its potency as an inhibitor of isolated CHO cell pol α (K_i = 10-15 μM) (Khan et al., 1985). The following is a description of some of the hypothetical explanations of the inhibitory properties of BuAdA, which together served as the rationale for the project at its outset:

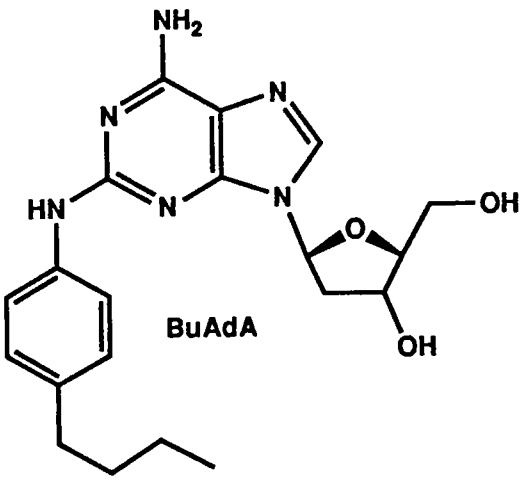
(i) Assuming that pol δ has a role in mammalian DNA replication, BuAdA might have inhibited that enzyme with significantly greater potency than pol α. If so, the investigation may have uncovered a selective inhibitor of pol δ, and it would have supported a role for the



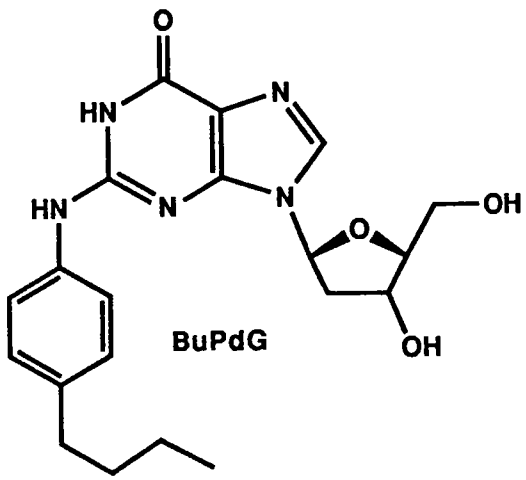
BuAA (R = NH_2)
BuAOMe (R = OMe)



BuPG



BuAdA



BuPdG

Figure III-1. Structures of BuAA, BuAOMe, BuAdA, BuPG and BuPdG.

participation of that enzyme in mammalian cell DNA replication.

(ii) BuAdA might have been metabolized in the cell to forms which inhibit pol α with greater potencies than BuAdA itself. Such an observation may have revealed useful enzymatic syntheses of potent DNA polymerase inhibitors, and it may have led to the discovery of DNA polymerase inhibitors of novel structure. The observation would also have exposed an important caveat in studies designed to determine the roles of DNA polymerases in DNA metabolism by the correlation of the *in vitro* and *in vivo* potencies of nucleobase and nucleoside analog inhibitors.

(iii) The investigation might have revealed cellular targets for inhibition other than DNA polymerases. If so, and if, for example, the enzyme(s) were potential chemotherapeutic targets, BuAdA might have served as a lead compound for the development of chemotherapeutic agents.

Cytotoxicity of compounds other than BuAdA. As the investigation progressed, I became interested in the cytotoxic mechanisms of some compounds other than BuAdA. In most cases, such as that of 2-anilinoadenine (2AA), the compounds were selected because of their structural relationship to others, and were included in certain

experiments as controls for the contributions of certain functional groups to their potencies. I studied the cytotoxicity of 2-(3,4-dichloroanilino)adenine (DCAA) and certain analogs thereof because of its potent cytotoxicity to mammalian cells[1] and its structural similarity to known pol α inhibitors such as the 2-(p-n-butylanilino)purines. These analogous compounds were tested in some but not all of the experiments described below.

B. Development of Novel Inhibitors Based on DCAA

I synthesized several analogs of DCAA during the course of the work described below, with the goal of finding compounds which display increased potency as inhibitors of cellular DNA synthesis, isolated DNA polymerase activity, or both, relative to DCAA. For clarity, I describe the syntheses of all of the DCAA analogs in this section, regardless of the order in which I synthesized them or the timing of the syntheses with respect to the experiments described in subsequent sections.

Nucleobase analogs of DCAA. Several analogs of the corresponding base analog of BuAdA, 2-(p-n-butylanilino)adenine (BuAA, Figure III-1), differing from BuAA in the nature of the substituents in the 6-position of the purine base, were more potent inhibitors of CHO cell pol

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[1] P. Medveczky and G. Wright, unpublished.

α than BuAA (Wright et al., 1987). The latter observation suggested by analogy that similar 6-substituted analogs of DCAA might possess greater inhibitory potency than DCAA. I therefore synthesized 6-methoxy [2-(3,4-dichloroanilino)-6-methoxypurine, DCMP, Figure III-2] and 6-methylthio [2-(3,4-dichloroanilino)-6-methylthiopurine, DCMTP, Figure III-2] analogs of DCAA, as well as the intermediate 6-thio [N^2-(3,4-dichlorophenyl)-6-thioguanine, DCTG, Figure III-2] and 6-chloro [2-(3,4-dichloroanilino)-6-chloropurine, DCACl, Figure III-2] analogs. Figure III-2 outlines the syntheses of the latter compounds from the corresponding guanine analog [N^2-(3,4-dichlorophenyl)guanine, DCPG, Figure III-2]; details of the syntheses are described in Chapter II. The presence of the methyl group resonances in the ^1H NMR spectra of DCMP and DCMTP, and the chemical shifts of other resonances, served to identify the latter reaction products.

Synthesis of 2AA. In several experiments described below, I wished to demonstrate the role of the substituents on the anilino rings of DCAA and BuAA (3,4-dichloro and p-n-butyl groups, respectively). To do so, I measured the inhibitory potency of 2AA, a compound which is structurally identical to DCAA except that it lacks the anilino ring substituents of DCAA and BuAA. I synthesized 2AA from 2-anilino-6-chloroadenine (ClAP, Figure III-3) (Focher et al., 1988b), as outlined in Figure III-3. The singlet at δ 6.74

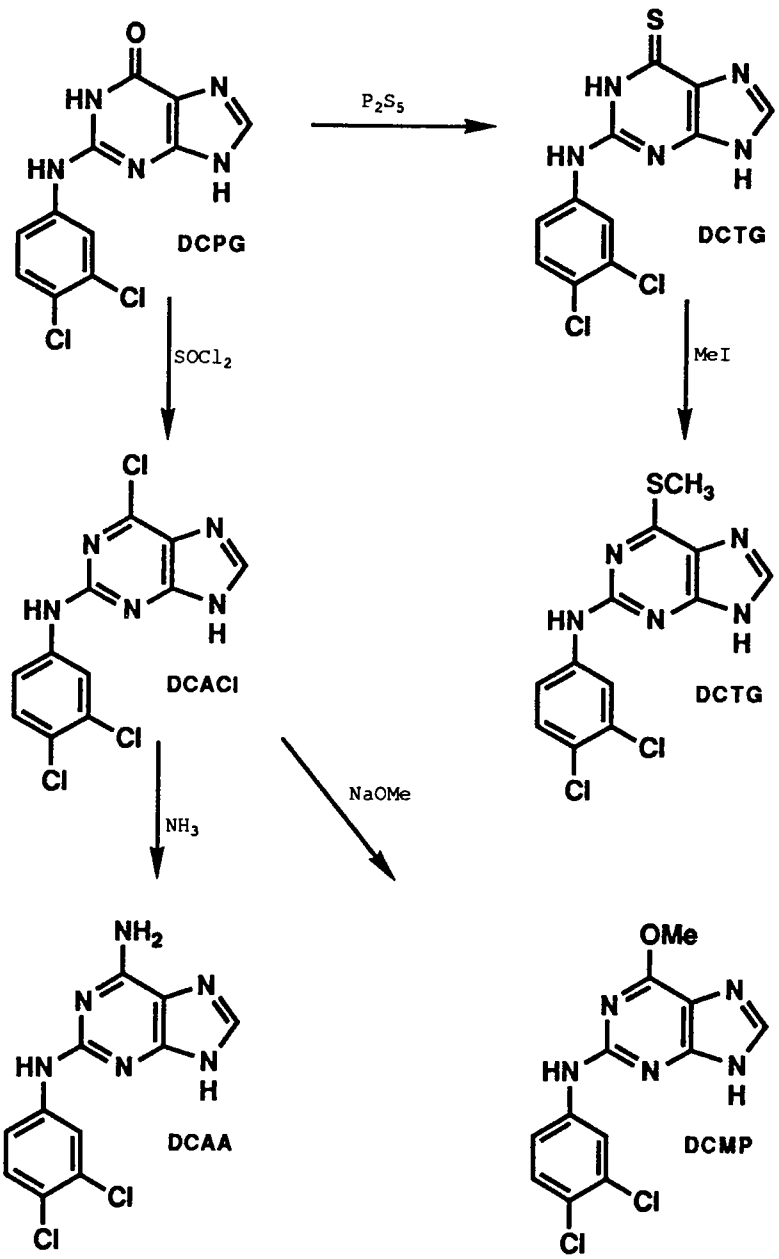


Figure III-2. Outline of the syntheses of DCAA and several DCAA analogs from the common intermediate DCPG. Each synthesis is described in detail in Chapter II.

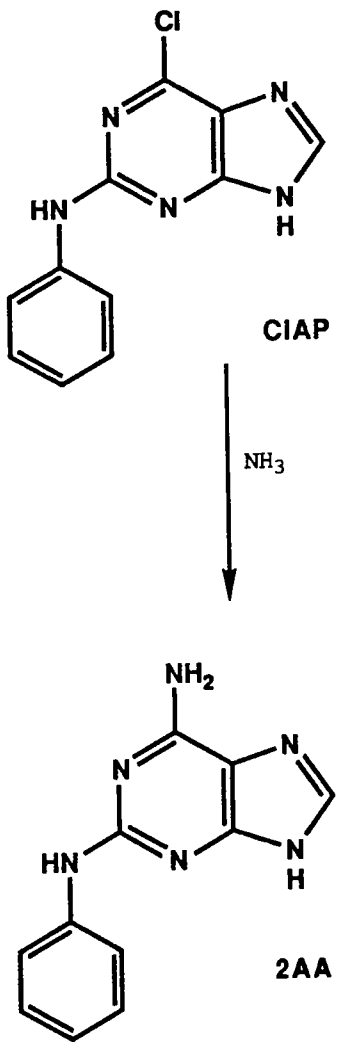


Figure III-3. Synthesis of 2AA from ClAP. The reaction is described in detail in Chapter II.

in the NMR spectrum of 2AA served to identify the compound as a 2-amino derivative of the starting material, ClAP.

Nucleoside analogs of DCAA. The observation that nucleoside analogs of BuAA, such as BuAdA, inhibit CHO cell growth and [^{3}H]TdR incorporation with at least an order of magnitude greater potency than BuAA suggested by analogy that nucleoside analogs of DCAA might also be more potent inhibitors than DCAA. I therefore synthesized 2-(3,4-dichloroanilino)-6-chloro-9-(2-deoxy-β-D-ribofuranosyl)purine (DCACldR) and 2-(3,4-dichloroanilino)-2'-deoxyadenosine (DCAdA); DCAdA served both as an inhibitor and as an intermediate in the synthesis of nucleotidyl forms of DCAA (see below). Figure III-4 describes in outline form the syntheses of DCACldR and DCAdA. Isolation of the major (66%) 9-β and minor (15%) 7-β isomers was consistent with the results of similar glycosylations of 6-chloropurines (Kazimierczuk et al., 1984; Wright et al., 1987). The blocked intermediates 2-(3,4-dichloroanilino)-6-chloro-9-(2-deoxy-3,5-di-p-toluoyl-β-$_D$-ribofuranosyl)purine (blocked DCACldR) and its 7-isomer were identified by proton NMR spectra. Resonances corresponding to the toluyl blocking groups were present. Characteristic pseudotriplet resonances at δ 6.56 and 6.76, respectively, due to the 1'-protons, identified the products as β-anomers. The relative chemical shifts of the 1'- and 8-position protons served to

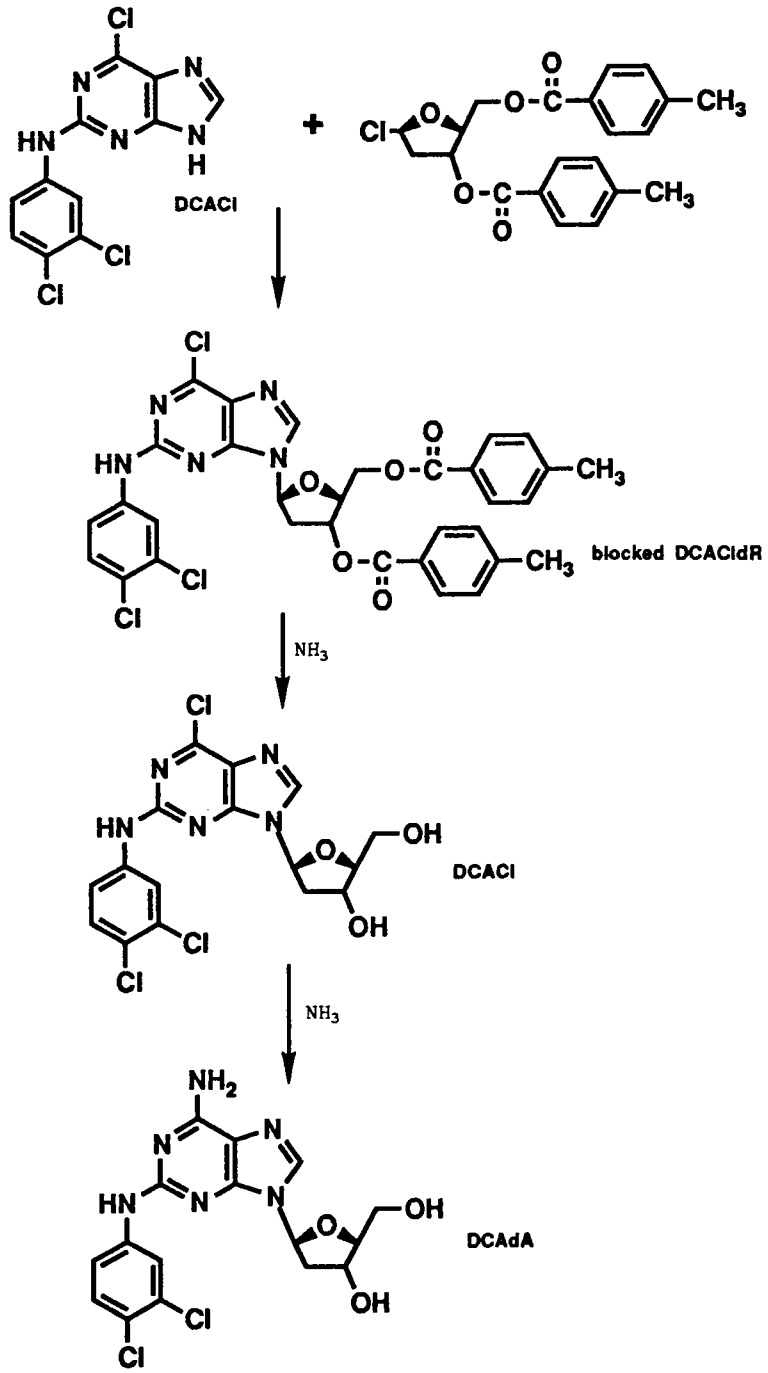


Figure III-4. Outline of the synthesis of DCAdA from its corresponding base analog DCAA. Each step is described in detail in Chapter II.

distinguish between the 9- and 7-isomers (Kazimierczuk et al., 1984; Wright et al., 1987). The pseudotriplets for the 1'-proton resonances of DCACldR and DCAdA confirmed the identity of the latter compounds as β-isomers.

Nucleotide analogs of DCAA. The deoxyribonucleoside 5'-triphosphate form of BuAA (BuAdATP) inhibited CHO cell pol α with a potency approximately four orders of magnitude greater than BuAA (Khan et al., 1985). The latter observation as well as similar observations on the inhibitory potencies of the guanine analogs of BuAA and BuAdATP [N^2-(p-n-butylphenyl)guanine, BuPG, Figure III-1, and BuPdGTP) prompted me to synthesize the corresponding nucleoside 5'-triphosphate of DCAA [2-(3,4-dichloroanilino)-2'-deoxyadenosine 5'-triphosphate, DCAdATP, Figure III-5] for testing as an inhibitor of purified DNA polymerases alpha and delta (see Chapter IV). The synthesis of DCAdATP from DCAdA, through the intermediate 5'-monophosphate [2-(3,4-dichloroanilino)-2'-deoxyadenosine 5'-monophosphate, DCAdAMP, Figure III-5] is outlined in Figure III-5. The presence of a triplet in the ^{31}P NMR spectrum of DCAdAMP, due to coupling of the phosphorus with the 5' and 5'' protons, served to confirm that the monophosphate product was the 5' phosphate isomer. The observation of a pseudotriplet in the β-phosphorus resonance of the ^{31}P NMR spectrum of DCAdATP confirmed that that compound was the

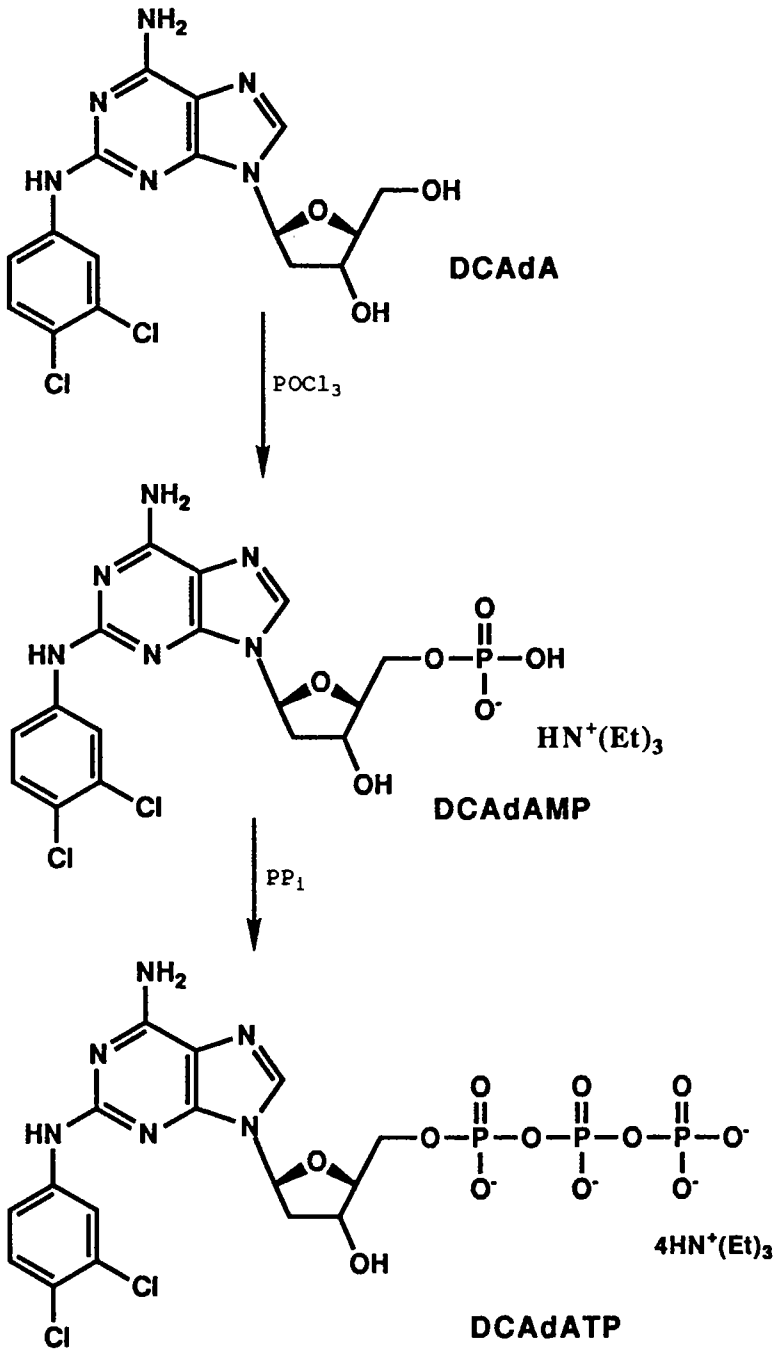


Figure III-5. Outline of the syntheses of nucleotidyl forms of DCAA. Each reaction is described in detail in Chapter II.

triphosphate analog of the starting material DCAdAMP, and, therefore, the 5'-triphosphate derivative of DCAdA.

C. Inhibition of CHO Cell Macromolecule Precursor Incorporation

Choice of CHO cells. I chose to study the cytotoxic mechanisms of BuAdA and related compounds in CHO cells rather than continue the work begun by Wright et al. (1987) in HeLa cells for two reasons. First, the availability in our lab of CHO cell pol α, as well as information on the inhibitory properties of BuAdA and analogs thereof toward that enzyme (Khan et al., 1984; Khan et al., 1985; Wright et al., 1987), would have allowed comparison between the inhibitory properties of compounds toward mammalian cells and pol α derived from the same source. Second, the availability of a variety of mutant CHO cell lines (for example, purine auxotrophs) may have facilitated studies designed to elucidate of cytotoxic mechanism(s) of BuAdA.

Potencies of adenine analogs as inhibitors of CHO cell macromolecule precursor incorporation. I measured the potencies of BuAdA and several other adenine analogs as inhibitors of incorporation of [^{3}H]thymidine ([^{3}H]TdR), [^{3}H]uridine ([^{3}H]Urd) and [^{3}H]leucine ([^{3}H]Leu) into CHO cell DNA, RNA and protein, respectively, by the techniques described in Chapter II. The results are summarized in

Table III-1, and are discussed below.

BuAA, BuAdA, DCAA and DCAdA. The pol α inhibitors BuAA and BuAdA inhibited [^{3}H]TdR incorporation by CHO cells with IC_{50} values of 15 and 3 μM, respectively (Table III-1), in rough agreement with the corresponding values of 23 and 1 μM previously determined by Wright et al. (1987) in HeLa cells. Neither BuAA nor BuAdA inhibited [^{3}H]TdR incorporation with significant selectivity relative to [^{3}H]Urd incorporation; both compounds inhibited incorporation of the latter precursor into cellular RNA with IC_{50} values of 10 μM. BuAA and BuAdA inhibited [^{3}H]Leu incorporation into protein weakly compared with their effects on [^{3}H]TdR and [^{3}H]Urd incorporation; BuAA displayed an IC_{50} value >100 μM, and BuAdA was inactive at concentrations up to 200 μM. DCAA, an inhibitor of pol α with a potency similar to that of BuAA, but with little selectivity compared to pol δ (see Chapter IV, esp. Table IV-1), and its deoxyribonucleoside derivatives DCAdA and DCAC1dR, inhibited [^{3}H]TdR incorporation by CHO cells with IC_{50} values similar to those displayed by BuAA and BuAdA. Like the butylanilino-substituted compounds, the nucleosides DCAdA and DCAC1dR were more potent inhibitors of [^{3}H]TdR incorporation than was the corresponding nucleobase, DCAA.

Structure-activity relationships governing the cytotoxic potencies of 2-anilinoadenine derivatives. I measured the

Table III-1. Inhibition of [^{3}H]TdR, [^{3}H]Urd and [^{3}H]Leu Incorporation into CHO Cell Macromolecules[1]

Inhibitor	IC_{50} (μM)[2]		
	[^{3}H]TdR	[^{3}H]Urd	[^{3}H]Leu
BuAA	15	10	>100
BuAdA	3	10	I
DCAA	20	N	N
DCAdA	7	N	N
DCACldR	7	N	N
2AA	150	N	N
DAP	I	S[3]	I
DAPdR	I	S[4]	I
dA	I	N	N

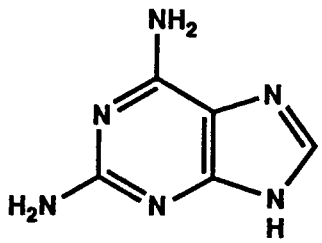
[1]Incorporation of [^{3}H]TdR, [^{3}H]Urd and [^{3}H]Leu into CHO cell DNA, RNA and protein, respectively, was measured as described in Chapter II. CHO cells were incubated with inhibitors (or solvent control) for 1 h, then [^{3}H]TdR, [^{3}H]Urd or [^{3}H]Leu was added, and incubation was continued for 30, 45 or 180 min, respectively.

[2]IC_{50} values were determined on the basis of inhibitor concentration *vs.* percent activity curves. I = inactive at 200 μM, the highest concentration tested. N = not measured. S = stimulation of macromolecule precursor incorporation.

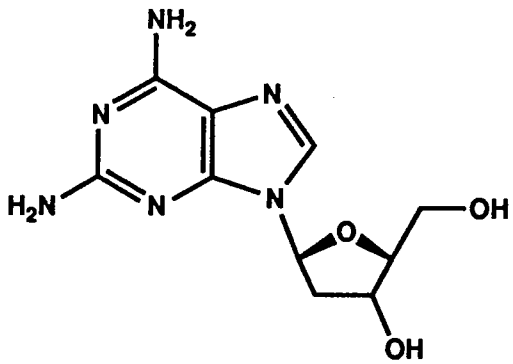
[3]DAP at 200 μM stimulated incorporation of [^{3}H]Urd 74% relative to controls.

[4]DAPdR at 40 μM stimulated incorporation of [^{3}H]Urd 152% relative to controls.

inhibitory properties of several compounds chosen to demonstrate the significance of the 2-substituents of BuAA, DCAA and derivatives thereof. 2AA is a nucleobase analog structurally identical to BuAA and DCAA, except that it lacks substituents on the phenyl ring of its anilino group. The relative weakness of 2AA as an inhibitor of CHO cell [^{3}H]TdR incorporation (IC_{50} = 150 μM; Table III-1) compared to the corresponding values for BuAA and DCAA, demonstrated the importance of the p-n-butyl and the 3- and/or 4-chloro groups of BuAA and DCAA, respectively, to the potencies of the latter compounds. The cytotoxic drug 2,6-diaminopurine (DAP, Figure III-6) (Burchenal et al., 1949), and its corresponding 2'-deoxyribonucleoside (2-amino-2'-deoxyadenosine, DAPdR, Figure III-6), represent structural controls for the contribution of the phenyl rings and their substituents in BuAA, DCAA and their derivatives to their inhibitory potencies. DAP, once considered for clinical use as an anticancer agent (Burchenal et al., 1949) most likely exerts its short term cytotoxicity (over a few hours) by inhibition of purine de novo biosynthesis (Heidelberger & Keller, 1955; Gots & Golub, 1959) after metabolism to the corresponding ribonucleoside 5'-monophosphate (Steglich & DeMars, 1982). The mechanism of cytotoxicity of DAPdR is unknown. Both DAP and DAPdR were ineffective as inhibitors of CHO cell [^{3}H]TdR incorporation under conditions where



DAP



DAPdR

Figure III-6. Structures of DAP and DAPdR.

BuAA and DCAA were potent (Table III-1), and both caused significant stimulation of [^{3}H]Urd incorporation (Table III-1). The observations of stimulation of [^{3}H]Urd incorporation are consistent with the proposed cytotoxic mechanism of DAP; in the short term, the cells might adapt to inhibition of de novo purine biosynthesis by relying more heavily than usual on polynucleotide precursors taken up from the cell culture medium, resulting in stimulation of incorporation. It is unclear why [^{3}H]TdR incorporation is not similarly stimulated. Clearly, however, the inhibitory properties of DAP and DAPdR argue that the substituted phenyl groups of BuAA, DCAA and their derivatives cause the latter compounds to act on cells by mechanisms distinct from those of DAP and DAPdR. The contribution of the 2-anilino substituents of BuAdA and DCAdA to their cytotoxicity was demonstrated by the lack of effect of deoxyadenosine (dA) on CHO cell [^{3}H]TdR incorporation at concentrations up to 200 μM (Table III-1).

Time course of inhibition of CHO cell [^{3}H]TdR incorporation by BuAA, BuAdA, and DAP. To gain insight into the mechanism of cytotoxicity of BuAA and BuAdA, I measured the degree of inhibition of CHO cell [^{3}H]TdR incorporation by BuAA, BuAdA and, for comparison, DAP, as a function of time of exposure to the inhibitors. The cells were incubated in the presence of inhibitors for various times,

after which [^{3}H]TdR was added and incubation continued for 15 min. Incorporation was then quenched, and measured as described in Chapter II. The results are summarized in Figure III-7. Inhibition of [^{3}H]TdR incorporation by BuAA and BuAdA was rapid in onset, and the degree of inhibition did not significantly change with time during 320 min of inhibitor exposure. In contrast, a lag time of about 2 h preceded inhibition by DAP. In addition, the effect of DAP appeared gradually during several hours, whereas the initial effects of BuAA and BuAdA were maximal. The absence of an analogous lag time for inhibition by BuAA and BuAdA argues that the latter compounds inhibit CHO cell [^{3}H]TdR incorporation by mechanisms substantially different from that of DAP; for example, BuAA and BuAdA may not require metabolism to active forms.

Reversibility of inhibition of CHO cell [^{3}H]TdR incorporation by BuAA and BuAdA. I determined the reversibility of the inhibitory effects of BuAA and BuAdA on CHO cell [^{3}H]TdR incorporation by measuring cumulative [^{3}H]TdR incorporation before, during and after exposure of cells to inhibitors. CHO cells were incubated in the presence of [^{3}H]TdR for 5 h. Inhibitors (or solvent control) were added to the culture medium at t = 1 h, and were removed at t = 2 h by twice washing the cells with PBS (pH = 7.0) and replacing the culture medium with fresh

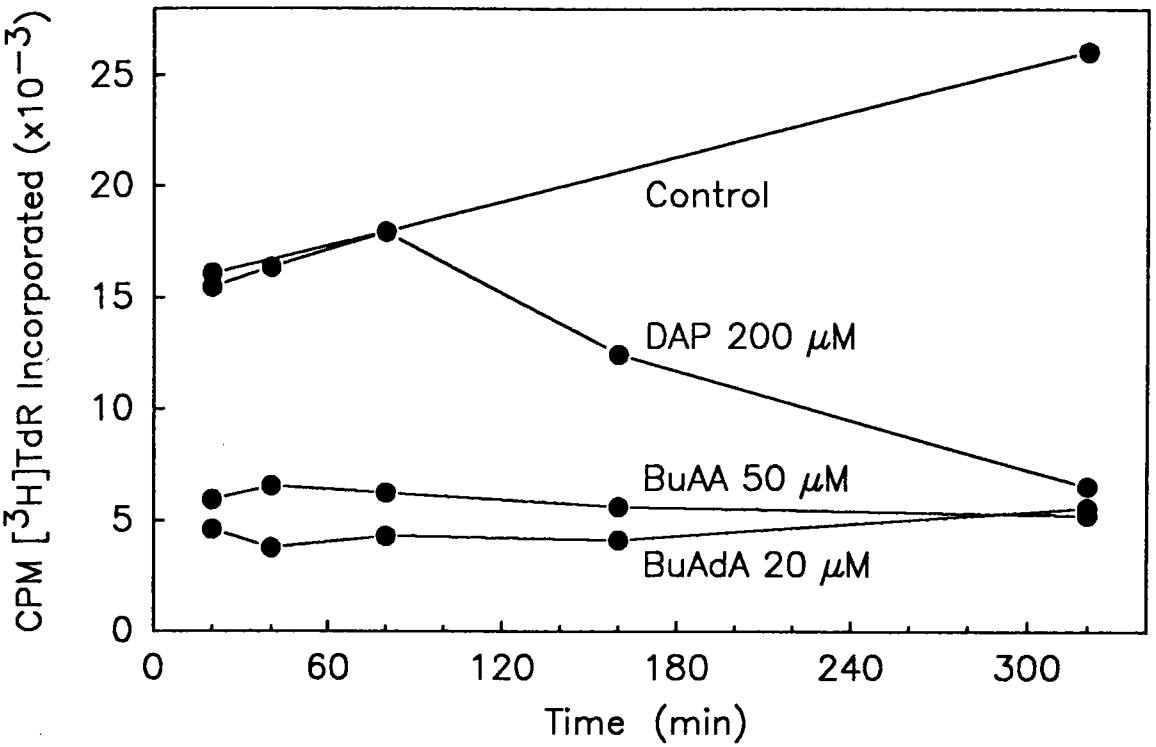


Figure III-7. Time course of inhibition by BuAA, BuAdA and DAP of CHO cell [^{3}H]TdR incorporation. Cells were incubated with inhibitors for various times. At the indicated times, [^{3}H]TdR was added, and incubation was continued for 15 min. Incorporation was then quenched, and measured as described in Chapter II.

culture medium. Incubation was then continued for 3 h. At various times, aliquots of cells were removed, incorporation was quenched and [^{3}H]TdR incorporation was measured as described in Chapter II. The results are presented in Figure III-8. At t = 2 h, the end of the hour of drug exposure, significant inhibition of [^{3}H]TdR incorporation by both BuAA and BuAdA was apparent, as expected. After t = 2 h, the rate of [^{3}H]TdR incorporation by mock-treated cells (controls) had decreased substantially. The decrease was likely due to the sudden change from conditioned to fresh culture medium when the inhibitors were removed. At t = 5 h, 3 h after the inhibitors were removed, the cells previously exposed to BuAA or BuAdA were incorporating [^{3}H]TdR at rates at least as great as that of the mock-treated cells. That observation indicated that there was no residual effect of exposure to the inhibitors; inhibition of [^{3}H]TdR incorporation was completely reversible on removal of the inhibitors. The latter conclusion served as evidence against the hypothesis that BuAA, BuAdA or both are metabolized within CHO cells to more potent forms. For example, if BuAdA were metabolized to any of its 5'-phosphate forms, which inhibit DNA polymerase alpha with 10 to 1000-fold greater potency than BuAdA (Khan et al., 1985), the membrane impermeability to those metabolites would likely not have resulted in the observed reversibility.

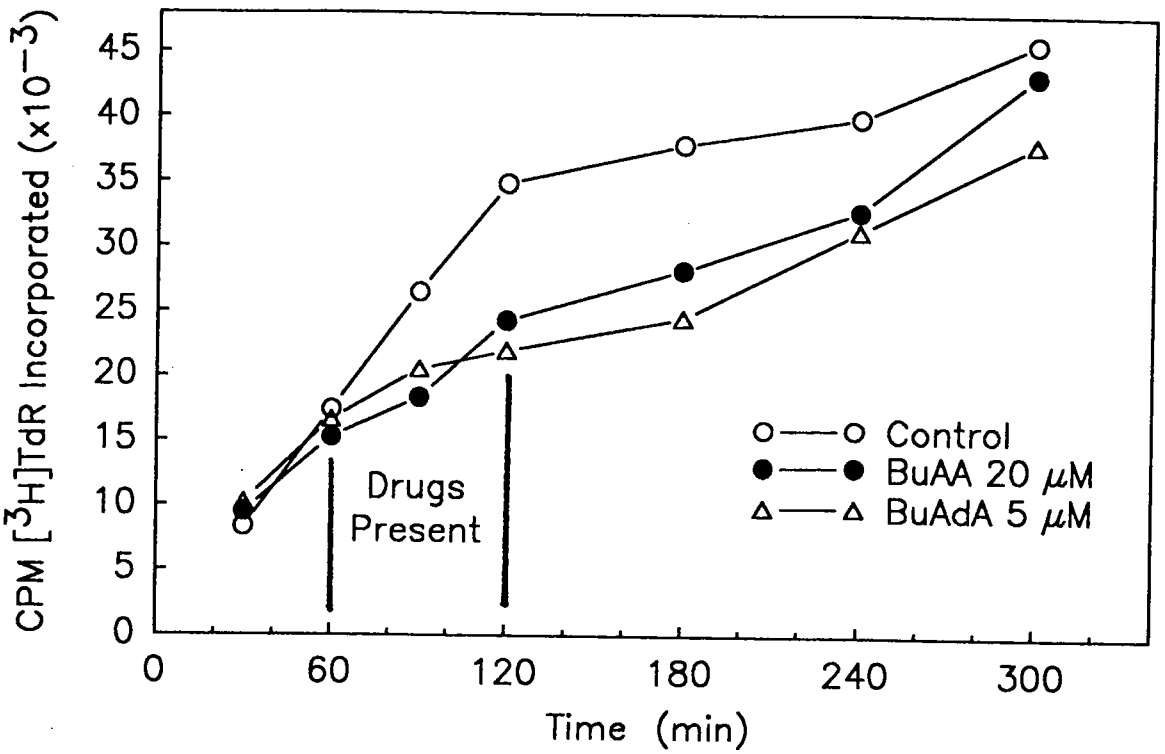


Figure III-8. Reversibility of inhibition by BuAA and BuAdA of CHO cell [^{3}H]TdR incorporation. Cells were incubated in the presence of [^{3}H]TdR. At t = 1 h, inhibitors were added, and removed at t = 2 h by twice washing the cells with phosphate buffered saline, and resuspending in fresh medium. Samples of cells were removed at the indicated times, and incorporation was quenched and measured as described in Chapter II.

D. Mechanism of Inhibition of Thymidine Incorporation

Inhibition of [^{3}H]TdR uptake. I began investigating the mechanism of nucleobase and nucleoside analog inhibition of [^{3}H]TdR incorporation by examining the inhibitory effects of BuAA, BuAdA, DCAA, DCAdA and other compounds on the metabolic steps required for [^{3}H]TdR incorporation. As the first mediated step in the overall process of incorporation of extracellular [^{3}H]TdR into DNA, I first considered [^{3}H]TdR transport across the cell membrane as a potential target for inhibition. I adapted the technique of Aronow and Ullman (1985) to measure short term [^{3}H]TdR uptake by CHO cells in the absence of replicative DNA synthesis. Uptake is defined as the sum of transport across cell membranes plus metabolism to forms which cannot diffuse out of the cell, such as nucleotides and polymers (Cass and Paterson, 1977). Thus, nucleoside transport per se can only be measured in the absence of nucleoside metabolizing enzymes, for example in a reconstituted system. I, therefore, measured nucleoside uptake, and controlled for the likely possibility that one or more of the inhibitors in question block [^{3}H]TdR incorporation at least partially as a result of inhibition of DNA polymerases by doing so in the absence of replicative DNA synthesis. I completely inhibited replicative DNA synthesis by adding 10 μM aphidicolin to the cells before measuring [^{3}H]TdR uptake

(Huberman, 1981). At this concentration, total measured uptake decreased by 10 to 15%. Finally, background counts were estimated as the residual counts observed in the presence of both aphidicolin and the nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR) at 10 μM, a concentration several orders of magnitude above its IC_{50} (Wohlhueter et al., 1978). The background so measured typically represented 20% of the total apparent uptake.

Using the experimental conditions described above, I examined the potencies of several compounds as inhibitors of [^{3}H]TdR uptake; the results are presented in Table III-2. Both of the deoxyadenosine analogs, BuAdA and DCAdA, strongly inhibited [^{3}H]TdR uptake, the latter with an IC_{50} value (4 μM) comparable to that which it displayed for inhibition of [^{3}H]TdR incorporation (7 μM; Table III-1). These observations suggest that much of the inhibitory potencies displayed by BuAdA and DCAdA in the [^{3}H]TdR incorporation assay (Table III-1) may be a result of inhibition of [^{3}H]TdR transport and phosphorylation, i.e. uptake, rather than inhibition of the replicative DNA polymerase(s). The requirement of the 2-anilino substituents of BuAdA and DCAdA to their potencies as inhibitors of [^{3}H]TdR uptake was demonstrated by the inactivity of dA in the uptake assay. Uptake inhibition also was strongly dependent on the presence of the

Table III-2. Inhibition of [^{3}H]TdR Uptake by CHO Cells in the Absence of Replicative DNA Synthesis[1]

Inhibitor	IC_{50} (μM)[2]
BuAA	200
BuAdA	23
DCAA	100
DCAdA	4
BuPdG	150
2AA	I
dA	I

[1][^{3}H]TdR uptake by CHO cells during 15 sec exposure was measured as described in Chapter II. Net uptake was that which occured in the presence of 10 μM aphidicolin and could be blocked by 10 μM NBMPR.

[2]IC_{50} values were determined on the basis of inhibitor concentration vs. percent activity curves. I = inactive at 200 μM, the highest concentration tested.

deoxyribofuranosyl group, as shown by the weak inhibitory potencies of the nucleobase analogs BuAA and DCAA, and the inactivity of 2AA (Table III-2). The relatively weak activity of the corresponding deoxyguanosine analog of BuAdA [N^2-(p-n-butylphenyl)-2'-deoxyguanosine, BuPdG, Figure III-1] in the uptake assay demonstrated the importance of the 6-substituent of BuAdA to its potency.

E. Inhibition of [^{32}P]Phosphate Incorporation into CHO Cell DNA

The results described in the previous section indicated that [^{3}H]TdR was an unfortunate choice as a DNA precursor for the estimation of inhibition of CHO cell DNA synthesis by 2-substituted purine nucleobase and nucleoside analogs. The ability of BuAdA and DCAdA to inhibit CHO cell [^{3}H]TdR uptake (IC_{50} values of 23 and 4 μM, respectively; Table III-2) may substantially contribute to or explain the potencies of these compounds as inhibitors of CHO cell [^{3}H]TdR incorporation. Thus, BuAdA and DCAdA may be significantly weaker inhibitors of cellular DNA synthesis than previously believed. I therefore chose to reassess the potencies of those and related compounds as inhibitors of cellular DNA synthesis.

In order to bypass the effects of inhibitors on nucleoside uptake, I used the method of Robichaud and Fram

(1987), which is described in Chapter II, to measure [^{32}P]phosphate incorporation into CHO cell DNA. The use of phosphate as the radiolabelled DNA precursor has the advantage that it does not require the activity of several potential inhibitory targets such as nucleoside transport proteins. The disadvantages of [^{32}P]phosphate incorporation include the requirement of several hours of incubation before cellular DNA is significantly labeled, as demonstrated by the data of Figure III-9, in which [^{32}P]phosphate incorporation into CHO cell DNA is plotted as a function of time of exposure to the label. The curve clearly demonstrates that there is a lag time of about 60 min before DNA is significantly labeled. Therefore, cells were exposed to inhibitors (or solvent control) for 1 h, after which [^{32}P]phosphate was added. Three hours later, incorporation was quenched, and samples were prepared for scintillation counting as described in Chapter II. That portion of base stable, acid precipitable counts whose formation could be blocked by 10 μM aphidicolin (typically 80% of the total) was taken as representing replicative DNA synthesis.

The results of the inhibition studies are presented in Table III-3. The purine nucleobase analogs BuAA and DCAA were significantly more potent than their 2'-deoxyribonucleoside counterparts (BuAdA and DCAdA) as

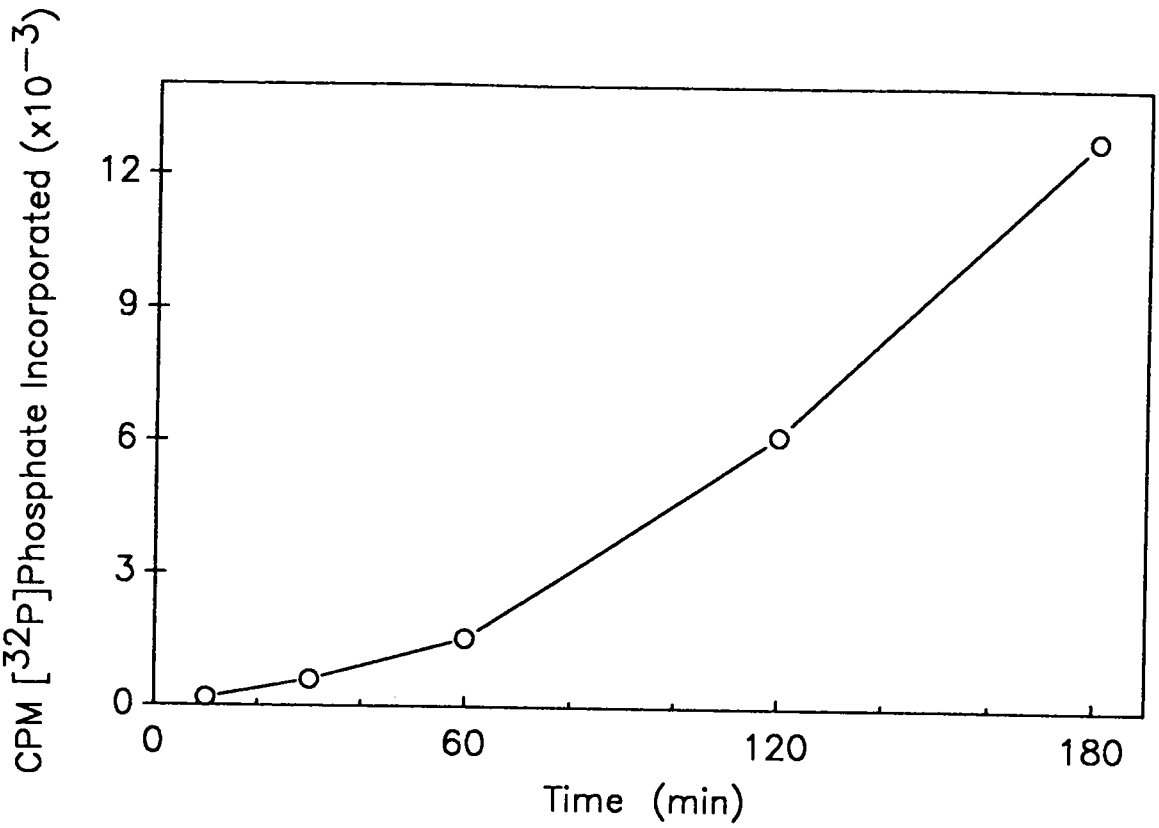


Figure III-7. Incorporation of [^{32}P]phosphate into CHO cell DNA as a function of time of exposure to the label. [^{32}P]Phosphate incorporation was measured as described in Chapter II, except that incubation time was varied as indicated.

Table III-3. Inhibition of Incorporation of [^{32}P]Phosphate into CHO Cell DNA[1]

Inhibitor	IC_{50} (μM)[2]
DCAA	12
BuAOMe	17
DCMTP	20
DCMP	30
DCACl	30
BuAA	30
DCAdA	35
DCTG	50
BuPTG	50
BuACl	65
BuAdA	80
BuASMe	100
DCPG	I
BuPG	I
BuPdG	I

[1][^{32}P]Phosphate incorporation into CHO cell DNA was measured as described in Chapter II. Cells were incubated with inhibitors (or solvent control) for 1 h, [^{32}P]phosphate was added, and incubation was continued for 3 h. That portion of radiolabelled, base stable, acid precipitable macromolecules whose synthesis could be blocked by aphidicolin was taken as representing net DNA synthesis.

[2]IC_{50} values were determined on the basis of inhibitor concentration vs. percent activity curves. I = inactive at 100 μM, the highest concentration tested.

inhibitors of [^{32}P]phosphate incorporation. The activities of BuAdA and DCAdA in the [^{32}P]phosphate incorporation assay (with IC_{50} values of 80 and 35 μM, respectively), compared to their activities in the [^{3}H]TdR incorporation assay (IC_{50} values of 3 and 7 μM, respectively), were consistent with the proposal made earlier that one or both of the compounds inhibits CHO cell [^{3}H]TdR incorporation largely as a result of inhibition at targets not directly related to DNA synthesis, for example, nucleoside transport proteins. The most potent inhibitor in the [^{32}P]phosphate incorporation assay was DCAA (IC_{50} 12 μM; Table III-3), a compound that inhibited [^{3}H]TdR uptake with an IC_{50} value of 100 μM (Table III-2) and inhibited [^{3}H]TdR incorporation into CHO cell DNA with an IC_{50} value of 20 μM. The latter results suggest that DCAA, unlike DCAdA and BuAdA, inhibited [^{32}P]phosphate and [^{3}H]TdR incorporation by CHO cells as a result of inhibition of DNA synthesis, possibly by direct inhibition of the replicative DNA polymerase(s).

I tested several 6-substituted analogs of DCAA for inhibition of [^{32}P]phosphate incorporation by CHO cells. The rationale for testing these compounds was that the members of an analogous series of BuAA derivatives were more potent inhibitors of CHO cell pol α than BuAA itself (Wright et al., 1987); 6-substitution of DCAA might have resulted in one or more compounds with greater inhibitory potency toward

pol α (or perhaps pol δ), or toward DNA synthesis. The results (Table III-3) indicated that none of the DCAA analogs was a more potent inhibitor than DCAA itself. The guanine analog of DCAA (DCPG) was completely inactive in the [^{32}P]phosphate incorporation assay; in fact, all of the guanine analogs tested were weaker inhibitors than their adenine analog counterparts. The data of Table III-3 are consistent with the observations of Wright et al. (1987), who found that, in the case of BuAA analogs, adenine analogs were generally more potent than their guanine analog counterparts, and that 6-substituted BuAA analogs, despite their increased potency as inhibitors of pol α, were generally less potent inhibitors of CHO cell [^{3}H]TdR incorporation than BuAA.

CHAPTER IV

INHIBITION OF MAMMALIAN DNA POLYMERASES ALPHA AND DELTA

A. Introduction

In Chapter III, I described the investigation of the mechanism of inhibition of CHO cell [^{3}H]TdR incorporation by BuAdA, a compound which inhibited CHO cell [^{3}H]TdR incorporation more potently than pol α (Wright et al., 1987). During that investigation, I found that the potency of inhibition of [^{3}H]TdR incorporation by BuAdA could largely be explained by its ability to inhibit [^{3}H]TdR uptake rather than DNA synthesis. I reassessed inhibition of cellular DNA synthesis using [^{32}P]phosphate rather than [^{3}H]TdR as the labeled precursor, and found that BuAdA, like all of the BuAA analogs which have been tested (Wright et al., 1987), is a weaker inhibitor of cellular DNA synthesis than of pol α. During the course of the work described in Chapter III, it was reported that BuPdGTP, an inhibitor of pol α with nanomolar range potency (Khan et al. 1984), and a degree of selectivity of about three orders of magnitude for pol α compared to pol δ (Lee et al., 1985), inhibited permeable cell DNA synthesis with a potency two to three orders of magnitude less than its potency as an inhibitor of pol α (Dresler & Frattini, 1986; 1988). The latter result fit the pattern that inhibitors selective for pol α relative

to pol δ, unlike inhibitors such as aphidicolin which do not discriminate between the two enzymes (Goscin & Byrnes, 1982), inhibit cellular DNA synthesis significantly more weakly than they do pol α. This observation suggested (i) a role for pol δ in cellular DNA synthesis, and (ii) that inhibition of pol δ, perhaps in addition to inhibition of pol α, is the major requirement for inhibition of cellular DNA synthesis. An inhibitor of pol δ selective for that enzyme relative to pol α could be used to test both of the latter hypotheses, although such a compound was not known. I therefore began a search for a selective inhibitor of pol δ. The search for, discovery of, and characterization of the inhibitory mechanism of one such compound is the topic of the present chapter. The subsequent chapter, V, which completes the "results" section of the thesis, describes experiments designed to reveal the effects of the selective pol δ inhibitor on cellular DNA synthesis.

B. Enzymes.

Calf thymus DNA polymerase alpha. Pol α from calf thymus was prepared by Drs. Kimberly Foster and Russell Hammond (University of Massachusetts Medical School), by the immunoaffinity chromatography method described by Chang et al. (1984). Pol α so prepared was composed of four major peptides, as judged by SDS-polyacrylamide gel

electrophoresis. The preparation contained two enzyme activities: a DNA polymerase and a DNA primase.

Calf thymus DNA polymerase delta. Pol δ from calf thymus DNA was prepared by Drs. Federico Focher and Ulrich Hübscher (University of Zürich-Irchel). I used two enzyme preparations, which differed in their degree of purity. One, designated HAP-δ, was purified as described (Focher et al., 1988c), through step III, hydroxyapatite chromatography. HAP-δ was composed of four major peptides, and several others of lower abundance, as judged by a SDS-polyacrylamide gel electropherogram stained with Coomassie blue. The second preparation, designated FPLC-δ, was purified as described (Focher et al., 1989), through step V, FPLC-monoS chromatography. FPLC-δ was composed of four major peptides. FPLC-δ became available to me late in the course of the experiments described here, and I used it only in the analysis of the mechanism of inhibition of pol δ by carbonyldiphosphonate (COMDP) (section IV-E below). Both HAP-δ and FPLC-δ contained two enzyme activities: a DNA polymerase and a 3' → 5' exonuclease. Neither FPLC-δ (Focher et al., 1988c) nor HAP-δ[1] were stimulated by PCNA. Western blots using a PCNA-specific heteroantiserum (Almendral et al., 1987) indicated that PCNA was not present in either HAP-δ (Focher et al., 1988c) or FPLC-δ (Focher et

[1] U. HÜbscher, personal communication.

al., 1989). Calf thymus is the only tissue from which both PCNA stimulated (Lee et al., 1984) and PCNA insensitive pol δ forms (Crute et al., 1986; Focher et al., 1988c) have been isolated.

C. Screening for Inhibitors of DNA Polymerases Alpha and Delta.

DNA polymerase assay conditions. To screen for inhibition of pol α and pol δ, I used activated DNA as the template:primer. Because activated DNA is comprised of all four dNMP monomers, its use allowed omission (truncation) of any of the dNTP substrates, an approach which increases the sensitivity of the DNA polymerase reaction to inhibition by compounds which bind to the enzyme competitively with the omitted dNTP (Wright & Brown, 1976). Another advantage of the truncated assay is that, in the absence of the competitive dNTP, the K_i value of an inhibitor is measured directly as the IC_{50} value obtained from a drug concentration vs. enzyme activity curve (Wright & Brown, 1976). The disadvantage of activated DNA as the template:primer for the present studies is that it directs a level of DNA synthesis by pol δ which is only about 25% that directed by the template:primer preferred by pol δ, poly(dA):oligo(dT) (Focher et al., 1988c). In addition, truncation of the assay decreases DNA synthesis catalyzed by

pol δ to about 60% of the value which occurs under full assay conditions (all four dNTPs present). In order to increase the activity of pol δ on activated DNA under truncated conditions, I doubled the amount of enzyme used per assay tube and modified the reaction conditions. The modifications were: (i) an increase in KCl concentration from 10 to 100 μM, (ii) an increase in the reaction time from 30 to 90 min, and (iii) addition of 1 mM GMP or AMP, in order to inhibit the 3' → 5' exonuclease activity of pol δ (Byrnes et al., 1977). With these changes, DNA synthesis catalyzed by pol δ under truncated conditions gave incorporation of >2000 cpm of the radiolabel per assay tube; enough to give a usable signal to noise ratio (>10) when the enzyme was significantly inhibited.

Screening of compounds for inhibition of DNA polymerases alpha and delta. I assayed pol α under the same conditions as pol δ. I found that pol α catalyzes a level of DNA synthesis under pol δ conditions similar to that which it catalyzes under typical pol α conditions. I measured the inhibitory activity against pol α and pol δ of several nucleotide, nucleoside, purine base and aphidicolin analogs at 100 μM in appropriate truncated assays, and pyrophosphate analogs at 100 μM in a full assay. The results are presented in Table IV-1, and are discussed below.

dATP analogs. None of the dATP analogs tested displayed

Table IV-1. Screen of Compounds for Inhibition of DNA Polymerases Alpha and Delta Assayed on Activated DNA[1]

Inhibitor	% Inhibition pol α	pol δ
-dATP		
araATP	72	54
BuAdATP	90	40
BuAA	71	0
BuAOMe	78	16
DCAA	67	40
DCACl	48	0
DCMP	35	0
DCMTP	53	29
DCAdA	16	0
DCAdAMP	63	17
DCAdATP	90	79
-dGTP		
BuPdGTP	92	74
EMPdGTP	81	16
PhdGTP	73	15
HexdGTP	80	0
ACGTP	90	79
-dCTP		
araCTP[2]	5	60
Aphidicolin[3]	90	96
17-Acetylaphidicolin	48	43
17,18-Diacetylaphidicolin	20	32
3-Epiaphidicolin	57	47
"16-Ketoaphidicolin"	74	64
-dTTP		
AZTTP	24	28
ddTTP	57	62
Full Assay		
araCTP[2]	40	71
PP_i	20	40
PFA	20	15
F_2MDP	8	36

[1]Enzyme activities (pol α and HAP-δ) were assayed under

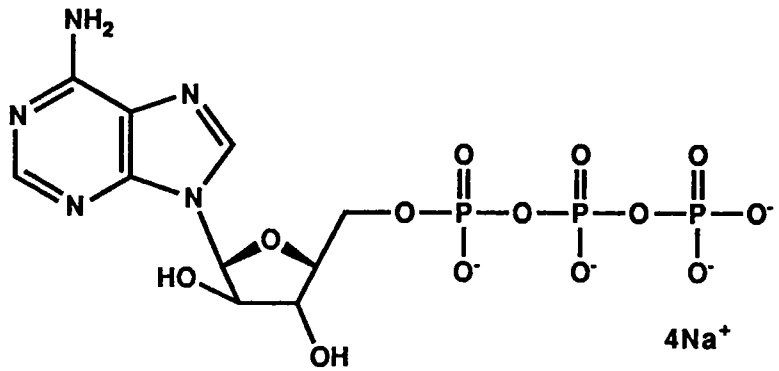
the conditions described in Chapter II for activated DNA as the template:primer. Enzymes were assayed under full (all four dNTP substrates present) or truncated (one dNTP omitted) conditions, as indicated. Inhibitors were present at 100 μM unless noted otherwise.

[2]araCTP was assayed in the presence and absence of dCTP.

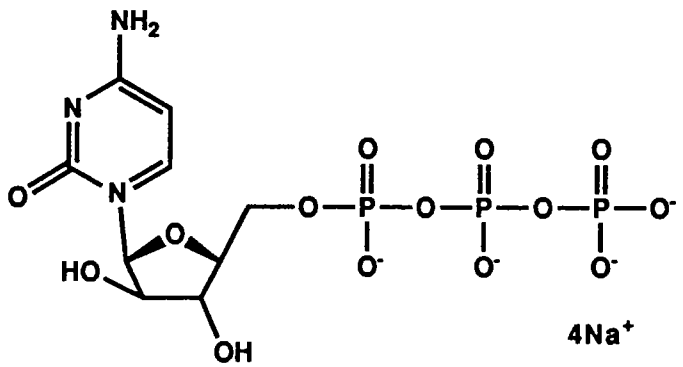
[3]Inhibitor present at 10 μM.

selective inhibition of pol δ compared to pol α (Table IV-1). Because of the high concentration used in the screen, the selectivity of BuAdATP for pol α compared to pol δ is grossly understated by the results presented in Table IV-1. The K_i values for BuAdATP for the two enzymes, determined on the basis of concentration *vs.* enzyme activity curves, are presented in Table IV-2, and indicate that BuAdATP inhibited pol α with a potency more than four orders of magnitude greater than that with which it inhibited pol δ. The base form of BuAdATP, BuAA, and its 6-substituted derivative BuAOMe [2-(p-n-butylanilino)-6-methoxypurine, Figure III-1], were weaker inhibitors of both enzymes than was BuAdATP, and both base analogs displayed significant selectivity for pol α compared to pol δ (Table IV-1). The ATP isomer araATP [9-β-$_D$-arabinofuranosyl)adenine 5'-triphosphate, Figure IV-1] inhibited both enzymes without significant selectivity (Table IV-1).

The adenine analog, DCAA, and all of the DCAA derivatives, inhibited pol α with greater potency than pol δ (Table IV-1). Table IV-2 presents the K_i values for DCAdATP; pol α is inhibited by DCAdATP with a K_i value of 3 μM, while the corresponding value for pol δ is 66 μM. Thus, the pol α selectivity of DCAdATP, a close structural analog of BuAdATP, is more than three orders of magnitude less than that of BuAdATP. The latter observation suggests that there



araATP



araCTP

Figure IV-1. Structures of araATP and araCTP.

Table IV-2. Potencies of Selected Inhibitors of DNA Polymerases Alpha and Delta Assayed on Activated DNA[1]

Inhibitor	K_i (μM)[2] pol α	pol δ
BuPdGTP	0.005	25
BuAdATP	0.005	150
DCAdATP	3	66
ddTTP	60	25

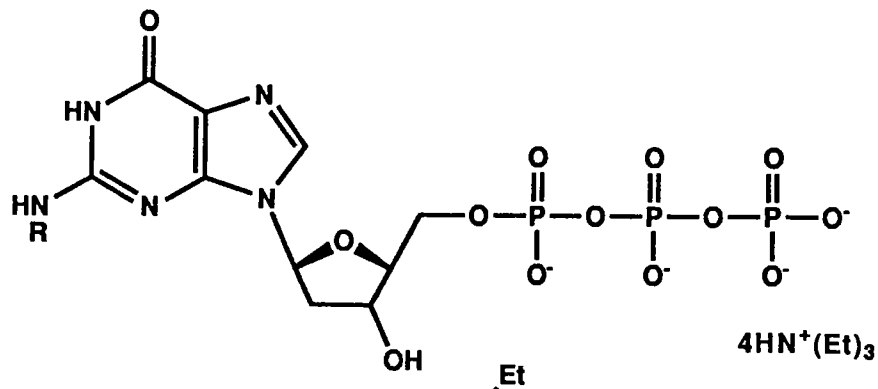
[1]Enzyme activities (pol α and HAP-δ) were assayed under the conditions described in Chapter II for activated DNA as the template:primer. Assay mixes were truncated with respect to the dNTP expected to be competitive with each inhibitor.

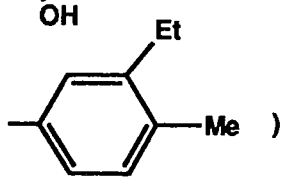
[2]K_i values were determined on the basis of inhibitor concentration *vs.* enzyme activity curves (Wright & Brown, 1976).

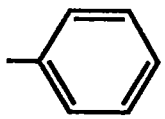
is considerable potential for the development of selective inhibitors of pol δ based on the DCAdATP motif; this potential is discussed in Chapter VI.

dGTP analogs. None of the dGTP analogs tested displayed selectivity for pol δ compared to pol α (Table IV-1). BuPdGTP, the dGTP analog of BuAdATP, appeared only weakly selective for pol α compared to pol δ when tested at 100 μM. However, like BuAdATP, this observation strongly understates the selectivity of BuPdGTP for pol α; the K_i values of BuPdGTP for pol α and pol δ, presented in Table IV-2, indicated that, like BuAdATP, BuPdGTP inhibited pol α with a potency several orders of magnitude greater than that of pol δ. Three other N^2-substituted dGTP analogs, EMPdGTP [N^2-(3-ethyl-4-methylphenyl)-2'-deoxyguanosine 5'-triphosphate, Figure IV-2], PhdGTP [N^2-(phenyl)-2'-deoxyguanosine 5'-triphosphate, Figure IV-2] and HexdGTP [N^2-(n-hexyl)-2'-deoxyguanosine 5'-triphosphate, Figure IV-2] were tested; all three displayed inhibitory selectivity for pol α compared to pol δ, and all were considerably weaker inhibitors of both enzymes than was BuPdGTP (Table IV-1).

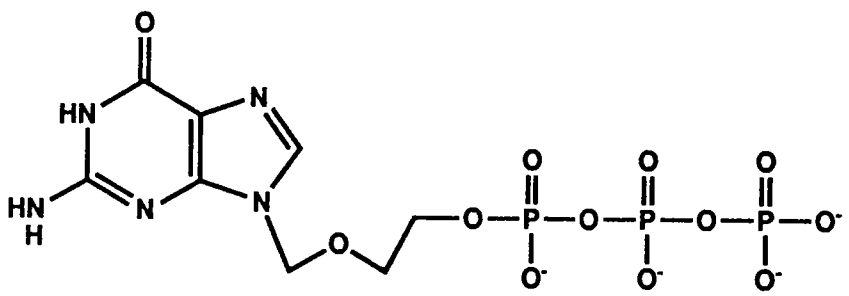
ACGTP {9-[(2-hydroxyethoxy)methyl]guanine triphosphate, Figure IV-2}, the triphosphate analog of the antiviral agent acyclovir and likely one of the active metabolites of that agent in vivo (Elion et al., 1977), did not display significant inhibitory selectivity for either enzyme (Table



EMPdGTP (R = )

PhdGTP (R = )

HexdGTP (R = $-(CH_2)_5CH_3$)



ACVTP

Figure IV-2. Structures of EMPdGTP, PhdGTP, HexdGTP and ACVTP.

IV-1). It is important to note that if pol δ is involved in DNA metabolism in mammalian cells inhibition, of that enzyme as well as others such as pol α by agents such as acyclovir and AZT (see below) and their metabolites is a potential contributor to their in vivo toxicity.

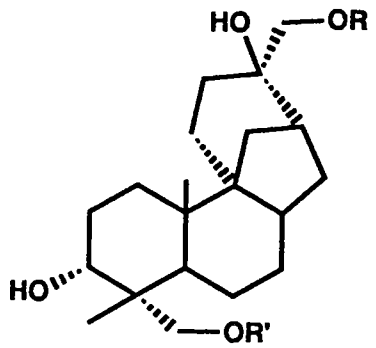
dCTP analogs. The arabinofuranosyl derivative araCTP [9-(β-$_D$-arabinofuranosyl)cytidine 5'-triphosphate, Figure IV-1], a CTP isomer, was an ineffective inhibitor of truncated (-dCTP) DNA synthesis catalyzed by pol α, while it inhibited the analogous reaction catalyzed by pol δ by 60% (Table IV-1). Most of this apparent selectivity disappeared with the addition of dCTP to the reaction mix; in the full assay, pol α and pol δ were inhibited by 40 and 71%, respectively (Table IV-1). The observation that DNA synthesis catalyzed by calf thymus pol α in the absence of dCTP was resistant to inhibition by araCTP is in accord with that of Yoshida et al. (1977), who also used pol α derived from calf thymus. However, it is not consistent with several observations that, as an inhibitor of pol α derived from a variety of sources, araCTP displayed simple competitive kinetics with respect to dCTP concentration (for a review, see Cozzarelli, 1977). Further, I have observed that 100 μM araCTP inhibits pol α derived from CHO cells 64% when assayed under truncated (-dCTP) conditions.[1] It is

[1] Results not shown.

possible that sensitivity of pol α to inhibition by araCTP in the absence of dCTP is a function of the source of the enzyme. My observations suggest that, with calf thymus as the enzyme source and truncated (-dCTP) polymerase reaction conditions, araCTP could be used to inhibit selectively pol δ and thereby distinguish it from pol α.

Aphidicolin analogs. I measured inhibition of pol α and pol δ by aphidicolin and its derivatives in reaction mixes lacking dCTP, because inhibition of pol α by aphidicolin is competitive with dCTP (Oguro et al., 1979; Pedrali-Noy & Spadari, 1979). The aphidicolin derivatives tested were 17-acetylaphidicolin, 17,18-diacetylaphidicolin, "16-ketoaphidicolin" (3,18-dihydroxy-17-noraphidicolan-16-one) and 3-epiaphidicolin (Figure IV-3); each was synthesized in the lab of Dr. George Wright by Dr. Lili Arabshahi as described (Arabshahi et al., 1988), by the methods of Dalziel et al. (1973). Aphidicolin was a potent inhibitor of both pol α and pol δ (Table IV-1), and, in agreement with previously reported results (Goscin & Byrnes, 1982) the compound did not display selective inhibition of either enzyme. Each of the four derivatives of aphidicolin tested (Table IV-1) inhibited both pol α and pol δ with less potency than the parent compound, and none displayed significant selectivity for either enzyme.

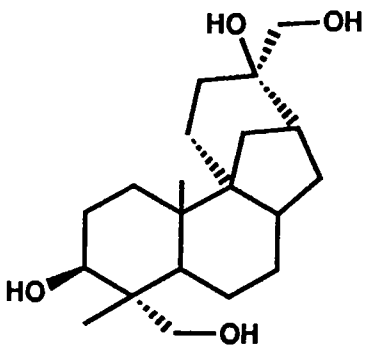
dTTP analogs. Neither of the dTTP analogs tested



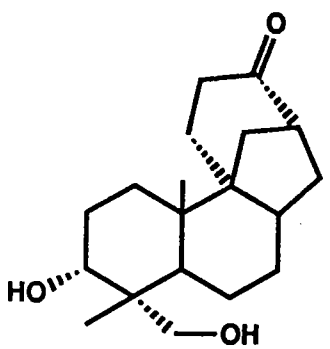
Aphidicolin (R = R' = H)

17-Acetylaphidicolin (R = Ac, R' = H)

17,18-Diacetylaphidicolin (R = R' = Ac)



3-Epiaphidicolin

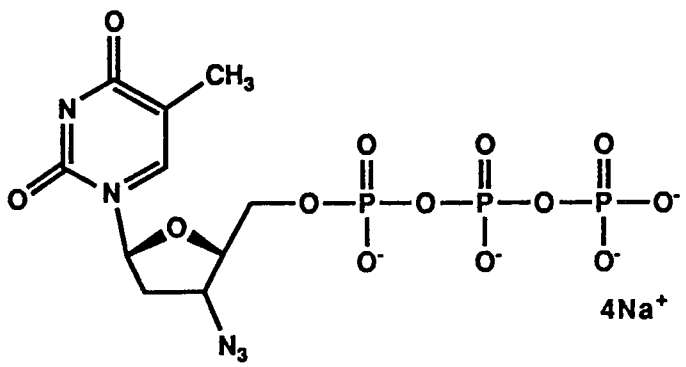


"16-Ketoaphidicolin"

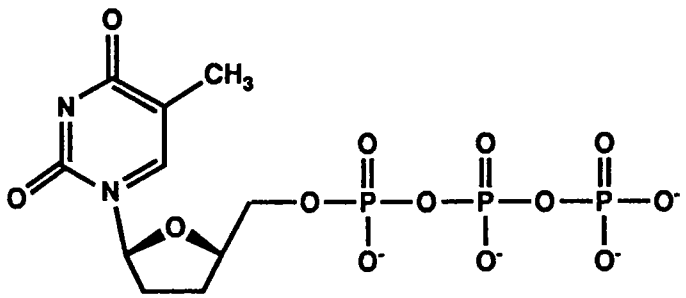
Figure IV-3. Structures of Aphidicolin and Aphidicolin derivatives.

displayed inhibitory selectivity for pol α or pol δ (Table IV-1). AZTTP [(3'-azido)-2'-deoxythymidine 5'-triphosphate, Figure IV-4], the 5'-triphosphate derivative of the human immunodeficiency virus inhibitor AZT, and likely its active metabolite in vivo (Mitsuya et al., 1985), was a weak inhibitor of both pol α and pol δ, and displayed selectivity for neither enzyme (Table IV-1). 2',3'-Dideoxythymidine 5'-triphosphate (ddTTP, Figure IV-4) displayed similar potency as an inhibitor of pol α and pol δ (Table IV-1). However, the K_i values of ddTTP, 60 and 25 μM for pol α and pol δ, respectively (Table IV-2), indicate that ddTTP displays some inhibitory selectivity for pol δ compared with pol α, an observation in accord with the results of Wahl et al. (1986). At least one attempt has been made to exploit the modest inhibitory selectivity of ddTTP for pol δ compared to pol α to determine a role for pol δ in DNA metabolism in vivo (Dresler & Kimbro, 1987).

Pyrophosphate analogs. I tested the inhibitory activity of pyrophosphate (PP_i, Figure IV-5) and two PP_i analogs in full assays rather than in truncated assays because the structure of PP_i did not suggest that it or its analogs would be competitive with any particular dNTP. Phosphonoformate (PFA, Figure IV-5) (Reno et al, 1978) was a weak inhibitor of both pol α and pol δ, and did not display selectivity for either enzyme (Table IV-1). The potency of

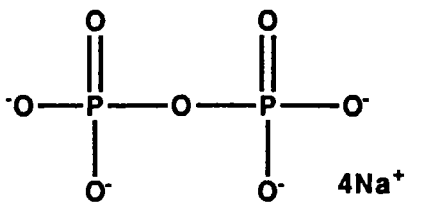


AZTTP

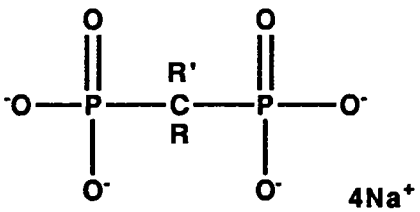


ddTTP

Figure IV-4. Structures of AZTTP and ddTTP.

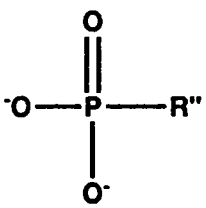


PP_i



MDP (R = R' = H)

F_2MDP (R = R' = F)



PFA (R = COOH)

PAA (R" = CH_2COOH)

Figure IV-5. Structures of PP_i, MDP, F_2MDP, PFA and PAA.

inhibition of pol α by PFA that I observed is less than that observed by others (for a review, see Öberg 1989), and it is significantly less than I observed when assaying pol α and pol δ using poly(A):oligo(dT) as the template:primer (see section IV-D below). PP_i and its isostere, difluoromethanediphosphonate (F_2MDP, Figure IV-5) were also weak inhibitors of both enzymes, but each inhibited pol δ selectively compared to pol α (Table IV-1). The latter observation prompted me to investigate the inhibition of pol α and pol δ by PP_i and F_2MDP in greater detail.

Concentration vs. enzyme activity curves, determined by Naseema Khan, (Figure IV-6) showed that F_2MDP inhibited pol δ with an IC_{50} value of 0.75 mM, while the compound was ineffective as an inhibitor of pol α at concentrations up to 2 mM (Figure IV-6A). Analogous experiments with PP_i (Figure IV-6B) showed that that compound was a more potent inhibitor of pol δ than was F_2MDP (IC_{50} = 0.15 mM), but was less selective relative to pol α, inhibiting the latter enzyme with an IC_{50} value of 0.6 mM. The observation of the selectivity of F_2MDP for pol δ compared to pol α prompted me to investigate its mechanism of inhibition of pol α and pol δ, and to survey the inhibitory potencies of the members of a larger series of PP_i analogs, with the goal of finding a compound with improved potency and selectivity for pol δ. The results of both sets of experiments are described below.

Figure IV-6.

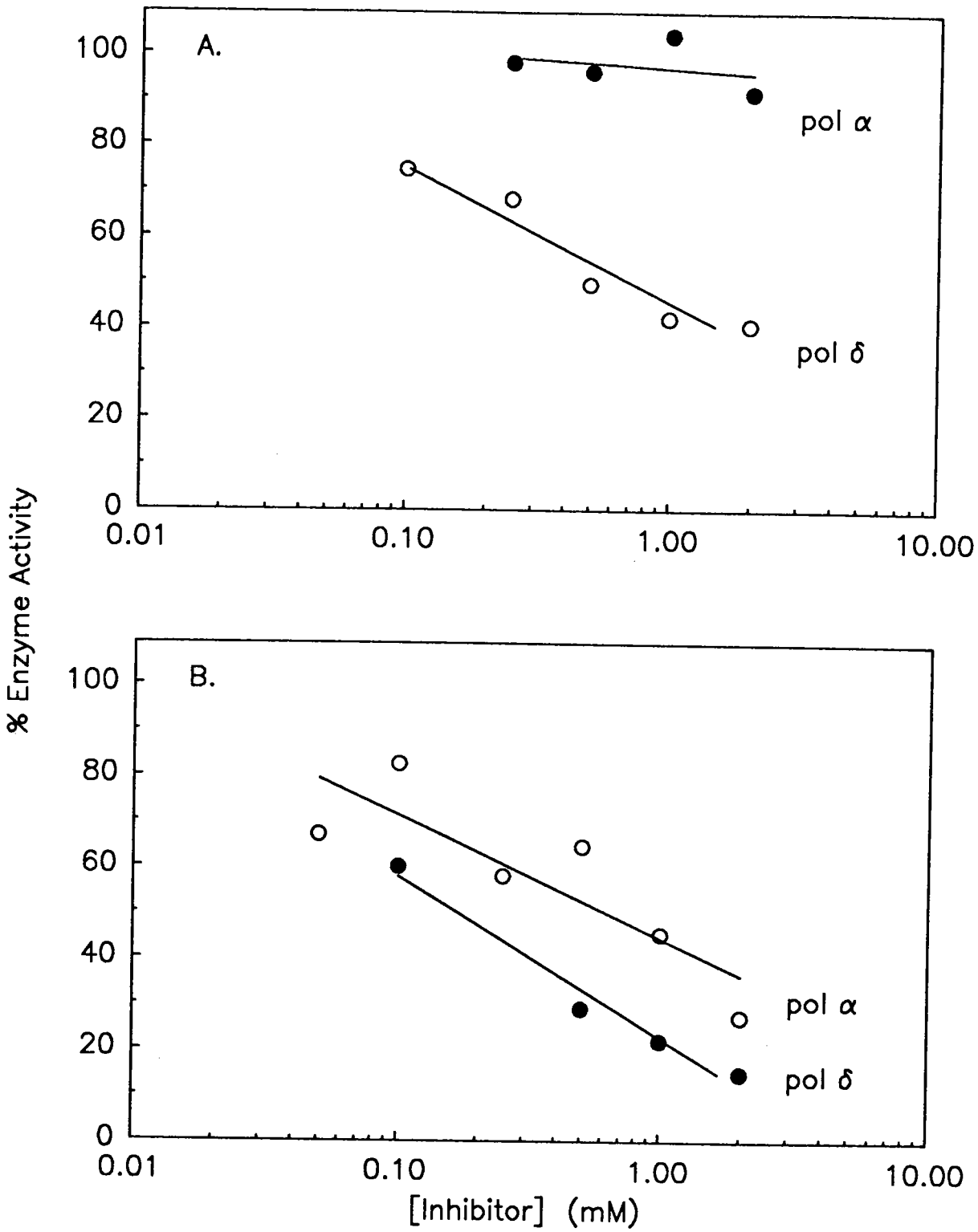

A.
100
80
60
40
20
0
0.01
0.10
1.00
10.00
pol α
pol δ
B.
100
80
60
40
20
0
0.01
0.10
1.00
10.00
pol α
pol δ
% Enzyme Activity
[Inhibitor] (mM)

Figure IV-6. Concentration *vs.* enzyme activity curves for inhibition of pol α and pol δ by PP_i and F_2MDP. Enzyme activities (pol α and HAP-δ) were assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer. (A) Inhibition of pol α and pol δ by F_2MDP. (B) Inhibition of pol α and pol δ by PP_i.

D. Pyrophosphate and Difluoromethanediphosphonate: Selective Inhibitors of DNA Polymerase Delta.

DNA polymerase assay conditions. As DNA polymerase inhibitors, neither PP_i nor its structural analogs were expected to be competitive with any particular dNTP, and, therefore, the use of truncated assays with activated DNA offered no advantage for measuring inhibition of pol δ activity. I therefore chose the template:primer preferred by pol δ, poly(dA):oligo(dT) (Focher et al., 1988c) for the investigation of the mechanism of inhibition of pol δ by PP_i and its analogs, and for further screening of PP_i analogs (described below). I found that poly(dA):oligo(dT) also is an effective template:primer for pol α, directing a level of synthesis comparable to that directed by activated DNA. GMP was included in all assays at a concentration of 2 mM, to inhibit the 3' → 5' exonuclease of pol δ (Byrnes et al., 1977) and thereby keep the polymerase reaction linear with time during the reaction period, even at the low dNTP concentrations used in some experiments.

Inhibition of DNA polymerases alpha and delta by PP_i and PP_i analogs using poly(dA):oligo(dT) as the template:primer. I determined the IC_{50} values for PP_i and four PP_i analogs on pol α and pol δ; the results are presented in Table IV-3. Both PP_i, with IC_{50} values of 1 mM and 250 μM for pol α and pol δ, respectively, and F_2MDP, with IC_{50} values of 10 mM

and 4 mM for pol α and pol δ, respectively (Table IV-3), were weaker inhibitors of both enzymes when assayed using poly(dA):oligo(dT) as the template:primer than they were when activated DNA was used (see above). The degree of selectivity of both compounds was not significantly altered by the change in template:primer. Methanediphosphonate (MDP, Figure IV-5), however, displayed steep concentration *vs.* enzyme activity curves as an inhibitor of both pol α and pol δ; neither enzyme was inhibited significantly by MDP at 3 mM, and both enzymes were inhibited almost completely at 10 mM. The phosphonates PFA and phosphonoacetate (PAA, Figure IV-5) (Leinbach et al., 1976) inhibited both enzymes with greater potency than the other PP_i analogs (Table IV-3), but neither displayed inhibitory selectivity for either enzyme.

Possible role of Mg^{2+} chelation in the mechanism of inhibition of DNA polymerase delta by PP_i, F_2MDP and MDP. The standard conditions which I used for the assay of pol δ included Mg^{2+} at 10 mM (Focher et al., 1989). Because the IC_{50} values of PP_i, F_2MDP and MDP as inhibitors of pol δ were within an order of magnitude of the Mg^{2+} concentration, I was concerned that one or more of the compounds inhibited pol δ primarily as a result of Mg^{2+} chelation. I tested that possibility by measuring the potencies of the inhibitors as a function of Mg^{2+} concentration. I first

Table IV-3. Potencies of PP_i and Selected PP_i Analogs as Inhibitors of DNA Polymerases Alpha and Delta Assayed on Poly(dA):oligo(dT)[1]

Inhibitor	IC_{50} (mM)[2] pol α	pol δ
PP_i	1	0.25
F_2MDP	10	4
MDP[3]	<10	<10
PFA	0.007	0.007
PAA	0.007	0.007

[1]Enzyme activities (pol α and HAP-δ) were assayed under the conditions described in Chapter II for activated DNA as the template:primer.

[2]IC_{50} values were determined on the basis of inhibitor concentration *vs.* enzyme activity curves of at least five points.

[3]This compound displayed an unusually steep inhibitor concentration *vs.* enzyme activity curve; see text, Chapter IV, section C.

measured the activity of pol δ as a function of Mg^{2+} concentration. I found, in rough agreement with the observations of Focher et al. (1989) that pol δ displayed a broad Mg^{2+} optimum centered at about 5 mM (Figure IV-7). Next, I measured inhibition of pol δ by PP_i, F_2MDP and MDP as a function of Mg^{2+} concentration, and found that with each compound, the degree of inhibition decreased with increasing Mg^{2+} concentration (Table IV-4). MDP, an inhibitor which displayed no selectivity for pol α or pol δ (Table IV-3), and an IC_{50} value for pol δ close to the Mg^{2+} concentration used in most experiments (10 mM), may inhibit pol δ at least partially as a result of Mg^{2+} chelation. Consistent with the hypothesis that MDP sequesters a significant fraction of the Mg^{2+} in the assay mixes is the observation that 3 mM MDP reversed inhibition of pol δ activity caused by high Mg^{2+} concentration, giving an apparent 81% stimulation of pol δ activity when the enzyme was assayed in the presence of 15 mM Mg^{2+} (Table IV-4; see also Figure IV-7). PP_i and F_2MDP inhibited pol δ with IC_{50} values of 0.25 and 4 mM, respectively; concentrations which are below the concentration of Mg^{2+} in pol δ assay mixes. Since the data of Figure IV-7 indicates that 10 mM Mg^{2+} is two to three times the optimum of that ion for pol δ, it seems unlikely that that either PP_i or F_2MDP chelates enough Mg^{2+} at inhibitor concentrations near their IC_{50} values to

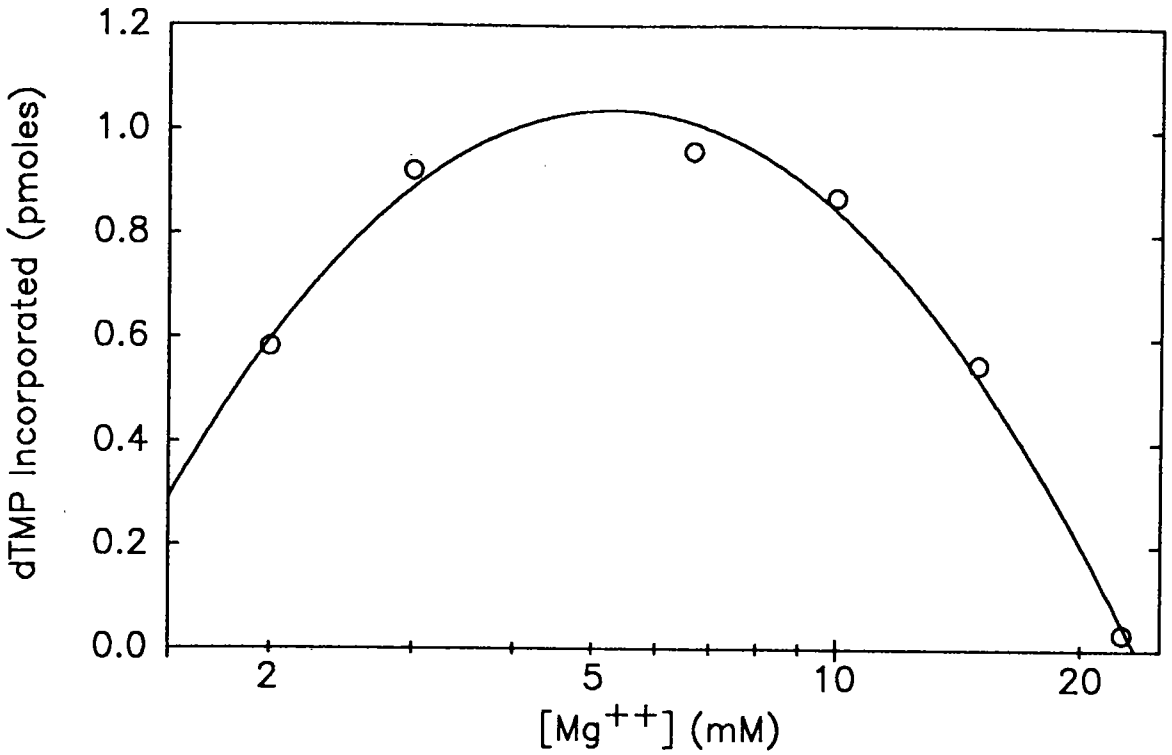


Figure IV-2. Activity of DNA polymerase delta as a function of Mg^{2+} concentration. HAP-delta was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer, except that the Mg^{2+} concentration was varied as indicated.

Table IV-4. Inhibition of DNA Polymerase Delta by PP_i, F_2MDP and MDP as a function of Mg^{2+} Concentration[1]

	% Inhibition[2]			
$[Mg^{2+}]$, mM:	2.5	5	10	15
Inhibitor (conc.)				
PP_i (300 µM)	63	56	39	30
F_2MDP (3 mM)	63	41	46	36
MDP (3 mM)	80	2	(33)	(81)

[1]Pol δ (HAP-δ) was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer.

[2]Numbers represent percent inhibition of enzyme activity by inhibitors present at indicated concentrations relative to control activity, which was measured at the same Mg^{2+} concentration. Numbers in parentheses indicate percent stimulation relative to controls.

inhibit pol δ activity. Indeed, the data of Figure IV-7 indicate that sequestration of up to 70% of the free Mg^{2+} in the assay mixes would have no effect on the activity of pol δ. Mg^{2+} chelation might account for the increased potency of PP_i and F_2MDP at Mg^{2+} concentrations below 10 mM (Table IV-4).

Patterns of inhibition of DNA polymerase delta by PP_i, F_2MDP and PFA. To gain insight into the mechanism of inhibition of pol δ by the weakly selective inhibitor F_2MDP, I examined the nature of its inhibitory effect on that enzyme as a function of dTTP and poly(dA):oligo(dT) concentrations. For comparison, I performed analogous experiments using PP_i and the more potent but nonselective analog PFA.

Analysis of PP_i action. The data summarized by the Lineweaver-Burk plots of Figure IV-8 show that inhibition of pol δ by PP_i is competitive with both dTTP and poly(dA):oligo(dT). From the data of Figure IV-8A, I calculated a K_i value for PP_i of 540 ± 250 μM (the method of calculation for this and analogous experiments described below is detailed in Chapter II). The patterns of inhibition are consistent with a mechanism in which PP_i binds the enzyme at a site which overlaps the site(s) of both dTTP and poly(dA):oligo(dT) binding; occupancy of this site interferes with the binding of both substrates, and

Figure IV-8.

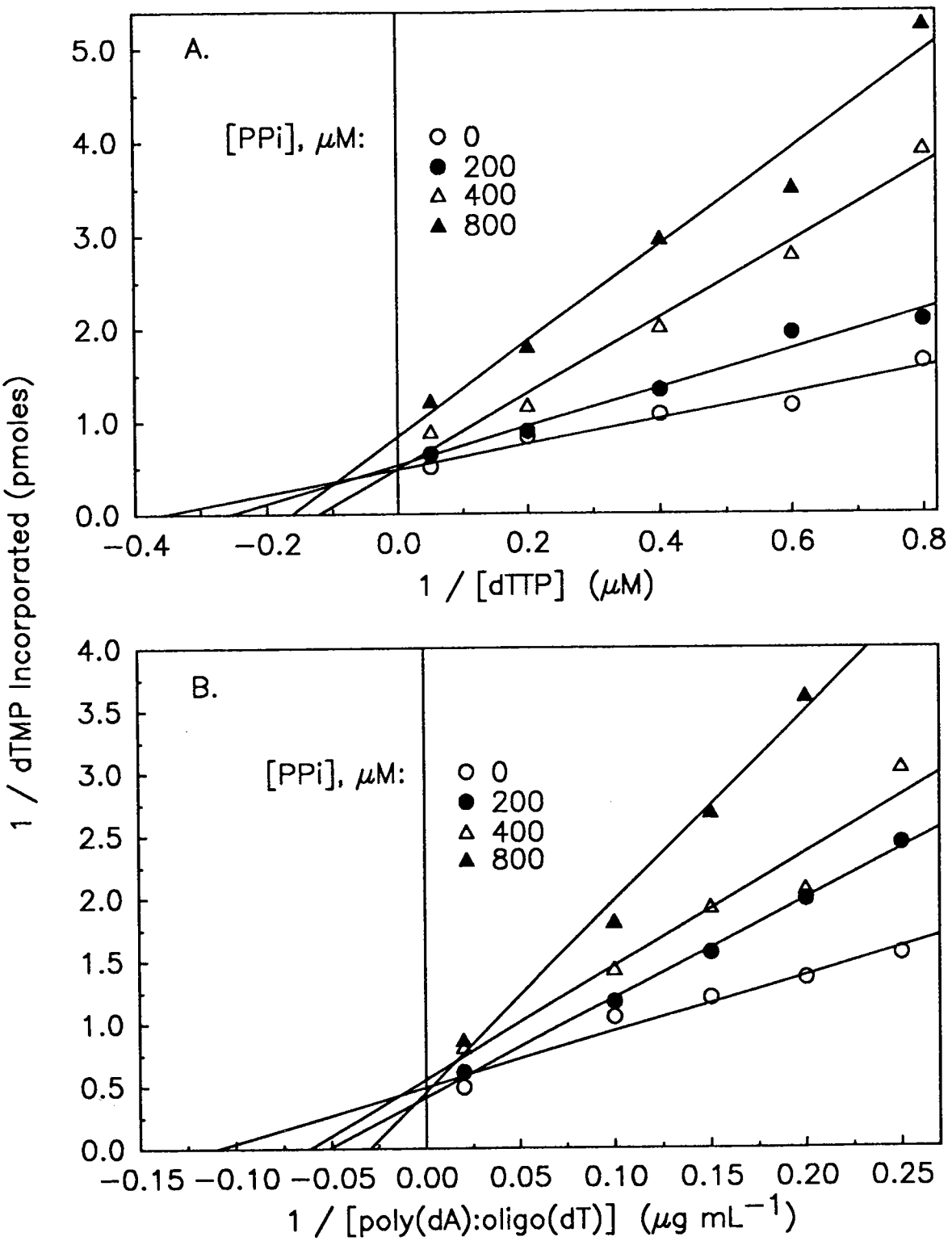

A.
[PPi], μM:
0
200
400
800
1 / dTMP Incorporated (pmoles)
1 / [dTTP] (μM)
B.
[PPi], μM:
0
200
400
800
1 / [poly(dA):oligo(dT)] (μg mL⁻¹)

Figure IV-8. Lineweaver-Burk analysis of inhibition of DNA polymerase delta by PP_i as a function of dTTP and poly(dA):oligo(dT) concentrations. HAP-δ was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer, except that dTTP and poly(dA):oligo(dT) concentrations were varied as indicated. (A) dTTP as the variable substrate. (B) Poly(dA):oligo(dT) as the variable substrate.

therefore is subject to competition by either. Alternatively, the PP_i binding site may be distinct from those of one or both substrates, and the binding of PP_i and the substrates may be mutually exclusive by an allosteric mechanism.

Analysis of F_2MDP action. Lineweaver-Burk plots analogous to those described above, but using F_2MDP as the inhibitor, show that this compound , like PP_i, was competitive with dTTP (Figure IV-9A). However, unlike PP_i, F_2MDP was noncompetitive with poly(dA):oligo(dT) (Figure IV-9B). The observed patterns of inhibition of pol δ with respect to the two substrates suggest that F_2MDP binds a site on pol δ which overlaps the dNTP site but not the template:primer site. As a result, F_2MDP and dTTP compete for binding, but the inhibitor and poly(dA):oligo(dT) bind without effect on one another. F_2MDP might bind the allosteric site proposed above, but if so, it fails to induce the PP_i-specific conformational change that results in competitive inhibition with respect to template:primer. Alternatively, F_2MDP may not bind the same site as PP_i, but a unique one, independent of the template:primer binding site but overlapping the dNTP site. From the data of Figure IV-9A I calculated a K_i value for F_2MDP of 1.0 ± 0.2 mM.

Analysis of PFA action. Lineweaver-Burk plots analogous to those described above, but using PFA as the inhibitor, show

Figure IV-9.

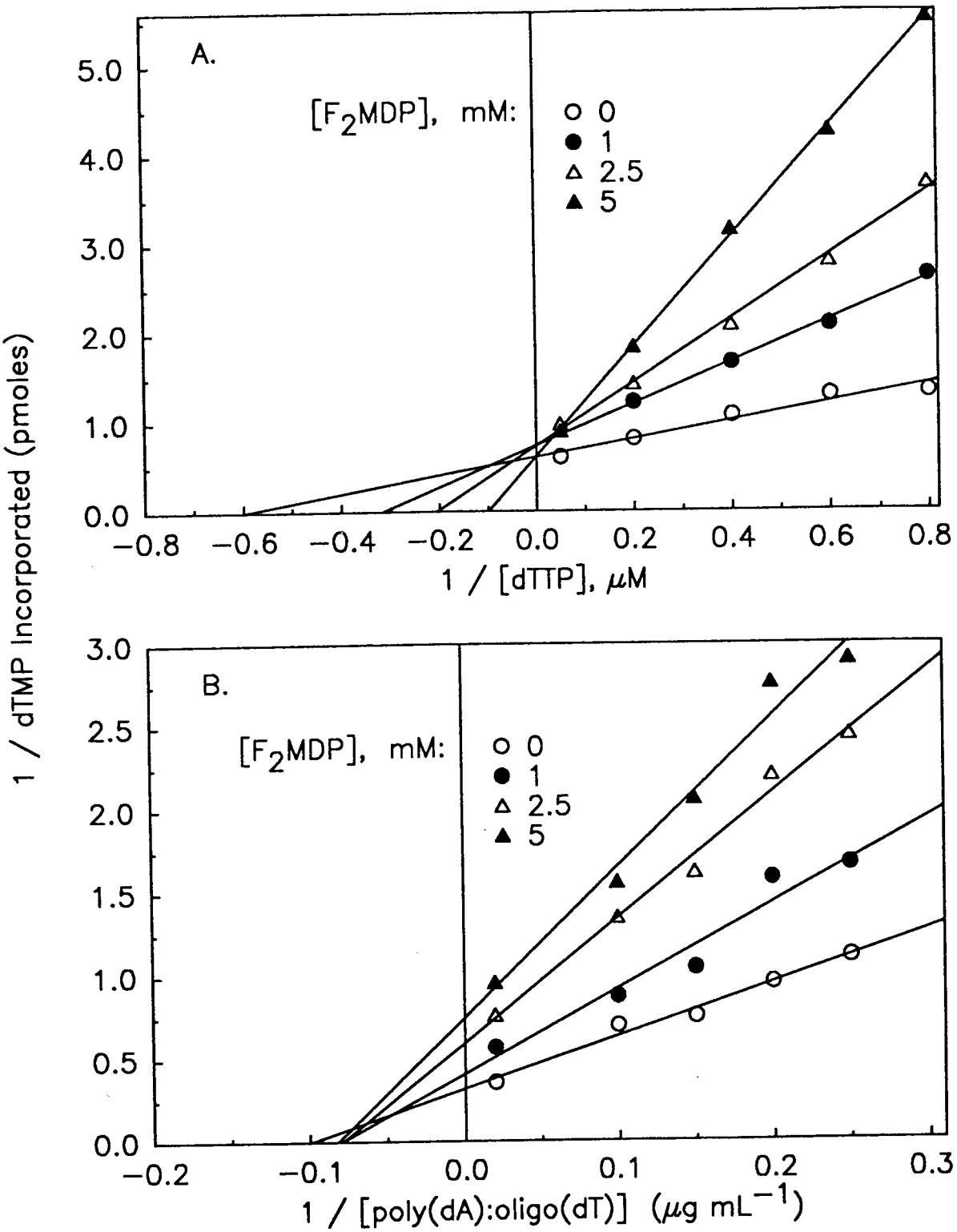

A.
[F2MDP], mM:
0
1
2.5
5
5.0
4.0
3.0
2.0
1.0
0.0
−0.8
−0.6
−0.4
−0.2
0.0
0.2
0.4
0.6
0.8
1 / [dTTP], μM
1 / dTMP Incorporated (pmoles)
B.
[F2MDP], mM:
0
1
2.5
5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
−0.2
−0.1
0.0
0.1
0.2
0.3
1 / [poly(dA):oligo(dT)] (μg mL−1)

Figure IV-9. Lineweaver-Burk analysis of inhibition of DNA polymerase delta by F_2MDP as a function of dTTP and poly(dA):oligo(dT) concentrations. HAP-δ was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer, except that dTTP and poly(dA):oligo(dT) concentrations were varied as indicated. (A) dTTP as the variable substrate. (B) Poly(dA):oligo(dT) as the variable substrate.

that this compound inhibits pol δ uncompetitively with both dTTP and poly(dA):oligo(dT) (Figure IV-10, panels A and B). The results are in accord with previous observations (for a review, see Öberg, 1989) which indicate that PFA inhibits viral DNA polymerases and pol α derived from HeLa cells uncompetitively or noncompetitively with respect to both dNTP and template:primer concentrations. My observations suggest that the binding site of PFA on pol δ, unlike those (or that) of PP_i and F_2MDP, is optimized or perhaps created by the binding of the substrates. I calculated a K_i value from the data of Figure IV-10A of 9.5 ± 3 μM.

Promise of PP_i and F_2MDP as probes of the function of DNA polymerase delta. Neither PP_i nor F_2MDP was suitable as an *in vivo* probe of the function(s) of pol δ. PP_i did not inhibit pol δ with sufficient selectivity compared to pol α, and it had the inherent disadvantage of susceptibility to hydrolysis by phosphatases. F_2MDP, although a more selective inhibitor of pol δ than PP_i, was still not selective enough to clearly discriminate the functions of pol δ and pol α *in vivo*. In addition, F_2MDP was such a weak inhibitor of pol δ that at concentrations expected to significantly inhibit the latter enzyme, it may act indiscriminately on important cell targets other than pol δ. The inhibitory properties of PP_i and F_2MDP did indicate, however, that the latter compounds represented a promising

Figure IV-10.

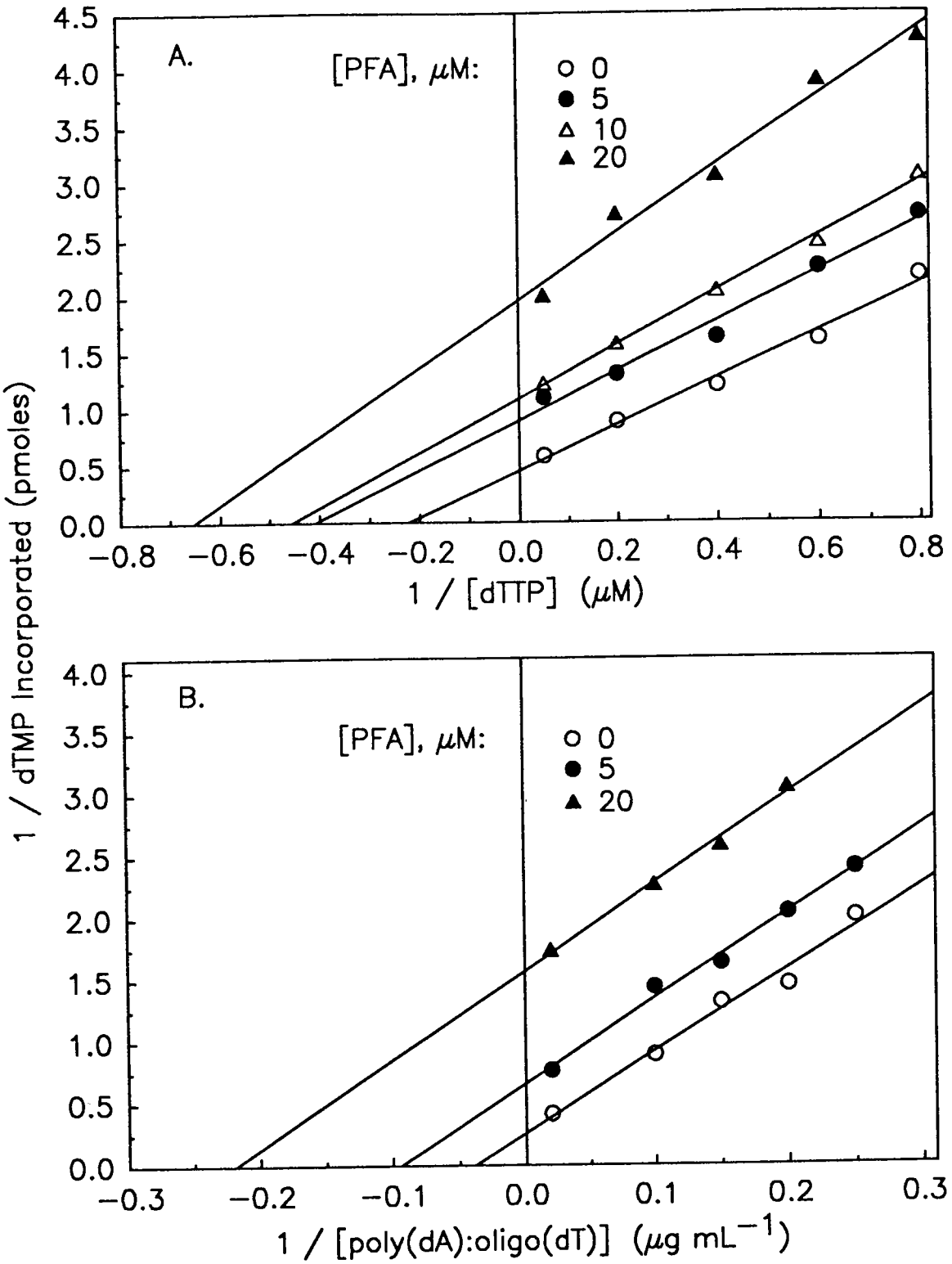

A.
[PFA], μM:
0
5
10
20
1 / dTMP Incorporated (pmoles)
1 / [dTTP] (μM)
B.
[PFA], μM:
0
5
20
1 / [poly(dA):oligo(dT)] (μg mL⁻¹)

Figure IV-10. Lineweaver-Burk analysis of inhibition of DNA polymerase delta by PFA as a function of dTTP and poly(dA):oligo(dT) concentrations. HAP-δ was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer, except that dTTP and poly(dA):oligo(dT) concentrations were varied as indicated. (A) dTTP as the variable substrate. (B) Poly(dA):oligo(dT) as the variable substrate.

structural basis for the development of a more ideal inhibitor of pol δ. In the work described below, I continued the search for a selective inhibitor of pol δ, screening a new series of PP_i analogs for inhibition of pol α and pol δ.

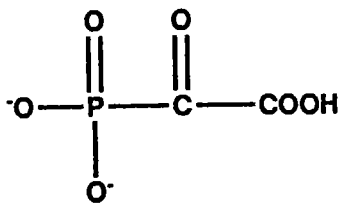
E. Carbonyldiphosphonate: A Selective Inhibitor of DNA Polymerase Delta

Enzymes and assay conditions. The experiments with pol δ described in the previous sections were performed with HAP-δ (see section IV-B). During the course of the experiments described below, a second, highly purified preparation of pol δ, called FPLC-δ, became available (see section IV-B). In the experiments described below, the former preparation was used for inhibitor screening, and the latter for the subsequent analysis of the mechanism of inhibition of pol δ by COMDP. In the cases where I tested the potencies of inhibitors with both HAP-δ and FPLC-δ, I did not find a significant difference between the two; therefore, comparison of results obtained with the two enzymes seems warranted. Whenever possible, poly(dA):oligo(dT) was used as the template:primer in assays of pol α and pol δ activities.

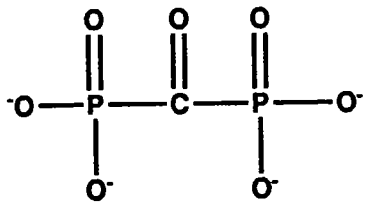
Screening of a larger set of PP_i analogs for inhibition of DNA polymerases alpha and delta. The observation that

PP_i and F_2MDP inhibited pol δ weakly, but selectively relative to pol α, suggested that other PP_i analogs might inhibit pol δ with greater potency and greater selectivity with respect to pol α. Numerous PP_i analogs have been synthesized because of their potential as antiviral agents (Öberg, 1989). I obtained a series of PP_i analogs, specifically MDP and PAA analogs, from Dr. Charles McKenna (University of Southern California); the structures and acronyms of the latter compounds are given in Figure IV-11. I tested the PP_i analogs shown in Figure IV-11 as inhibitors of pol α and pol δ, and the results are summarized in Table IV-5. The most potent inhibitors were the monohalogenated PAA analogs FPAA, BrPAA and ClPAA, none of which displayed significant inhibitory selectivity for either pol α or pol δ. Two MDP analogs, COMDP and FBrMDP, displayed significant selectivity for pol δ, the former with a potency as an inhibitor of pol δ (but not pol α) close to the potencies of the monohalogenated PAA analogs on either enzyme.

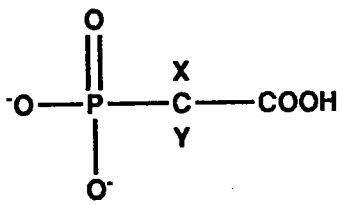
Potencies of selected PP_i analogs as inhibitors of DNA polymerases alpha and delta assayed using activated DNA. In order to test the template:primer dependencies of the inhibitory potencies of ceratin PP_i analogs, I measured inhibition of pol α and pol δ using activated DNA rather than poly(dA):oligo(dT) as the template:primer. The compounds tested were the five most potent inhibitors of pol

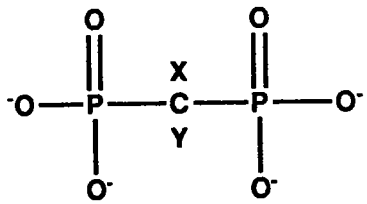


COPAA



COMDP





X	Y	
F	H	FPAA
Br	H	BrPAA
Cl	H	ClPAA
F	F	F_2PAA
F	Br	FBrPAA
F	Cl	FClPAA
Cl	Cl	ClBrPAA
Cl	Br	Br_2PAA
Br	Br	FPPA
F	Me	ClPPA
Cl	Me	BrPPA
Br	Me	

X	Y	
F	Br	FBrMDP
F	H	FMDP
F	F	F_2MDP
F	Cl	FClMDP
Cl	Br	ClBrMDP
Cl	H	ClMDP
Cl	Cl	Cl_2MDP
Br	H	BrMDP
Br	Br	Br_2MDP

Figure IV-11. Structures and acronyms of analogs of PAA and MDP.

Table IV-5. Potencies of an Expanded Set of PP_i Analogs as Inhibitors of DNA Polymerases Alpha and Delta Assayed on Poly(dA):oligo(dT)[1]

Inhibitor	IC_{50} (μM)[2]	
	pol α	pol δ
FPAA	1.2	0.9
BrPAA	8	3
ClPAA	5	4
COMDP	60	6
COPAA	22	22
F_2PAA	300	200
FBrMDP	>2000	400
FBrPAA	1000	900
FClPAA	2000	900
FMDP	1000	1000
FClMDP	1000	2000
Cl_2PAA	2000	2000
ClBrPAA	2000	2000
Br_2PAA	2000	2000
ClBrMDP	I	2000
FPPA	2000	>2000
ClPPA	>2000	>2000
BrPPA	I	>2000
ClMDP	I	>2000
Cl_2MDP	I	>2000
BrMDP	I	>2000
Br_2MDP	I	>2000
Dicyclohexylamine[3]	I	I
Pyridine[3]	I	I

[1]Enzyme activities (pol α and HAP-δ) were assayed under the conditions described in Chapter II for poly(dA):oligo(dT) as the template:primer.

[2]IC_{50} values were determined on the basis of inhibitor concentration *vs.* enzyme activity curves of up to 8 points, depending on the potency of each compound. I = inactive at 2 mM, the highest concentration tested.

[3]Dicyclohexylamine and pyridine were the bases forming the counterions of several inhibitors, and had no inhibitory effect at 5 mM on either enzyme.

α and pol δ assayed using poly(dA):oligo(dT) as the template:primer (Table IV-5). The results are summarized in Table IV-6. Activated DNA-directed DNA synthesis catalyzed by either enzyme was less sensitive to inhibition by each of the compounds than was poly(dA):oligo(dT)-directed synthesis (compare the data of Tables IV-5 and IV-6). Further, with activated DNA as the template:primer, all five compounds inhibited pol δ with greater potency than pol α. Because of its unique combination of potency and selectivity for pol δ compared to pol α, as well as the template:primer-independence of that selectivity, I chose to study the properties of COMDP as an inhibitor of poly(dA):oligo(dT)-directed DNA synthesis in greater detail.

Patterns of inhibition of DNA polymerases alpha and delta by COMDP. Lineweaver-Burk analyses of inhibition of pol α and pol δ (FPLC-δ; see above) assayed on poly(dA):oligo(dT) by COMDP, as a function of dTTP concentration, are presented in Figure IV-12, panels A and B, respectively. The results indicated that, with respect to dTTP, COMDP inhibited pol α uncompetitively and pol δ competitively. From the data of Figure IV-12, I calculated K_i values for COMDP of 40 ± 5 and 1.8 ± 0.3 μM for pol α and pol δ, respectively. Experiments analogous to those of Figure IV-12 were performed, in which the concentration of poly(dA):oligo(dT) rather than dTTP was varied; Lineweaver-

Table IV-6. Potencies of Selected PP_i Analogs as Inhibitors of DNA Polymerases Alpha and Delta Assayed on Activated DNA[1]

Inhibitor	IC_{50} (μM)[2]	
	pol α	pol δ
FPAA	20	2
BrPAA	160	20
ClPAA	120	70
COMDP	300	40
COPAA	170	50

[1]Enzyme activities (pol α and HAP-δ) were assayed under the conditions described in Chapter II for activated DNA as the template:primer.

[2]IC_{50} values were determined on the basis of inhibitor concentration vs. enzyme activity curves of 5 to 7 points.

Burk analyses of the resulting data are presented in Figure IV-13. The patterns of inhibition indicated that COMDP inhibited both pol α and pol δ in a mixed noncompetitive / uncompetitive mode with respect to poly(dA):oligo(dT). Clearly, inhibition of neither enzyme was competitive with poly(dA):oligo(dT).

The observation that COMDP bound pol δ competitively with dTTP suggests that it bound part of the dNTP binding site (this suggestion was further supported by results described below, which indicated that COMDP is competitive only with the dNTP specified by the template in the polymerase reaction). The similarity between the structure of COMDP and the triphosphate group of a dNTP suggested further that its binding site either overlaps or is identical with the region of the dNTP site that binds the triphosphate group of dNTPs. The observation that COMDP binds pol α uncompetitively with dTTP suggests that, because the dNTP site is occupied by dNTP when COMDP binds (the definition of uncompetitive binding), the COMDP binding site on pol α must necessarily be different from that of dNTP.

<u>COMDP competes only with the dNTP specified by the template.</u> I performed experiments designed to determine whether competitive binding of pol δ between COMDP and dTTP was specific to dTTP, was not specific (that is, whether it would be competitive with any dNTP), or whether COMDP

Figure IV-12.

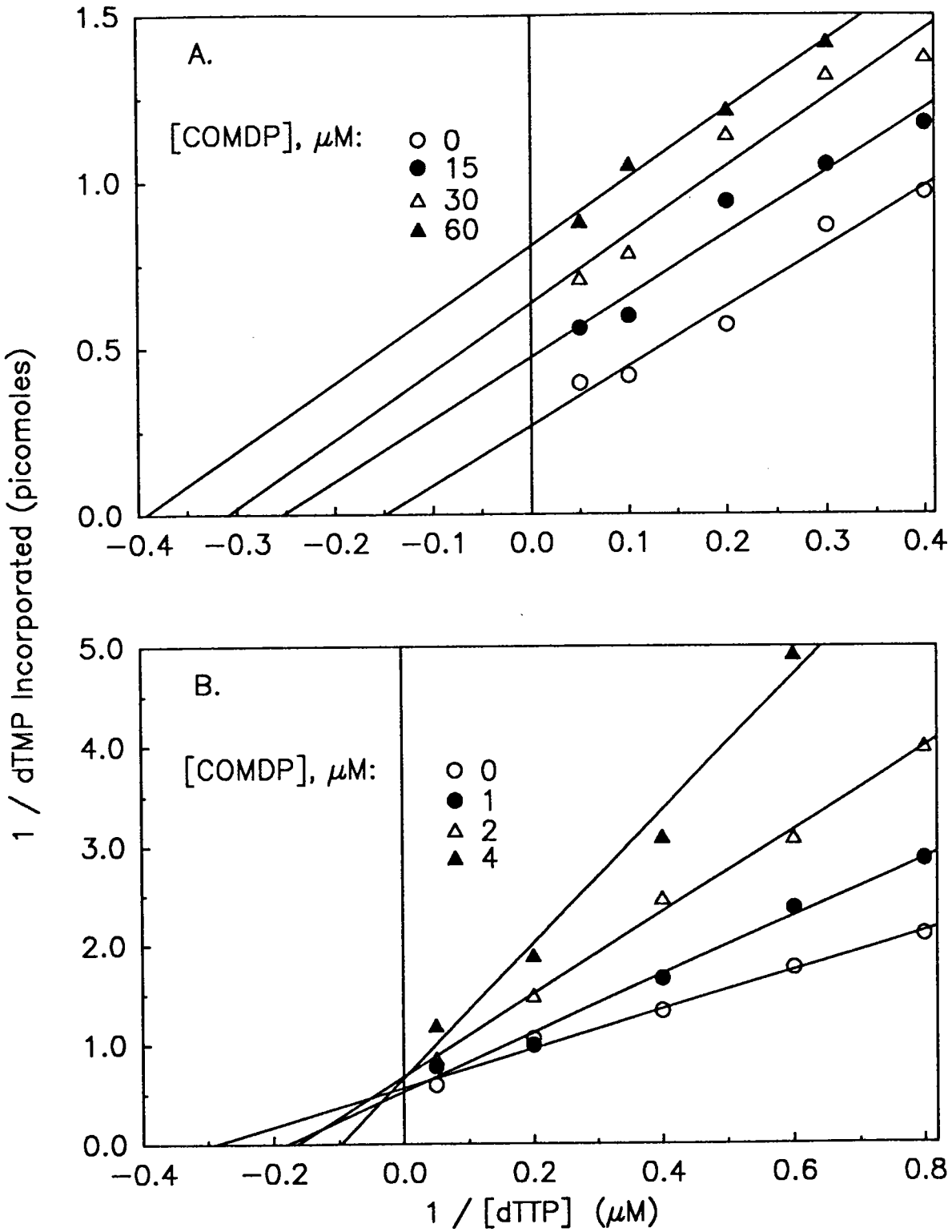

A.
[COMDP], μM:
0
15
30
60
1.5
1.0
0.5
0.0
−0.4
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3
0.4
B.
[COMDP], μM:
0
1
2
4
5.0
4.0
3.0
2.0
1.0
0.0
−0.4
−0.2
0.0
0.2
0.4
0.6
0.8
1 / dTMP Incorporated (picomoles)
1 / [dTTP] (μM)

Figure IV-12. Lineweaver-Burk analysis of inhibition of DNA polymerases alpha and delta by COMDP as a function of dTTP concentration. FPLC-δ and pol δ were assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer, except that the dTTP concentration was varied as indicated. (A) Inhibition of pol α. (B) Inhibition of pol δ.

Figure IV-13.

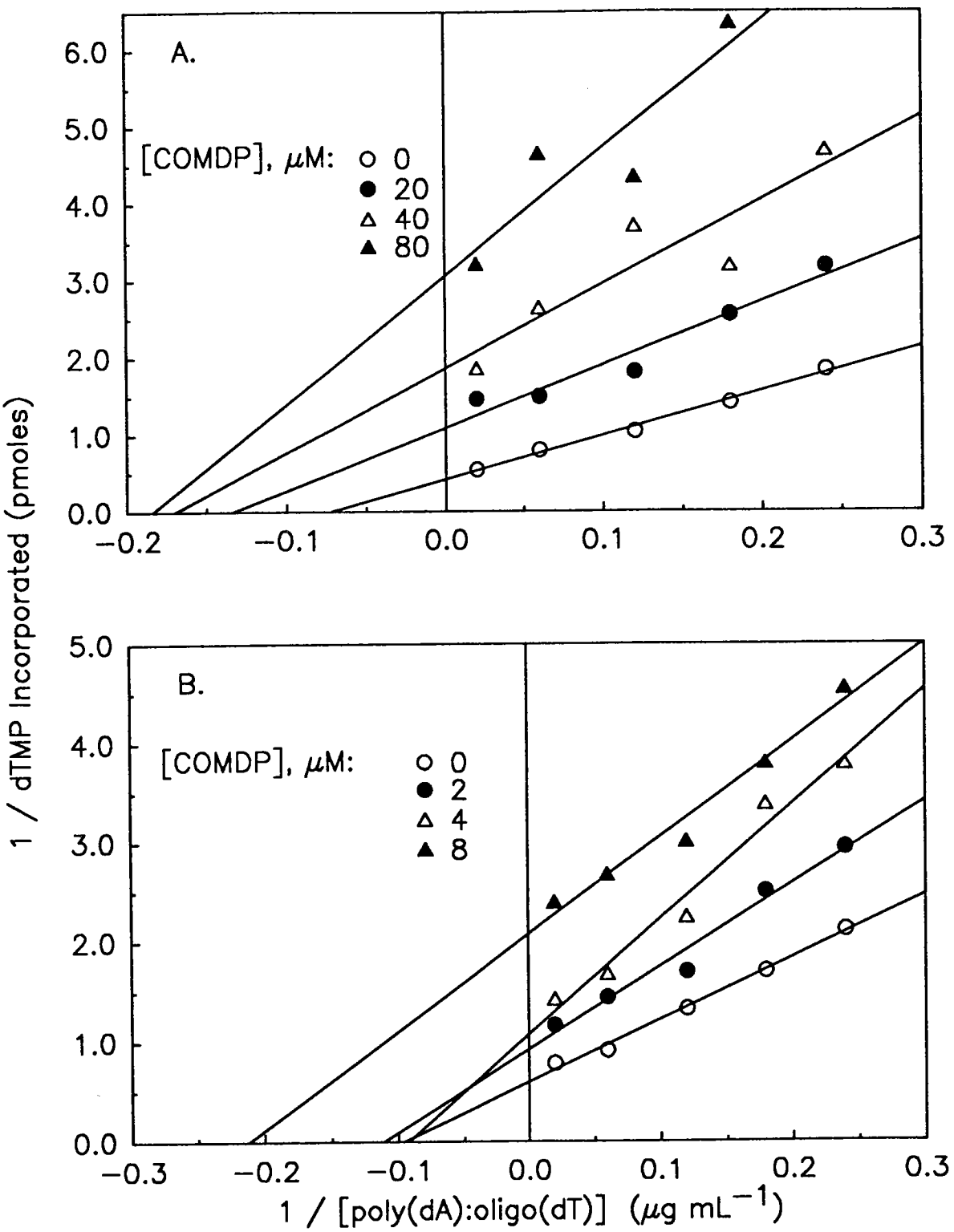

A.
[COMDP], μM:
0
20
40
80
B.
[COMDP], μM:
0
2
4
8
1 / dTMP Incorporated (pmoles)
1 / [poly(dA):oligo(dT)] (μg mL⁻¹)

Figure IV-13. Lineweaver-Burk analysis of inhibition of DNA polymerases alpha and delta by COMDP as a function of poly(dA):oligo(dT) concentration. FPLC-δ and pol δ were assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer, except that the poly(dA):oligo(dT) concentration was varied as indicated. (A) Inhibition of pol α. (B) Inhibition of pol δ.

competes only with the dNTP specified by the template. With poly(dA):oligo(dT) as the template:primer, only dTTP competed with COMDP; addition of a high concentration (800 μM) of dATP, dCTP or dGTP to the assay mix did not have a significant effect on the potency of the inhibitor (Table IV-7). Poly(dC):oligo(dG), when substituted for poly(dA):oligo(dT) as the template:primer, directed a level of DNA synthesis by pol δ about 20% of that directed by poly(dA):oligo(dT). With poly(dC):oligo(dG) as the template:primer, the pattern of inhibition with respect to varying dGTP concentration determined by Lineweaver-Burk analysis (Figure IV-14) indicated that COMDP was competitive with dGTP. From the data of Figure IV-14, I calculated a K_i value for COMDP of 45 μM, significantly higher than that observed with poly(dA):oligo(dT) as the template:primer. Addition of 800 μM dATP, dCTP or dTTP to the assay mix in which poly(dC):oligo(dG) was the template:primer had no significant effect on DNA synthesis catalyzed by pol δ (Table IV-8). The latter observations support the hypothesis that COMDP binds a site on pol δ which is occupied by the dNTP about to be incorporated onto the growing primer chain during DNA synthesis.

COMDP is a readily reversible inhibitor of pol δ. I observed that COMDP gradually lost inhibitory activity when stored at -20 °C in Tris buffer (a primary amine), whereas

Table IV-7. Effect of dATP, dGTP and dCTP on Inhibition by COMDP of DNA Polymerase Delta Assayed on Poly(dA):oligo(dT)[1]

% Inhibition[2]			
Control	dATP	dGTP	dCTP
38	45	35	26

[1]Pol δ (FPLC-δ) was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer, with 800 μM of a dNTP (or solvent control) added as indicated.

[2]Determined by comparing enzyme activity between samples containing 800 μM of the indicated dNTP (or solvent control), with either 4 μM COMDP or solvent control.

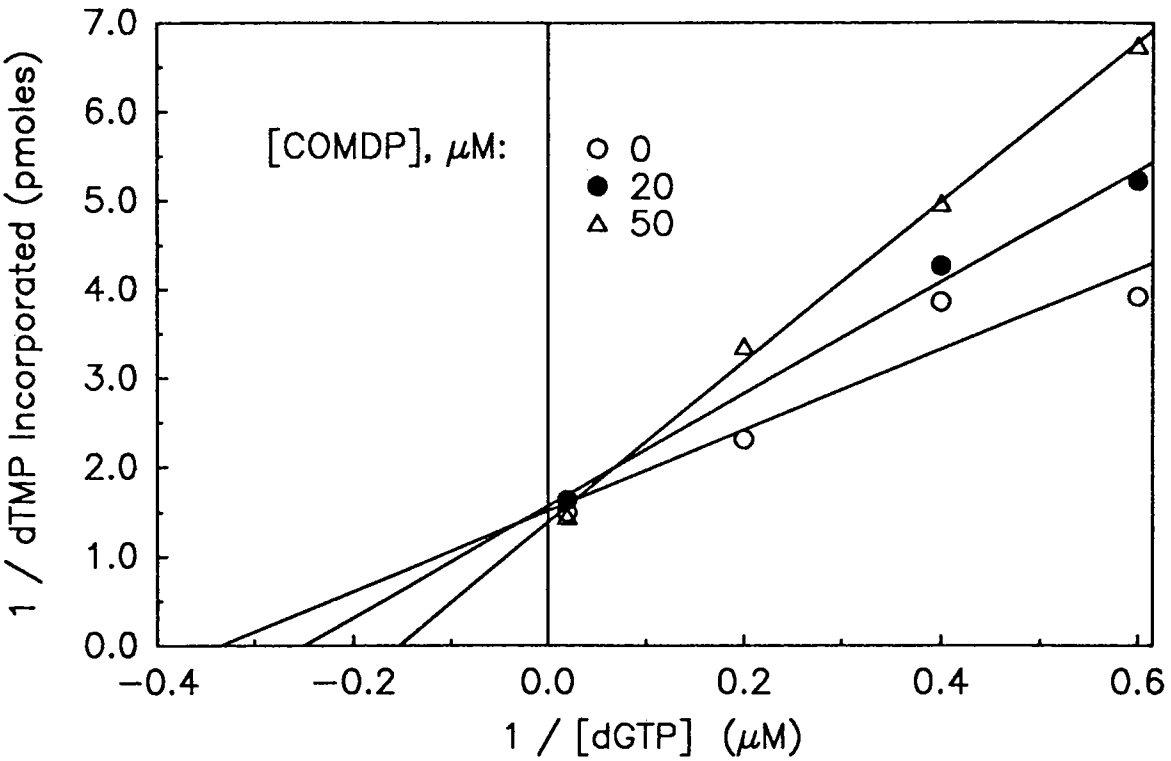


Figure IV-14. Lineweaver-Burk analysis of inhibition by COMDP of DNA polymerase delta assayed on poly(dC):oligo(dG) as a function of dGTP concentration. FPLC-delta was assayed as described in Chapter II, for poly(dA):oligo(dT) as the template:primer, except that poly(dC):oligo(dG) was substituted for poly(dA):oligo(dT) and [^{3}H]dGTP was substituted for [^{3}H]dTTP.

it was stable in HEPES buffer (a tertiary amine). The latter observations and consideration of the structure of COMDP suggested that its carbonyl group might bind pol δ by forming a Schiff base intermediate with a primary amine on the surface of the enzyme. Such a reaction scheme is depicted in Figure IV-15. A Schiff base intermediate may be stable, and thus may not readily dissociate. I tested the latter possibility by performing the following experiment: FPLC-δ was incubated for up to 45 min at 37 °C with 8 μM COMDP (a concentration approximately four times its K_i for pol δ; see above) in a polymerase reaction mix [(including poly(dA):oligo(dT)] lacking only dTTP. The inhibitor was then separated from the enzyme by Sephadex G-25 chromatography, and its activity and that of mock-treated enzyme was measured. The results, which are summarized in Table IV-9, indicated that preincubation of pol δ with COMDP had no permanent effect on the enzyme activity. This result is inconsistent with the notion that COMDP inhibited pol δ by forming a stable complex with it.

Treatment of a COMDP-pol δ complex with a reducing agent results in irreversibly inactivated enzyme. The results described above did not eliminate the possibility that COMDP formed a reversible Schiff base intermediate with a primary amine on the surface of pol δ. Such an intermediate might be reducible, resulting in a covalent enzyme-inhibitor

Table IV-8. Effect of dATP, dTTP and dCTP on Inhibition by COMDP of DNA Polymerase Delta Assayed on Poly(dA):oligo(dT)[1]

% Inhibition[2]			
Control	dATP	dTTP	dCTP
39	39	42	32

[1]Pol δ (FPLC-δ) was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer, except that poly(dC):oligo(dG) was substituted for poly(dA):oligo(dT), and [^{3}H]dGTP for [^{3}H]dTTP, with 800 μM of a dNTP (or solvent control) added as indicated.

[2]Determined by comparing enzyme activity between samples containing 800 μM of the indicated dNTP (or solvent control), with either 4 μM COMDP or solvent control.

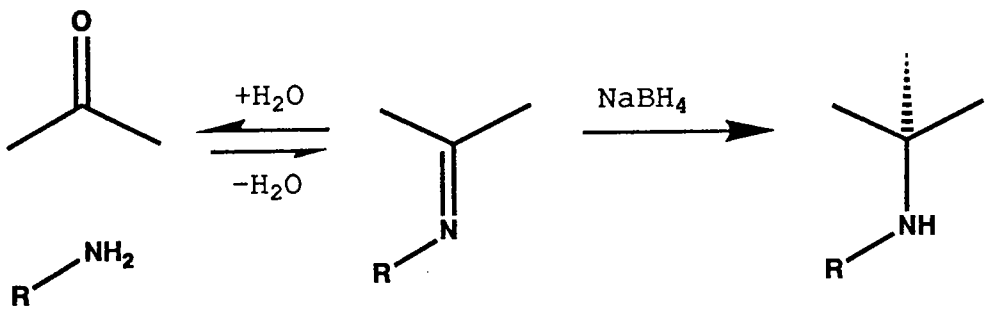


Figure IV-15. Schiff base formation between a carbonyl group and a primary amine, and its subsequent reduction by $NaBH_4$.

Table IV-9. Reversibility of Inhibition of DNA Polymerase Delta by COMDP[1]

Preincubation time (min)	[^{3}H]dTMP Incorporation (pmoles)	
	Control	8 μM COMDP
15	12.6	13.2
30	13.4	13.7
45	10.2	9.8

[1]Pol δ (FPLC-δ) was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer. The enzyme was preincubated for the indicated times with COMDP (or solvent). COMDP was then separated from the enzyme by Sephadex chromatography, [^{3}H]dTTP was added, and enzyme activity was measured.

complex (see Figure IV-15), perhaps thus in the vicinity of the active site. I tested the latter possibility in an experiment modeled after those of Basu and Modak (1987), in which sodium borohydride ($NaBH_4$) was used to effect irreversible inactivation of E. coli DNA polymerase I with pyridoxal phosphate. FPLC-δ was incubated at 37 °C with 8 μM COMDP in a reaction mix including poly(dA):oligo(dT) and lacking only dTTP. After 20 min, $NaBH_4$ was added to a final concentration of 5 mM. Incubation was continued for 10 min, and the enzyme was separated from the inhibitor and reducing agent by Sephadex G-25 chromatography. Enzyme activity was then measured as described in Chapter II. Analogous samples in which $NaBH_4$, COMDP or both were omitted were included as controls. The results, which are summarized in Table IV-10, indicated that $NaBH_4$ promoted COMDP-dependent inactivation of pol δ. In subsequent experiments, I found that (i) when poly(dA):oligo(dT) was omitted from the preincubation mix, COMDP was still capable of irreversible inhibition of pol δ on treatment with $NaBH_4$ (Table IV-11), and (ii) the degree of COMDP-dependent inactivation after $NaBH_4$ treatment was not changed by the presence of 4 or 8 μM dTTP (Table IV-12). If irreversible inhibition were a result of covalent binding to the dNTP site of pol δ, one would expect an active template:primer to enhance it and dTTP to decrease or prevent it. Therefore, observations (i)

Table IV-10. Activity of DNA Polymerase Delta by COMDP After Treatment with COMDP and $NaBH_4$[1]

	[^{3}H]dTMP Incorporation (picomoles)	
$NaBH_4$ (mM)	Control	8 μM COMDP
0	8.7	9.9
5	9.0	4.6

[1]Pol δ (FPLC-δ) was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer. The enzyme was preincubated with COMDP (or solvent) for 20 min, then $NaBH_4$ (or solvent) was added to a final concentration of 5 mM, and incubation continued for 10 min. $NaBH_4$ and COMDP were then separated from the enzyme by Sephadex chromatography, [^{3}H]dTTP was added, and enzyme activity was measured.

and (ii) suggest that irreversible inactivation of pol δ by COMDP is a result of covalent binding of the compound to a site on the enzyme distinct from its active site.

Effect of COMDP on the 3' → 5' Exonuclease Activity of Pol δ. I assayed the 3' → 5' exonuclease activity of FPLC-δ by measuring its ability to catalyze excision of the primer termini of the synthetic template:primers poly(dA):oligo(dT)$_{12-18}$·([^{32}P]dC)$_{0.33}$ and poly(dA):oligo(dT)$_{12-18}$·([^{3}H]dT)$_{0.3}$. I prepared the radiolabeled homopolymers as described in Chapter II. Exonuclease activity measured on the mismatched primer terminus was 15 to 20% that of polymerase activity measured under standard conditions; with the properly base paired 3'-OH terminus, it was only 2.5 to 4% that of polymerase activity. The ratio of the exonuclease activities that I observed with matched and mismatched primer termini is consistent with suggestions that the 3' → 5' exonuclease is a "proofreading" exonuclease, which is an important contributor to the DNA replication fidelity of pol δ (Kunkel et al., 1986). I found that COMDP inhibited the exonuclease activity only weakly, displaying IC_{50} values determined on the basis of concentration vs. enzyme activity curves (Figure IV-16) which were greater than 1 mM with either template:primer.

Table IV-11. Activity of DNA Polymerase Delta After Treatment with COMDP and $NaBH_4$ in the Presence or Absence of Poly(dA):oligo(dT)[1]

[poly(dA):oligo(dT)] ($\mu G\ mL^{-1}$)[2]	[^{3}H]dTMP Incorporation (picomoles)	
	Control	8 μM COMDP
0	1.4	0.8
20	6.6	3.5

[1]Pol δ (FPLC-δ) was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer. The enzyme was preincubated with COMDP (or solvent) for 20 min in the presence of the indicated concentration of poly(dA):oligo(dT), then $NaBH_4$ (or solvent) was added to a final concentration of 5 mM, and incubation continued for 10 min. $NaBH_4$ and COMDP were then separated from the enzyme by Sephadex chromatography, [^{3}H]dTTP and poly(dA):oligo(dT) were added, and enzyme activity was measured.

[2]Indicates concentration in the preincubation mix.

Table IV-12. Activity of DNA Polymerase Delta After Treatment with COMDP and $NaBH_4$ in the Presence or Absence of dTTP[1]

	[^{3}H]dTMP Incorporation (picomoles)	
[dTTP] (μM)[2]	Control	8 μM COMDP
0	7.9	5.2
4	6.9	4.6
8	6.4	3.6

[1]Pol δ (FPLC-δ) was assayed under the conditions described in Chapter II, for poly(dA):oligo(dT) as the template:primer, with the template:primer present. The enzyme was preincubated with COMDP (or solvent) for 20 min in the presence of the indicated concentration of (unlabeled) dTTP, then $NaBH_4$ (or solvent) was added to a final concentration of 5 mM, and incubation continued for 10 min. $NaBH_4$, COMDP and dTTP were then separated from the enzyme by Sephadex chromatography, [^{3}H]dTTP was added, and enzyme activity was measured.

[2]Indicates concentration of unlabelled dTTP in the preincubation mix.

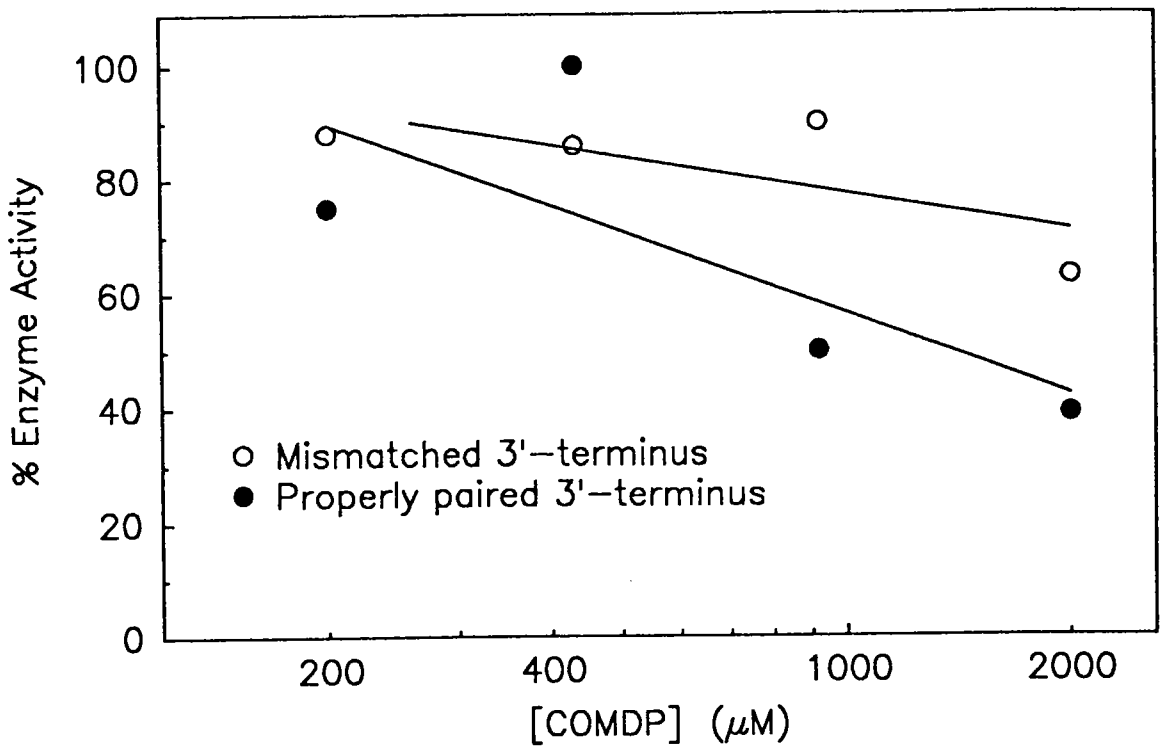


Figure IV-16. Concentration vs. enzyme activity curves for inhibition of the 3′ to 5′ exonuclease activity of pol delta (FPLC-delta) by COMDP. The exonuclease activity, using either a mismatched or a properly base paired template:primer substrate was assayed as described in Chapter II.

CHAPTER V

INHIBITION OF PERMEABLE CELL DNA SYNTHESIS

A. Introduction

COMDP is an ineffective inhibitor of intact cell growth and macromolecule synthesis. COMDP has a critical shortcoming as a discriminator of the function of pol δ *in vivo*; it is charged, and therefore likely penetrates intact cell membranes poorly if at all. Consistent with the notion of poor membrane penetration is the observation of Stenberg (1981) that 500 μM COMDP did not inhibit mammalian cell macromolecule synthesis. I measured CHO cell [^{3}H]TdR incorporation in the presence or absence of COMDP by the technique described in Chapter II. CHO cells were incubated at 37 °C for 3 h in the presence of 20 or 200 μM COMDP, or its solvent as a control. [^{3}H]TdR was added to each, incubation was continued for 30 min, and samples were prepared for scintillation counting as described in Chapter II. I found that there was no significant difference in the amount of [^{3}H]TdR incorporation between control samples and samples of cells treated with either concentration of COMDP. I also measured the effects of COMDP on CHO cell growth in monolayer culture. CHO cells were plated in a 24-well tissue culture plate at a low density (5 to 10% confluence). COMDP was added to some wells to a final concentration of

200 μM, and an equal volume of solvent was added to the others. The cells were incubated at 37 °C, and observed after 24, 48 and 72 h. At each time point, the cells treated with COMDP were indistinguishable from mock-treated cells, both in their appearance and in their density (percent confluence). After 72 h, all of the cultures, regardless of the presence or absence of COMDP, had achieved 30 to 50% confluence. Both sets of observations confirm those of Stenberg (1981) and support the notion that the charge of COMDP limits its use as a probe of the *in vivo* function of pol δ to measurement of its capacity to inhibit macromolecule synthesis in isolated nuclei or in membrane permeabilized cells. I measured the effect of COMDP on CHO cell DNA synthesis in experiments of the latter type; the results are described below.

B. Effect of Inhibitors on Permeable Cell DNA Synthesis

Permeabilization of CHO cells. I used the technique of Dresler et al. (1982), adapted for use with CHO cells[1], for measurement of [α-^{32}P]dATP incorporation into permeabilized CHO cells. I effected CHO cell permeabilization by incubating cells at 0 °C in a slightly hypotonic buffer, as detailed in Chapter II. In the cases of HeLa cells (Seki et

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[1] S. L. Dresler, Washington University School of Medicine, unpublished.

al., 1975) and mouse L cells (Berger & Johnson, 1976), a similar treatment caused cells to swell and release proteins, but their cell membranes maintained their integrity, and after permeabilization the cells incorporated exogenous dNTPs and NTPs into DNA and RNA, respectively. I measured incorporation of [α-^{32}P]dATP by permeabilized cells during an incubation at 37 °C of 8 min, which was within the linear portion of the reaction. I estimated background counts by the apparent incorporation of [α-^{32}P]dATP in samples of permeable cells which were not incubated at 37 °C. Samples incubated at 37 °C for 8 min in the presence of 200 μM aphidicolin, a concentration expected to inhibit replicative DNA synthesis in permeabilized cells almost completely (Dresler, 1984), displayed insignificant [α-^{32}P]dATP incorporation above background. Since aphidicolin selectively inhibits mammalian cell replicative DNA synthesis, and not RNA, (repair) DNA or protein synthesis (Huberman, 1981), the latter result indicated that the incorporation above background measured in the absence of inhibitors was due almost entirely to replicative DNA synthesis.

In an attempt to maximize the degree of inhibition by certain compounds, I changed the concentrations of the unlabeled dNTPs (dCTP, dGTP and dTTP) in the incubation mixtures from 50 to 1 μM (the dATP concentration was 1 μM in

all cases). COMDP inhibition was measured in this manner because, based on its effects on pol δ (Chapter IV, Figures IV-12 and IV-14), the compound most likely inhibits pol δ competitively with all four dNTP substrates, and, thus, minimizing the total dNTP concentration might have maximized the degree of inhibition by COMDP. In the case of FPAA, I did not know whether the compound would inhibit pol α or pol δ competitively with any dNTP; if so, it seemed unlikely that it would be competitive with any particular dNTP. I measured the potency of inhibition by aphidicolin at reduced dNTP concentrations because that compound inhibited DNA synthesis in vivo competitively with all four dNTPs (Ayusawa et al., 1980). I found that the change in dNTP concentration did not have a significant effect on the amount of incorporation of [α-^{32}P]dATP by the permeabilized cells during the assay period.

Potencies of selected compounds as inhibitors of permeable cell DNA synthesis. I tested the potencies of several compounds as inhibitors of DNA synthesis in permeabilized CHO cells, and the results are summarized in Table V-1. Aphidicolin inhibited permeable cell DNA synthesis with an IC_{50} value of 0.2 μM, a potency about an order of magnitude greater than that expected based on the potency of the compound as an inhibitor of pol α and pol δ (Lee et al., 1985). FPAA inhibited permeable cell DNA

Table V-1. Inhibition of [^{32}P]dATP Incorporation into Permeabilized CHO Cell DNA[1]

Inhibitor	IC_{50} (μM)[2]
Aphidicolin[3]	0.2
FPAA[3]	6
COMDP[3,4]	200
DCAA	>200
BuAdATP	>200
BuAA	I

[1][^{32}P]dATP incorporation into the DNA of permeabilized CHO cells was measured as described in Chapter II. Cells were made permeable, added to solutions containing inhibitors (or solvent control) and [α-^{32}P]dATP, and incubated at 37 °C for 8 min. Samples contained 7.5 to 15 x 10^5 permeabilized cells, contained 2000 to 2500 cpm of previously incorporated [^{3}H]TdR, and typically incorporated 1500 to 3000 cpm (17 to 34 picomoles) of [α-^{32}P]dATP during the assay period. Background counts were estimated as the counts in samples not incubated at 37 °C.

[2]IC_{50} values were determined on the basis of inhibitor concentration *vs.* percent activity curves. I = inactive at 200 μM, the highest concentration tested.

[3]Inhibition by aphidicolin, FPAA and COMDP was measured as described in Chapter II, except that the unlabelled dNTPs (dCTP, dGTP and dTTP) were present at 1 rather than 50 μM. The change in dNTP concntrations had little effect on incorporation of [^{32}P]dATP by permeabilized cells.

[4]COMDP displayed 50% inhibition of permeabilized cell DNA synthesis at 200 μM, the highest concentration tested. The statement that 200 μM is the IC_{50} value of COMDP as an inhibitor of permeabilized cell DNA synthesis is based on the assumption that 100% inhibition of permeabilized cell DNA synthesis can be achieved at a higher COMDP concentration.

synthesis with a potency (IC_{50} = 6 μM) about 5-fold less than that which it displayed for inhibition of pol α and pol δ when assayed using poly(dA):oligo(dT) as the template:primer (Table IV-5), and at the (logarithmic) midpoint of the values obtained in analogous experiments employing activated DNA as the template:primer (Table IV-6).

BuAA and DCAA were significantly weaker inhibitors of permeable CHO cell DNA synthesis (being inactive, and displaying an IC_{50} value >200 μM, respectively; Table V-1) than they were of either [^{32}P]phosphate incorporation into intact CHO cell DNA (IC_{50} values of 30 and 12 μM, respectively; Table III-3), or of pol α assayed using activated DNA and (-dATP) truncated conditions (71 and 67% inhibition at 100 μM; Table IV-1). BuAdATP inhibited permeable cell DNA synthesis with an IC_{50} value (>200 μM; Table V-1) greater than its K_i values as an inhibitor of pol α or pol δ (0.005 and 150 μM, respectively; Table IV-2). It is not clear why BuAA, DCAA and BuAdATP were such weak inhibitors of permeabilized cell DNA synthesis. In the case of BuAdATP, the result is surprising, since both BuPdGTP (Dresler & Frattini, 1986; 1988) and BuAdATP[2] inhibited DNA synthesis in permeabilized human fibroblasts with IC_{50} values of 3 μM. It should, however, be noted that in

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2 S. L. Dresler, Washington University School of Medicine, unpublished.

estimating the IC_{50} values of weak inhibitors (those with IC_{50} values $\geq$ 200 μM, the highest concentration tested in all cases), I assumed that 100% of the activity was susceptible to inhibition. It is conceivable that pol α catalyzes only one half of the total DNA synthesis in permeabilized cells, and as a result, a selective pol α inhibitor may only be capable of inhibiting 50% of the total activity. Thus, 50% of the maximum inhibition of permeabilized cell DNA synthesis that a compound is capable of may occur at inhibitor concentrations lower than the values given in Table V-1.

COMDP inhibited permeable cell DNA synthesis with an IC_{50} value of 200 μM (Table V-1). That value is significantly higher than its K_i values as an inhibitor of pol α and pol δ, assayed using poly(dA):oligo(dT) as the primer:template (40 and 1.8 μM, respectively; Chapter IV, Figure IV-12), but between its IC_{50} values as an inhibitor of pol α and pol δ using activated DNA as the template:primer (300 and 40 μM, respectively; Table IV-6). If, as mentioned above, 100% inhibition of permeabilized cell DNA synthesis is not achievable at a higher COMDP concentration, the IC_{50} of the compound may be closer to 40 μM, and may therefore reflect <u>in vivo</u> inhibition of pol δ. Another possibility is that if the inhibitor concentration <u>vs.</u> percent activity curve were extended to higher COMDP

concentrations, the curve might indicate biphasic inhibition, reflecting in vivo inhibition of pol δ at lower concentrations, and pol α at higher concentrations.

BuAdATP and COMDP do not inhibit permeable cell DNA synthesis synergistically. I tested the possibility that BuAdATP and COMDP, selective inhibitors of pol α and pol δ, respectively (Chapter IV), inhibit permeabilized cell DNA synthesis synergistically as a result of their combined action on pol α and pol δ. The hypothesis that simultaneous inhibition of pol α and pol δ results in increased potency of inhibition of DNA synthesis is speculative, but is supported by some observations. For example, aphidicolin, a nonselective inhibitor of pol α and pol δ (Goscin & Byrnes, 1982; Lee et al., 1985) inhibited permeable cell DNA synthesis with significantly greater potency than those with which the compound inhibited pol α and pol δ (see above). I measured permeable cell DNA synthesis in the presence or absence of various concentrations of BuAdATP, COMDP or both, at reduced concentrations of dNTPs (see above), and the results are summarized in Table V-2. The potency of BuAdATP as an inhibitor of permeabilized cell DNA synthesis was not changed by decreasing the concentrations of the unlabelled dNTPs from 50 to 1 μM; the data of Table V-2 indicate that the IC_{50} value of BuAdATP was still >200 μM (Table V-1)

Table V-2. Inhibition of [^{32}P]dATP Incorporation into Permeabilized CHO Cell DNA by BuAdATP, COMDP and combinations thereof[1]

Inhibitor concentration (μM)		
BuAdATP	COMDP	% Inhibition
200	0	31
0	200	53
100	100	51
50	0	9
0	50	24
25	25	13
20	0	0
0	20	21
10	10	22

[1][^{32}P]dATP incorporation into the DNA of permeabilized CHO cells was measured as described in Chapter II, except that the unlabelled dNTPs (dCTP, dGTP and dTTP) were present at 1 rather than 50 μM. Cells were made permeable, added to solutions containing inhibitors (or solvent control) and [^{32}P]dATP, and incubated at 37 °C for 8 min. Background counts were estimated as the counts in samples not incubated at 37 °C.

under the conditions of reduced dNTP concentrations. The degree of inhibition by BuAdATP and COMDP when present together (Table V-2) was clearly no more than the sum of their individual actions; no synergism between the two inhibitors was apparent.

Assuming the validity of the hypothesis that simultaneous inhibition of pol α and pol δ should result in synergistic inhibition of cellular DNA synthesis, there are several possible reasons for the observed lack of synergism. One derives from that postulated above for the weakness of COMDP as an inhibitor of permeabilized cell DNA synthesis. The potencies of inhibition of pol α and pol δ are a function of the composition of the template:primer, and, further, a change in the template:primer does not necessarily change the sensitivities of pol α and pol δ equally. Thus, COMDP might by template:primer effects alone might not inhibit that enzyme selectively <u>in vivo</u> relative to pol α. If so, synergistic inhibition of DNA synthesis by BuAdATP and COMDP would not be expected.

CHAPTER VI

DISCUSSION

A. Correlation of Inhibition of DNA Polymerases Alpha and Delta and Cellular DNA Synthesis

The present study represents progress toward the long term goal of using selective inhibitors of pol δ to probe and define the role of that enzyme in mammalian cell replicative DNA synthesis. I succeeded in finding a compound (COMDP) which inhibited pol δ selectively with respect to pol α, and I characterized its action on pol δ in detail. However, the results of the limited experiments that I performed using permeable cells and COMDP, in an attempt to find evidence for the participation of pol δ in DNA replication, were inconclusive.

Inhibition of cellular DNA synthesis by COMDP. COMDP was a weak inhibitor of permeable cell DNA synthesis, displaying an IC_{50} value of 200 μM. That value is considerably higher than its K_i values as an inhibitor of pol α (40 μM; Figure IV-12) and pol δ (1.8 μM; Figure IV-12), using poly(dA):oligo(dT) as the template:primer, but between its IC_{50} values when the enzymes were assayed using activated DNA as the template:primer (300 and 40 μM, respectively; Table IV-6). From these results, it is difficult to draw conclusions about the role of pol δ in the

aphidicolin sensitive DNA synthesis in permeabilized cell system. The most likely source of uncertainty is the variation in the potency of COMDP as a function of the composition of the template:primer used in the DNA polymerase assay. In addition to the differences in COMDP potencies when the enzymes were assayed using poly(dA):oligo(dT) *vs.* activated DNA, I found that when poly(dC):oligo(dG) was the template:primer (Figure IV-12), the K_i value of COMDP for inhibition of pol δ was 45 μM, twenty-five times that observed when the template:primer was poly(dA):oligo(dT). The latter observation serves to point out the difficulty in correlating the potency of inhibition of pol δ by a compound with the characteristics of the template:primer used in the assay. Poly(dC):oligo(dG) presents a DNA polymerase enzyme with the same primer density and the same long (compared with activated DNA) stretches of single stranded DNA as does poly(dA):oligo(dT), yet the activity of pol δ and its sensitivity to inhibition by COMDP are significantly different between the two. It is equally questionable to attempt to correlate the activity of an enzyme on a particular template:primer with its sensitivity to inhibition, since, for example, pol α is equally active on poly(dA):oligo(dT) and activated DNA, yet is much less sensitive to inhibition by COMDP when it is assayed using the latter (K_i = 40 *vs.* IC_{50} = 300 μM,

respectively). Even if the relationship between the characteristics of a template:primer and sensitivity to inhibition were known, it would still be impossible to know how well any particular template:primer combination approximates the template:primer substrate that a replicative DNA polymerase uses in vivo. In addition to template effects, one must consider that COMDP seems capable of reversibly forming a Schiff base with primary amines (Chapter IV). It is possible that in permeabilized cells enough COMDP is in such a state that the concentration of (free) COMDP is significantly less than would otherwise be expected. The inhibitory potency in the permeabilized cell assay of FPAA, which, like COMDP, is a charged pyrophosphate analog, argues against the possibility that the replicative enzymes in the nuclei of permeabilized cells are somehow physically inaccessible to COMDP.

In view of the results and arguments presented above, it is clear that pol δ-specific inhibitors with much greater potency and selectivity than COMDP must be developed to be of use in defining the role of pol δ in replicative DNA synthesis in vivo. Such a compound would not, however, guarantee success. Even BuPdGTP, an inhibitor of pol α with nanomolar range potency (Khan et al., 1984) and three to four orders of magnitude selectivity relative to pol δ (Lee et al., 1985; Table IV-2), inhibited permeable mammalian

cell DNA synthesis with an IC_{50} value of 3 μM (Dresler & Frattini, 1986; 1988), and thus did not clearly indicate a role for pol α in mammalian cell DNA synthesis.

B. Inhibition of DNA Polymerases by COMDP

COMDP inhibits DNA polymerases alpha and delta by different mechanisms. The properties of COMDP as an inhibitor of pol α and pol δ are significant both because the compound is the first known small molecule which inhibits pol δ with significantly greater potency than pol α, and because the apparent mechanisms of inhibition of the two enzymes by the compound are different. The results of Lineweaver-Burk analyses (Chapter IV, Figures IV-12 and IV-14) indicate that COMDP binds part of the dNTP binding site of pol δ, whereas the same is not true in the case of pol α (Figure IV-12). As described below, these properties of the inhibitor carry important implications both for the development of more potent inhibitors of pol δ based on COMDP, and also for the use of COMDP and COMDP derivatives as probes of the structure and function of pol δ.

dNTP analogs of COMDP. Based on the observation that COMDP inhibits pol δ competitively with dNTPs, and in consideration of the structure of the compound, I propose that the compound binds pol δ at the part of its active site which binds the 5'-triphosphate group of the "incoming"

dNTP. If COMDP binds the dNTP site as a dNTP analog, then a COMDP analog which more closely resembles a dNTP, for example, a dNTP in which the β-γ-phosphoanhydride oxygen is replaced with a carbonyl group (Figure VI-1), might bind that site with greater affinity than COMDP. Further, the kinetic studies suggest that COMDP does not bind the analogous site on pol α, and if so, a dNTP analog of COMDP might not bind that site either. As a result, a dNTP analog of COMDP might display greater inhibitory selectivity for pol δ compared to pol α than the parent compound COMDP.

Irreversible inhibition of DNA polymerase delta by COMDP and COMDP derivatives. The observation that COMDP gradually lost inhibitory potency in a buffer containing the primary amine Tris, along with a consideration of the inhibitor's structure, suggested that COMDP might form a stable Schiff base with a primary amine in the active site of pol δ. An attempt to reduce this hypothetical complex with $NaBH_4$ caused irreversible inactivation of the enzyme; however, subsequent experiments revealed that inactivation was not dependent on the presence of poly(dA):oligo(dT), and was not prevented by excess dTTP. The latter observation suggested that $NaBH_4$-dependent inactivation of pol δ by COMDP was due at least in part to Schiff base formation outside of the active site of the enzyme. Thus, the experiments neither demonstrated nor ruled out Schiff base formation by COMDP in

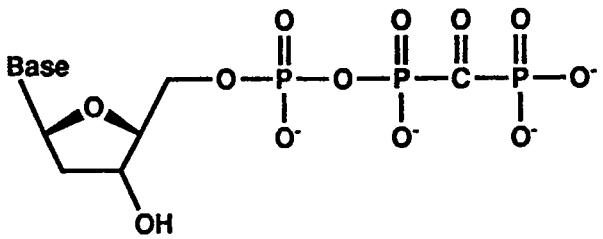

Figure VI-1. Proposed dNTP analog of COMDP.

the active site of pol δ. dNTP analogs of COMDP such as those proposed above might fail to form Schiff bases with residues of pol δ outside of its active site, and therefore might serve as irreversible pol δ active site labelling reagents. The observation of Basu and Modak (1987) that pyridoxal phosphate can be used to irreversibly label the active site of E. coli DNA polymerase I suggests that an analogous derivative of COMDP, in which a group such as pyridoxal is linked to it, might likewise serve as an irreversible active site probe of pol δ (Figure VI-2).

COMDP as a probe of the structure of DNA polymerase delta. At least two conclusions can safely be drawn concerning the structure of pol δ based on studies with COMDP. One is that the difference in potency and mechanism of inhibition of pol δ by the compound each argue that there are at least some structural differences in the active site regions of the two enzymes. The other is that, based on the observation that the 3' → 5' exonuclease activity of pol δ is resistant to inhibition by COMDP, the 3' → 5' exonuclease activity and DNA polymerase sites of pol δ may be structurally and functionally distinct. Freemont et al.

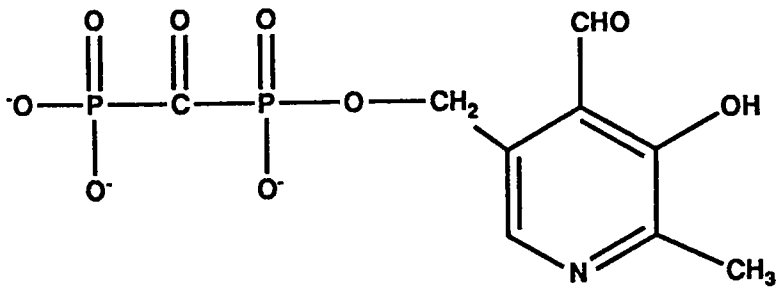

Figure VI-2. Proposed pyridoxylated derivative of COMDP.

(1988) have demonstrated by X-ray crystallography that the DNA polymerase and 3' → 5' exonuclease sites of the Klenow fragment of E. coli DNA polymerase I are spaced about 15 Å apart; perhaps the same is true of pol δ.

Inhibition of other DNA polymerases by COMDP. COMDP is a weak inhibitor of mammalian cell pol β and pol γ.[1] The compound is, however, a potent inhibitor of several viral DNA polymerases, displaying IC_{50} values ≤ 10 μM as an inhibitor of the DNA polymerases of Herpes Simplex Virus Type 1 (Eriksson et al., 1980), Human Cytomegalovirus (Eriksson et al., 1982), Epstein-Barr Virus (Öberg, 1989) and the reverse transcriptase of the Human Immunodeficiency Virus (HIV) (Vrang & Öberg, 1986). In the latter case, the potency of inhibition was, as I observed in the case of pol δ, strongly a function of the composition of the template:primer used in the reaction; the compound inhibited reverse transcriptase with IC_{50} values of 4 μM when assayed on poly(A):oligo(dT), 110 μM with poly(C):oligo(dG) and >200 μM with poly(dC):oligo(dG) (Vrang & Öberg, 1986). COMDP inhibited HIV reverse transcriptase in a mixed uncompetitive / noncompetitive mode with respect to dTTP concentration when the enzyme was assayed using poly(A):oligo(dT) (Vrang & Öberg, 1986), in contrast with my results in the cases of pol α (uncompetitive) and pol δ (competitive). The

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[1] C. E. McKenna, personal communication.

observations that the potency of COMDP as an inhibitor of pol δ and of several viral polymerases raises an important point made in Chapter IV in the context of the inhibitors AZTTP and ACGTP; if pol δ is involved in mammalian cell DNA metabolism, then inhibition of that enzyme by putative antiviral agents must not be overlooked as a potential contributor to their cytotoxicity.

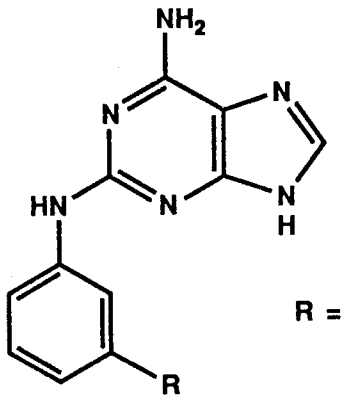
C. Future Directions

Development of potent and selective inhibitors of DNA polymerase delta based on COMDP. As a lead toward the development of inhibitors of increased potency and selectivity for pol δ, the structure of COMDP suggests two approaches. One, discussed above, is the development of dNTP analogs of COMDP. The other is the development of other PP_i analogs. Because of their potential for clinical use as antiviral agents, a large number of phosphate and PP_i analogs have been synthesized (Öberg, 1989). The simplicity of the structures of PP_i and COMDP, and a consideration of the large number of their analogs in existence, suggests that most or all of the COMDP analogs that one might imagine as potential pol δ inhibitors have already been synthesized. The task remains to collect samples of a number of such compounds and test their inhibitory potencies toward pol δ.

Development of selective inhibitors of DNA polymerase

delta based on DCAdATP. As noted in Chapter IV, both BuAdATP and DCAdATP are selective inhibitors of pol α relative to pol δ; however, the selectivity of the latter compound is more than three orders of magnitude less than that of the former. That observation, along with a consideration of the structural similarities and differences between BuAdATP and DCAdATP, suggests a rational basis for the development of other 2-substituted dATP analogs which might display potent and selective inhibition of pol δ. As a first step, I would suggest that derivatives of the purine base analog 2AA be synthesized in which the 3-position of its anilino ring is substituted with a series of halogen and alkyl groups, starting with chloro, bromo, methyl and ethyl groups (Figure VI-3). The compounds could be tested for inhibition of pol α and pol δ, and ones with promising inhibitory properties could be derivatized to their 2'-deoxyribonucleoside 5'-triphosphate forms for further testing. Although this approach would be more difficult than screening an expanded series of PP_i analogs, it seems to hold a greater potential for the discovery of an extremely potent and selective inhibitor of pol δ, analogous to BuAdATP and BuPdGTP in the case of pol α.

Use of selective inhibitors of DNA polymerase delta to discern a role for that enzyme in cellular DNA metabolism. In the present study, I attempted to use COMDP to detect the



R = Cl, Br, Me, Et.

Figure VI-3. Proposed 2AA analogs.

participation of pol δ in permeabilized cell replicative DNA synthesis, and as described above, the results were inconclusive. Despite the potential pitfalls noted above, the technique of cell permeabilization seems to hold considerable promise for the study of the cellular role of pol δ, especially if compounds are developed which possess sufficient potency and selectivity for pol δ to diminish the problem of the variability of inhibitor potency with template:primer composition. A unique advantage of the permeabilized cell assay compared to studies with intact cells is that, with permeable cells, the dNTP concentration can be varied at will. For example, the observation that a compound with the properties of COMDP inhibited permeable cell DNA synthesis competitively with dNTPs would constitute good evidence that the compound inhibited permeable cell synthesis by means of inhibition of pol δ rather than pol α. It seems unlikely that inhibitory mechanism is so strongly a function of the template:primer composition as inhibitor potency is.

A selective inhibitor of pol δ, perhaps COMDP itself, might be used to test the hypothesis of Focher et al. (1988a) and Downey et al. (1988) that pol α and pol δ catalyze lagging and leading strand synthesis, respectively. For example, Dresler and Frattini have found that BuPdGTP preferentially inhibited Okazaki fragment synthesis in

permeabilized human fibroblasts.[1] It remains to be shown whether a selective pol δ inhibitor such as COMDP preferentially inhibits leading strand synthesis, which would be indicated by a preference by the compound for inhibition of synthesis of long DNA strands.

Role of DNA polymerase delta in repair synthesis. I have limited the present discussion to the controversial role of pol δ in mammalian cell DNA replication. For completeness, I wish to note that there is also evidence for a role for pol δ in certain types of mammalian cell DNA repair synthesis (Dresler & Frattini, 1986; Dresler & Kimbro, 1987; Nishida et al., 1988; Dresler & Frattini, 1988; Dresler et al., 1988). Selective inhibitors of pol δ such as COMDP might prove to be important tools in experiments designed to define more precisely the role of pol δ in DNA repair.

Inhibitor studies: One step toward defining the role of DNA polymerase delta in DNA replication. In conclusion, I shall point out that several avenues must be pursued in order to establish the function of pol δ. When, in addition to inhibitor studies, the gene for mammalian pol δ is cloned and its relationship to that of pol α defined, when the expression of the pol δ gene as a function of the cell cycle is determined, and when cells carrying a conditionally

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[1] M. Frattini, University of Chicago, personal communication.

mutant pol δ gene are found and some deficiency in the cells' DNA replication under nonpermissive conditions is found, a strong statement on the role of pol δ in mammalian cell DNA replication will be possible.

CHAPTER VII

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