Design and characterization of N²-arylamino purines which selectively inhibit replicative DNA synthesis and replication-specific DNA polymerases: guanine derivatives active on mammalian DNA polymerase alpha and bacterial DNA polymerase III

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ABSTRACT

The 2-amino substituted derivatives of guanine, N²-(p-n-butylphenyl)guanine (BuPG) and N²-(3',4'-trimethylenephanyl) guanine (TMPG), were synthesized and found to selectively inhibit, respectively, HeLa cell DNA polymerase alpha (pol α) and *B. subtilis* DNA polymerase III (pol III). Both purines, like their corresponding uracil analogs, BuAU and TMAU (2,9), were specifically competitive with dGTP in their inhibitory action on their target polymerases. BuPG, the pol α -specific purine, was also toxic for HeLa cells *in vivo*, selectively inhibiting DNA synthesis. These N²-substituted purines, in contrast to the 6-substituted uracils, provide a structural basis for the synthesis of nucleosides and nucleotides with considerable potential as probes for the analysis of the structure of specific replicative DNA polymerases and their function in cellular DNA metabolism.

INTRODUCTION

6-Substituted uracils of the prototype depicted in Figure 1, when appropriately constructed, are potent and selective inhibitors of replicative DNA synthesis in bacteria and in mammalian cells (1,2). Their target in each of these systems is a replication-specific DNA polymerase (2,3,4). Their inhibitory activity is based in part on their capacity to mimic dGTP (5,6). Their credentials as dGTP derive from the 1 and 2 ring moieties and the 6-NH group of the base domain (cf Fig 1); the latter substituents endow the molecule with the capacity to form hydrogen (H) bonds with template cytosine. The inhibitory potency of the 6-substituted pyrimidines, although dependent on the base domain, is heavily dependent on the substituents in the aryl ring, which binds strongly to the enzyme in the area of its catalytic site, sequestering it in a catalytically non-productive drug:enzyme:template:primer complex. Figure 2 (from ref. 2; with permission) schematically depicts this complex and the roles of the base and aryl domains in its formation. The X and Y constituents of the aryl moiety (Fig 1) determine the enzyme specificity of the inhibitors (2,7). For example, a molecule carrying substituents such as CH₃ and Cl at X or Y are potent

Fig. 1. Uracil-based, dGTP-specific DNA polymerase inhibitor.

inhibitors of *B.subtilis* DNA polymerase III (pol III) and have no discernible action on mammalian DNA polymerase alpha (pol α). In contrast, an aryl moiety carrying a bulky *p*-*n*-butyl group at X(Y=H) destroys the drug's action against pol III and endows it with the capacity to inhibit pol α (2). We have sought to design more potent and more useful derivatives of the 6-(arylamino) uracils - in particular nucleoside and nucleotide derivatives.

Fig. 2. Inhibitory mechanism of 6-substituted aminouracils-formation of an inhibitor: primer-template complex.

The chemistry of the N-1 of the pyrimidine and consideration of the specific role of N-1 in H bonding makes manipulation/substitution at that position impossible. Therefore, we have taken the approach to design a similar inhibitor using the 2-aminopurine, guanine, as the drug nucleus. The basic structure of the purine prototype (cf Fig 3) involves placement of the aryl moiety on the 2-amino group of guanine. This design has had the three following objectives: (i) to preserve the base domain containing the specific base pairing substituents of guanine, i.e., the 2 NH, the 6 oxo group, and the H at N-1; (ii) to provide an appropriate site and configuration for placement of the enzyme-specific aryl group, and (iii) to provide, in the form of the ring nitrogen at position 9, a site for construction of nucleosides. In this paper we describe the synthesis and characterization of two prototypes: N²-(p-n-butylphenyl)guanine (BuPG), hypothetically selective for mammalian pol α , and N²-(3',4'-trimethylenepheryl) guanine (TMPG), hypothetically selective for bacterial pol III. The results, which are summarized below, indicate that the inhibitory properties of BuPG and TMPG were entirely analogous to, and in certain respects, superior to those of the corresponding uracil derivatives on which they were based.

METHODS AND MATERIALS

Growth of Cells. HeLa S₃ cells were grown in suspension culture in F-13 medium as described previously (2). Cells were counted with either an hemocytometer or Royco cell counter. Cells were synchronized by the double hydroxyurea block technique (8) using 0.5 mM hydroxyurea at each step and an initial cell density of 2-3 x 10⁵ cells per ml.

Materials. Radioactive materials were purchased from New England Nuclear. Nucleotides and synthetic polynucleotides were obtained from P-L Laboratories; calf thymus DNA was from Worthington. 6-Anilinouracils were

Fig. 3. Guanine-based, dGTP-specific DNA polymerase inhibitor.

prepared by the method described by Brown et al. (9). Elemental analyses were done by Schwarzkopf Microanalytical Laboratory, Woodside, NY.

N²-(*p*-*n*-butylphenyl)guanine (BuPG) was prepared in the following manner.

2-Chlorohypoxanthine (10) and an excess of *p*-*n*-butylaniline were heated at reflux in 2-methoxyethanol for 4 hr. An equal volume of H₂O was added, and the suspension was chilled overnight. The beige solid was filtered with suction, suspended in hot acetic acid, filtered again and washed extensively with hot acetic acid. BuPG was crystallized from either 2-methoxyethanol or acetic acid. Yield: 50%; m.p. 346–347°. Analysis: Calcd. for C₁₅H₁₇N₅O: C, 63.59; H, 6.05; N, 24.72. Found: C, 63.34; H, 6.15; N, 24.50.

N²-(3',4'-trimethylenephenyl)guanine (TMPG), was synthesized and purified by the above method using 5-aminoindane in lieu of butylaniline; the yield was 60%; m.p. 349–351°. Analysis: Calcd. for C₁₄H₁₃N₅O: C, 62.91; H, 4.90; N, 26.20. Found: C, 62.66; H, 5.00; N, 25.91.

Enzymes. HeLa cell DNA polymerases α , β , and γ were purified from synchronously dividing cells according to published procedures (11,12,13). B.subtilis pol III was purified by the immunoadsorbent-based method of Barnes and Brown (14). B.subtilis pol II was a DEAE-cellulose fraction produced in a conventional method of pol III purification (5). E.coli pol I was purchased from P-L Laboratories, and E.coli pol III was provided by Dr. Charles McHenry.

DNA Polymerase Assay. Enzymes of bacterial origin were assayed in the conditions described by Clements et al (5) for the assay of B.subtilis pol III. The assay conditions for HeLa cell DNA polymerases were those described by Novak and Baril (12). Unless otherwise specified, all assays employed [³H]TTP as the labeled DNA precursor and DNAase-activated calf thymus DNA as template-primer. Apparent inhibitor constants (K_i) for each inhibitor were determined in the appropriate polymerase assay conditions by employing the truncated, dGTP-deficient assay method described by Wright and Brown (15). Conditions of the truncated assay were devised to ensure that incorporation of [³H]TMP was linear throughout the assay period.

Assay of DNA, RNA, and Protein Synthesis in Intact Cells. The methods of assay of incorporation of [³]uridine into RNA, [³H]thymidine into DNA, and [(³H)]leucine into protein were performed as described previously (2).

RESULTS

BuPG: definition of the selective effect on pol α . Considering the structure

Fig. 4. Effect of BuPG on the activity of HeLa pol α . Pol α was assayed as described in the methods section in the presence of several concentrations of BuPG in the presence and absence of dGTP (truncated assay). 100 per cent activity in the absence and presence of dGTP (800 μ M) was represented by the incorporation, respectively, of 11 and 41 pmoles of [3 H]TMP.

of BuPG and its similarity chemically to BuAU, we hypothesized that it should be a potent and selective inhibitor of mammalian pol α and, further, that its effect should be reversed specifically by dGTP. This hypothesis was essentially correct, as indicated in the data of Figure 4 and Table 1.

Figure 4 depicts the activity of HeLa pol α in the truncated polymerase assay

TABLE I ENZYME SPECIFICITY OF BuPG

ENZYME	BuPG CONCENTRATION	% OF CONTROL ACTIVITY
HeLa cell pol α	0.1mM	11
HeLa cell pol β	2mM	100
HeLa cell pol γ	2mM	100
<i>B. subtilis</i> pol III	0.5mM	99
<i>B. subtilis</i> pol II	0.5mM	100
<i>E. coli</i> pol I	0.5mM	98

Each enzyme was assayed with activated DNA using the truncated, dGTP-deficient assay in conditions indicated in the methods section. Incorporation of [3 H] TMP into DNA in the controls ranged from 1.5 pmoles for pol γ to 16 pmoles for pol α .

(-dGTP) as a function of the concentration of BuPG and BuAU. Both inhibitors act on pol α - BuAU with an apparent K_i of 45-60 μ M and BuPG with an apparent K_i of 6-9 μ M. Addition of 0.8mM dGTP reversed the effects of both. The results of additional experiments (data not shown), designed to probe the effects of the other three deoxyribonucleotides, indicated that dATP, dCTP, and dTTP, each at a concentration of 0.8mM, were not competitive with BuPG.

The selectivity of BuPG is shown in the results of experiments summarized in Table 1. At concentrations of 0.5-2mM and in the truncated, dGTP-deficient assay, the inhibitor had no discernible effect on the activities of HeLa pol β and γ , *B.subtilis* pol II and III, and *E.coli* pol I.

Another experiment employing HeLa pol α , the synthetic primer:templates, oligo (dT): poly (dA), oligo (dG): poly (dC), and the appropriate monomeric substrates, questioned whether the action of BuPG, like that of BuAU, also required a template containing cytosine. The answer (results are not shown) was clearly yes; at 200 μ M BuPG had no effect on the incorporation of [3 H]TMP into oligo (dT), whereas it was a potent inhibitor of incorporation of [3 H]dGMP into oligo (dG).

BuPG action in vivo. Was BuPG, like BuAU, cytotoxic for the cells whose pol α it inhibited? Further, was it selective for DNA synthesis? The answers to these questions lie in the data of Figures 5 and 6. BuPG inhibited the division of HeLa cells with a potency approximately equal to that of BuAU (approximately 50% inhibition at a concentration of 100 μ M; ref 2).

The inhibitory effect of BuPG on the synthesis of macromolecules was essentially identical to that of BuAU (ref 2). As depicted in Fig 6, BuPG at 150 μ M inhibited incorporation of [3 H]thymidine almost completely and had little, if any, discernible effect on the incorporation of [3 H]uridine into RNA and [3 H]leucine into protein.

BuPG at concentrations as high as 250 μ M also had no effect on leucine and uridine incorporation by randomly dividing HeLa cells in which the processes of protein and RNA synthesis appear more active and longer lived than those apparent in cells released from hydroxyurea block (data not shown).

The above effects of BuPG also were consistent with those of other experiments (results also not shown) in which DNA, RNA, and protein were determined directly, by chemical assay, in cultures of synchronized cells.

BuPG at a concentration of 150 μ M did not significantly affect the increase of RNA and protein, whereas it inhibited formation of DNA by more than 90 per cent.

Fig. 5. Effect of BuPG on proliferation of HeLa cells in culture. Identical cultures in exponential growth were seeded as described in the methods section and exposed to BuPG at the indicated concentrations, and at intervals cell number was counted.

The aryl moiety determines polymerase specificity. In the case of the uracil-based inhibitors, the structure of the aryl moiety determines the spectrum of DNA polymerases on which a given inhibitor will act (2,7). The selectivity of BuPG for pol α strongly suggested that the aryl group, albeit attached to a purine, controlled specificity exactly as if it were a substituent of 6-aminouracil. To test this suggestion further, we characterized the spectrum of activity of TMPG, the BuPG analog containing the 3',4'-trimethylenepheryl moiety, a group which, in the 6-aminouracil series, confers selectivity for inhibition of pol III of Gram positive bacteria (9). The behavior of TMPG, examined in a truncated, dGTP-deficient assay (results not shown), confirmed this suggestion. TMPG, like TMAU was antagonized specifically by dGTP and was highly selective for B.subtilis pol III, yielding an apparent K_i of approximately 5 μ M; at a concentration of 200 μ M it had no discernible effect on the activity of B.subtilis pol II, E.coli Pol I, or HeLa pol α .

DISCUSSION

The results of the studies described above indicate that the 2-aminopurine nucleus can be exploited to construct DNA polymerase-specific inhibitors with

Fig. 6. Effect of BuPG on the synthesis of macromolecules by HeLa cells in culture. Synchronized cells were washed free of drug by centrifugation at 37° from fresh medium, resuspended in fresh medium at a density of 2.2×10^6 cells per ml, divided into six portions and exposed at 37° in the absence and presence of 150 μM BuPG to [^3H]thymidine (sp.act. ~ 50 Ci/mmol; 0.5 μCi/ml), [^3H]uridine (sp.act. ~ 30 Ci/mmol; 0.5 μCi/ml), and [^3H]leucine (sp.act. ~ 60 Ci/mmol; 0.5 μCi/ml) to assay the effect of the inhibitor on incorporation of isotope into respectively, DNA, RNA and protein. Samples were removed and analysed as described in the methods section.

a mechanism of action analogous to that of 6-(arylamino)pyrimidines. These guanine-based inhibitors, if appropriately designed, are indistinguishable from the uracil type. They compete specifically with dGTP (Fig 3); they require a template containing cytosine to exact their inhibitory effect, and their specificity for a given polymerase is determined by the structure of their aryl groups. The similarity of the inhibitory actions of the pyrimidine and guanine derivatives may also apply to their effects on intact growing cells, although the behavior of the purine is not always predictable. BuPG, the pol α -specific inhibitor, selectively inhibits proliferation and DNA synthesis of HeLa cells in culture (Fig 5), exactly like BuAU. In contrast, TMPG, for reasons which likely are related to inadequate transport and metabolic inactivation, is at least 40 times less potent than TMAU as an inhibitor of growth and DNA synthesis of *B. subtilis* (Brown and Wright, unpublished observations).

Relative merits of N²-arylamino purines and 6-(arylamino)pyrimidines as pol α specific inhibitors. Our research on polymerase-specific inhibitors is currently directed to the systematic development of purine and/or pyrimidine-based inhibitors specific for DNA polymerase α, an enzyme putatively involved in the replicative synthesis of mammalian DNA. Our objective is to produce – in the form of readily synthesized inhibitors of well characterized mechanism – reagents with which to probe the structure and mechanism of pol α, the nature of the structural gene which specifies pol α, and the process of replicative DNA synthesis in which pol α putatively serves (2).

At present it is not clear which inhibitor type best serves our objectives. The uracil nucleus, in the form of BuAU, provides a highly selective pol α inhibitor unlikely to be metabolized to forms which can attack secondary targets in the anabolic pathways to DNA or RNA. However, BuAU presents little opportunity for chemical manipulation. The unmodified purine, BuPG, albeit slightly more potent than BuAU in vitro and in vivo, does not per se have any well-established advantage over BuAU. However, it has an attractive, hypothetical advantage; it can be transformed readily into the 9-β-deoxyribonucleoside and the corresponding nucleoside triphosphate and thus, a putatively closer analog of the competitor substrate, dGTP. The higher 2'-deoxyribonucleoside derivatives, which can be synthesized chemically, and perhaps, via the action of various cellular kinases, may represent pol α-specific reagents which are considerably more potent and more specific than the unmodified purine per se.

As a first attempt to test and exploit the putative advantage of the purine-based inhibitor, we have synthesized the 9-β-2'-deoxyribonucleoside of BuPG (BuPGdR; Dudycz and Wright, unpublished results) and have begun to examine its effects on mammalian pol α in vitro and on mammalian cell growth and macromolecule synthesis. Against the isolated HeLa pol α, BuPGdR was at least equipotent with BuPG and in cultures of Chinese Hamster Ovary cells BuPGdR inhibited cell proliferation (Dudycz, Wright, and Brown, unpublished results). Experiments are in progress to determine if BuPGdR selectively inhibits DNA biosynthesis and whether such a selective effect, if observed, depends on the action of phosphorylated drug form(s) on pol α.

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