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ANALYSIS OF BUTYLPHENYL-GUANINE, BUTYLPHENYL-DEOXYGUANOSINE, AND BUTYLPHENYL-DEOXYGUANOSINE TRIPHOSPHATE INHIBITION OF DNA REPLICATION AND ULTRAVIOLET-INDUCED DNA REPAIR SYNTHESIS USING PERMEABLE HUMAN FIBROBLASTS

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Abstract—The purine base and nucleoside analogues N^2-(*p-n*-butylphenyl)-guanine (BuPh-Gua) and N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine (BuPh-dGuo) are strong inhibitors of isolated mammalian DNA polymerase α, but are less potent than expected as inhibitors of DNA replication in intact cultured cells [G. E. Wright, L. W. Dudycz, Z. Kazimierczuk, N. C. Brown and N. N. Khan, *J. med. Chem.* **30**, 109 (1987)]. The mechanistic basis for these observations was explored using permeable human fibroblasts. DNA replication in the permeable cells was inhibited only slightly by BuPh-Gua and BuPh-dGuo at 100 μM, the highest concentration which could be attained. Similiar results were obtained for ultraviolet-induced DNA repair synthesis, a process which is though to involve the same DNA polymerase as replication. More detailed studies were performed using the corresponding nucleotide analogue, N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine-5′-triphosphate (BuPh-dGTP), which is much more water-soluble than the base and nucleoside. The apparent K_i values for BuPh-dGTP inhibition of both replication and ultraviolet-induced repair synthesis in permeable cells were approximately 3 μM. These values are several hundred-fold greater than the apparent K_i for BuPh-dGTP inhibition of isolated human DNA polymerase α, which is approximately 10 nM. We conclude that BuPh-Gua and BuPh-dGuo are poor inhibitors of DNA replication in intact cells not because of permeability barriers, but because, unlike polymerase α, cellular DNA synthesis is relatively insensitive to this group of inhibitors. These results suggest that polymerase α may not be a good general model for predicting the potency of base, deoxyribonucleoside and deoxyribonucleotide analogues as inhibitors of mammalian cellular DNA replication. The fact that the permeable cell systems accurately reflect the relative insensitivity to butylphenyl-guanine derivatives of mammalian DNA replication suggests that permeable cells may be useful tools in future studies of base and nucleoside analogues.

DNA polymerase α has long been regarded as the enzyme responsible for mammalian nuclear DNA replication [1] and, as a result, isolated polymerase α has been used as a model for the development of analogues of the purine and pyrimidine bases and deoxyribonucleosides for use as inhibitors of mammalian cellular DNA replication. The N^2-(*p-n*-butylphenyl) analogues of guanine (BuPh-Gua)† and deoxyguanosine (BuPh-dGuo) are, however, less potent as inhibitors of cellular DNA replication than one would expect based on their effects on isolated polymerase α [2, 3]. In an attempt to understand this phenomenon, we investigated the inhibition of cellular DNA synthesis by these compounds using previously described, well-characterized permeable human fibroblast systems [4–8]. We studied not only DNA replication, but also UV-induced DNA repair synthesis, which is thought to be mediated by the same DNA polymerase as that which mediates replication [5, 6, 9, 10]. Replication and UV-induced repair synthesis in permeable human cells were, like replication in intact cells [3], substantially less sensitive to the butylphenyl-guanine derivatives than is isolated DNA polymerase α. The permeable cell system appears to accurately reflect the inhibitor sensitivities of DNA replication in intact cells and may be useful for further development of base and nucleoside analogues.

MATERIALS AND METHODS

Chemicals. Concentrations of nucleotide solutions were determined by UV absorbance. Purity of all nucleotides, determined by thin-layer chromatography on polyetheleneimine cellulose [11], was greater than 95%. Purity of [α-^{32}P]dGTP was greater than 98%. Stock solutions of BuPh-Gua (20 mM) and BuPh-dGuo (40 mM) dissolved in dimethyl sulfoxide, and of the tetraammonium salt of BuPh-dGTP (5 mM) dissolved in 20 mM Tris, pH 7.6, were stored at −20°. The three inhibitors were provided by Dr. George Wright, University of Massachusetts Medical School, Worcester, MA.

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† Abbreviations: BuPh-dGTP, N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine-5′-triphosphate; BuPh-Gua, N^2-(p-*n*-butylphenyl)-guanine; BuPh-dGuo, N^2-(p-*n*-butylphenyl)-2′-deoxyguanosine; K_i, inhibitor constant; and dNTPs, the four common 2′-deoxyribonucleoside-5′-triphosphates.

Measurement of DNA replication and repair synthesis in permeable cells. The assays for DNA replication and repair synthesis have been described previously in detail [4–8]. Human diploid fibroblasts (IMR-90 and AG1518; Institute for Medical Research, Camden, NJ) were passed into glass roller bottles, prelabeled with [*methyl*-^{14}C]thymidine and either used during exponential growth for studies of semiconservative DNA replication or grown to confluence and used for studies of DNA repair synthesis as described. Cells were collected, made permeable in 10 mM Tris (pH 7.6 at 37°), 4 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 3 mM dithiothreitol, and washed twice in the same buffer to remove endogenous nucleotides. (The depletion of endogenous nucleotide pools by this washing procedure has been documented [12].) For studies of repair synthesis, the permeable confluent cell suspension was spread in a layer approximately 1 mm thick in a plastic dish on ice and exposed to 100 J/m^2 of UV radiation from a G15T8 germicidal lamp at a flux of 3 W/m^2. Permeable cell suspension was mixed with 0.5 vol. of reaction mix to yield 40 mM Tris (pH 7.6 at 37°), 8 mM $MgCl_2$, 5 mM ATP, 167 mM sucrose, 2 mM dithiothreitol, 0.67 mM EDTA, and the indicated concentrations of KCl, dATP, dCTP, dTTP, [α-^{32}P]dGTP, and inhibitor. Samples were incubated at 37° for either 5 min (for measurements of replication) or 15 min (for measurements of repair synthesis), and radioactivity was determined. Repair synthesis was calculated by taking the difference between specific incorporation (^{32}P/^{14}C) in corresponding irradiated and unirradiated samples, and replication was measured as total specific incorporation (^{32}P/^{14}C) in undamaged growth-phase cells.

Assay of the activity of isolated DNA polymerase α. The polymerase α used, prepared from HeLa cells [13], was inhibited completely by aphidicolin and by the anti-polymerase α antibody SJK 287–38 [14]. Polymerase activity was assayed by incubating the enzyme with 20 mM Tris (pH 7.6 at 37°), 10 mM $MgCl_2$, 30 mM KCl, 2 mM 2-mercaptoethanol, 100 μM dATP, 100 μM dCTP, 100 μM dTTP, 200 μg/ml bovine serum albumin, 200 μg/ml activated DNA [15], and the indicated concentrations of [α-^{32}P]dGTP (9 Ci/mmol) and BuPh-dGTP for 10 min at 37°. The reactions were stopped by adding 4 vol. of ice-cold 10% trichloroacetic acid, the precipitates were collected on glass fiber filters and washed, and radioactivity was determined by liquid scintillation counting. Nucleotide incorporation was linear for at least 15 min (data not shown).

RESULTS

Inhibition of DNA replication and UV-induced DNA repair synthesis by BuPh-Gua, BuPh-dGuo, and BuPh-dGTP. We have investigated the effects of BuPh-Gua, BuPh-dGuo, and BuPh-dGTP on replication and UV-induced repair synthesis using well-characterized permeable cell systems. The DNA synthesized by permeable growth-phase cells has been shown to be the product of semiconservative replication [5], to be attached to DNA synthesized immediately prior to permeabilization, and to be preferentially associated with the nuclear matrix of the permeable cells (Dresler, Robinson-Hill and Frattini, unpublished). Thus, replicative DNA synthesis in permeable cells yields a product like that synthesized in intact cells and appears to involve the same nuclear DNA replication sites as are active in intact cells. The characteristics of UV-induced DNA repair synthesis have likewise been shown to be essentially identical to those seen in intact cells [6–8].

The effects of BuPh-Gua, BuPh-dGuo, and BuPh-dGTP were investigated in permeable human fibroblasts of two different types (AG1518 and IMR-90) Inhibition of replicative and repair synthesis was seen only at very high concentrations of BuPh-Gua and BuPh-dGuo (Fig. 1, A and B). Both replication (Fig. 1, A and C) and UV-induced repair (Fig. 1, B and D) synthesis were totally inhibited by the nucleotide analog, BuPh-dGTP. The inhibition curves were simple and apparently single-component, consistent with the involvement of a single DNA polymerase in each process. When replicative synthesis was assayed under the reaction con-

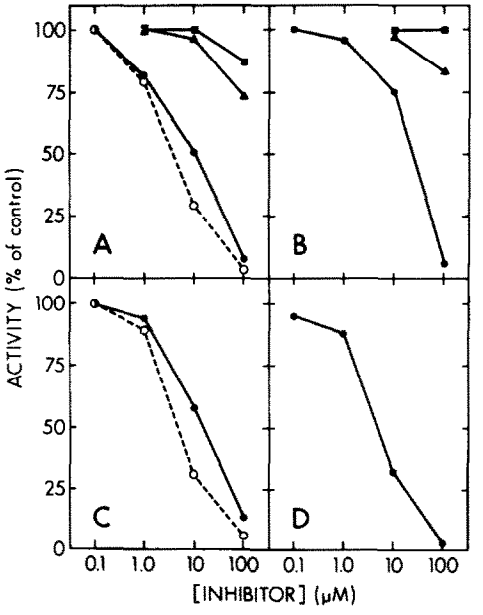

Fig. 1. Inhibition by BuPh-Gua, BuPh-dGuo, and BuPh-dGTP of DNA replication and UV-induced DNA repair synthesis in permeable IMR-90 (A and B) and AG1518 (C and D) fibroblasts. DNA replication (A and C) was measured in permeable growth-phase cells using either "replication conditions" (75 mM KCl, 5 μM dGTP, 50 μM dATP, 50 μM dCTP, 50 μM dTTP; ●, ▲, ■) or "repair synthesis conditions" (15 mM KCl, 0.3 μM dGTP, 3 μM dATP, 3 μM dCTP, 3 μM dTTP; ○). DNA repair synthesis (B and D) was measured in permeable confluent cells damaged with 100 J/m^2 UV using repair synthesis conditions (see above). The inhibitors BuPh-Gua (■), BuPh-dGuo (▲), and BuPh-dGTP (●, ○) were present at the indicated concentrations. The data are expressed as percentages of the replication or repair synthesis measured in the absence of inhibitor. Control values were as follows: (A) 1.74 for replication conditions and 0.96 for repair synthesis conditions; (B) 0.23; (C) 0.85 for replication conditions and 0.47 for repair synthesis conditions; and (D) 0.88. Each point is the average of two determinations.

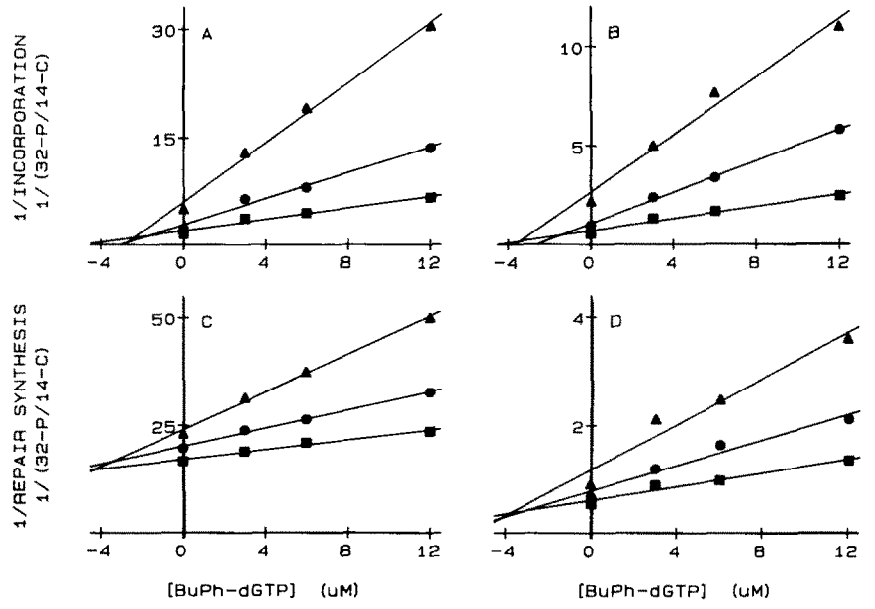

Fig. 2. Competitive inhibition by BuPh-dGTP of DNA replication and UV-induced DNA repair synthesis in permeable IMR-90 (A and C) and AG1518 (B and D) fibroblasts. Replication (A and B) was determined in permeable growth-phase cells using reaction mix containing 75 mM KCl, 100 μM dATP, 100 μM dCTP, 100 μM dTTP, and the indicated concentrations of BuPh-dGTP. The dGTP concentrations were 0.08 (▲), 0.16 (●), and 0.32 (■) μM. Repair synthesis (C and D), induced by 100 J/m^2 UV, was determined in permeable confluent cells using reaction mix containing 15 mM KCl, 100 μM dATP, 100 μM dCTP, 100 μM dTTP, and the indicated concentrations of BuPh-dGTP. The symbols are as described for replication. Each point is the average of two determinations. Lines were fitted by linear regression.

ditions usually used for the repair synthesis assay (Fig. 1, A and C), a small shift in the BuPh-dGTP inhibition curve was seen, probably as a result of the lower concentration of the competitive substrate, dGTP, present in the repair reaction mixture.

Mechanism of BuPh-dGTP inhibition of DNA replication and UV-induced DNA repair synthesis. Because of the limited water-solubility of BuPh-Gua and BuPh-dGuo, further studies were conducted only with BuPh-dGTP. The mechanism of inhibition was studied by assaying replication and repair synthesis in permeable AG1518 and IMR-90 cells at a number of different dGTP and BuPh-dGTP concentrations with dATP, dCTP, and dTTP held constant at a high level (100 μM). In Lineweaver–Burk plots of the data for replication (not shown), all lines intersected at single points on the ordinates, indicating that dGTP is competitive with BuPh-dGTP. From Dixon plots of these data (Fig. 2, A and B), it can be seen that, for both IMR-90 and AG1518 cells, the apparent K_i of DNA replication for BuPh-dGTP was approximately 3 μM. Lineweaver–Burk plots of the data for DNA repair synthesis (not shown) indicate that, for this process as well, dGTP was competitive with BuPh-dGTP. Dixon plots of the data (Fig. 2, C and D) show that, for both IMR-90 and AG1518 cells, the apparent K_i of UV-induced DNA repair synthesis for BuPh-dGTP was also approximately 3 μM. The similarity between the apparent K_i values for BuPh-dGTP of replication and repair synthesis supports the concept that the same DNA polymerase mediates both processes in human cells.

Inhibition by BuPh-dGTP of human DNA polymerase α. Khan *et al.* [2] found that inhibition of Chinese hamster DNA polymerase α by BuPh-dGTP is competitive with dGTP and that the K_i of the enzyme for the inhibitor is very low, about 10 nM. When we examined BuPh-dGTP inhibition of *in vitro* DNA synthesis catalyzed by DNA polymerase α isolated from the HeLa human tumor cell line, a K_i of 6 nM was observed (Fig. 3). Human DNA polymerase α, like the Chinese hamster enzyme, has

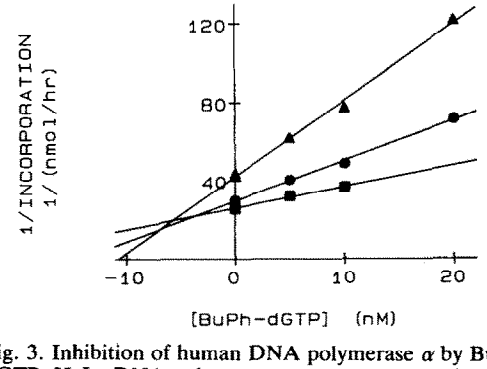

Fig. 3. Inhibition of human DNA polymerase α by BuPh-dGTP. HeLa DNA polymerase α was assayed in the presence of the indicated concentrations of BuPh-dGTP and dGTP concentrations of 1.2 (▲), 2.4 (●), or 3.6 (■) μM.

a K_i for BuPh-dGTP several hundred-fold lower than the K_i values of DNA replication and UV-induced DNA repair synthesis.

DISCUSSION

Because DNA polymerase α has been regarded as the enzyme responsible for replication of mammalian nuclear DNA [1], isolated polymerase α has been thought to be a good model for development of base and nucleoside analogue inhibitors of DNA replication. It has been reported recently, however, that BuPh-Gua and BuPh-dGuo, which are potent inhibitors of isolated polymerase α [2], are poor inhibitors of DNA replication in intact mammalian cells [3]. Using a permeable human fibroblast system which accurately reproduces the features of *in vivo* replicative DNA synthesis ([5]; Dresler, Robinson-Hill and Frattini, unpublished), we have investigated the basis for the relative resistance of cellular DNA replication to these butylphenyl-guanine derivatives. The effects of BuPh-Gua and BuPh-dGuo on replication in permeable cells were similar to those seen *in vivo*; only slight inhibition was seen at 100 μM, the highest concentration attainable. The corresponding nucleotide analogue, BuPh-dGTP, which is an extremely potent inhibitor of polymerase α isolated from human and other mammalian [2] sources (K_i of 10 nM or less), was also much less potent as an inhibitor of DNA replication in permeable human cells (K_i of approximately 3 μM). The nuclei of our permeable cells have been shown to be accessible to a 15,000 dalton nuclease [4] and to 150,000 dalton IgG molecules (Frattini and Dresler, unpublished), thus the relative insensitivity of DNA replication in permeable cells to these butylphenyl-guanine derivatives is not likely to result from a permeability barrier. We conclude that DNA replication in permeable human cells is substantially less sensitive to BuPh-Gua, BuPh-dGuo, and BuPh-dGTP than is DNA polymerase α. The consistency of our permeable cell results with results obtained in intact cells [3] suggests that permeable cell systems such as the one we employed may be useful as models for mammalian nuclear DNA replication.

We also examined the sensitivity of UV-induced DNA repair synthesis to inhibition by BuPh-Gua, BuPh-dGuo, and BuPh-dGTP. Like replication, UV-induced repair synthesis was only slightly inhibited even by high concentrations of BuPh-Gua and BuPh-dGuo. The apparent K_i for BuPh-dGTP of repair synthesis was approximately 3 μM, essentially the same as the value for replication. This result is consistent with our previous findings that replication and UV-induced repair synthesis have similar apparent K_i values for aphidicolin [5] and 2′,3′-dideoxythymidine-5′-triphosphate [10]. Taken together, our data suggest that replication and UV-induced repair synthesis are mediated by the same DNA polymerase. DNA polymerase inhibitors which are effective against replication may also be useful as DNA repair inhibitors to potentiate the cytotoxic effects of certain DNA damaging agents.

There are several possible explanations for our finding that the sensitivities of DNA replication and UV-induced DNA repair synthesis to BuPh-Gua, BuPh-dGuo, and BuPh-dGTP differ dramatically from that of isolated DNA polymerase α. Replication and UV-induced repair synthesis have been thought to be mediated by polymerase α primarily because both are sensitive to aphidicolin [1, 5], an agent initially regarded as a specific inhibitor of polymerase α [1]. It has lately become apparent, however, that a second aphidicolin-sensitive polymerase, δ, is also present in abundance in mammalian cells [16, 17]. Like replication and UV-induced repair synthesis in permeable human cells, DNA polymerase δ is several hundred-fold less sensitive to BuPh-dGTP than polymerase α [16–18]. We have also found recently that replication and UV-induced repair synthesis in human cells, like polymerase δ [19], are 5- to 10-fold more sensitive to dideoxythymidine triphosphate than is polymerase α [10]. Thus, the relative resistance of replication and UV-induced repair synthesis in butylphenyl-guanine derivatives may result from involvement of DNA polymerase δ in these processes.

The suggestion that DNA polymerase δ is involved in DNA replication seems to contradict a large body of literature which indicates that polymerase α is the replicative polymerase. Included in this previous work are studies of both intact cells [20–23] and subcellular systems [24–28]. In general, these studies were not designed to differentiate between polymerases α and δ in replication. Much of the previous work implicating polymerase α in replication has used inhibitors such as aphidicolin, *N*-ethylmaleimide, and arabinosyl nucleotides. Sensitivity to all three of these agents was thought to be a unique characteristic of DNA polymerase α [reviewed in Ref. 29]. It is now known, however, that polymerase δ is sensitive to all three of these agents as well [reviewed in Refs. 30 and 31]. Particularly compelling evidence supporting the involvement of polymerase α in replication comes from the demonstration that two monoclonal antibodies (SJK 287–38 and SJK 132–20) directed against polymerase α [14] inhibit DNA replication when added to the reaction mixture of permeable human fibroblasts ([32, 33]; Dresler and Frattini, unpublished) or when microinjected into the nuclei of intact human, hamster, and mouse cells [34]. Interestingly, complete inhibition of replication by either anti-polymerase α antibody has not been achieved in these studies; one group reports maximal inhibition of 60–70% [32–34], while a second group found maximal inhibition of 40–50% (Frattini and Dresler, unpublished). A further complication is introduced by the possibility that the anti-polymerase α antibodies used may not discriminate absolutely between DNA polymerases α and δ. Some authors [16, 35] report that the SJK 287–38 and SJK 132–20 antibodies do not inhibit polymerase, but Wahl *et al.* [19] found that both antibodies do inhibit one of the two forms of polymerase δ which they isolated from calf thymus. The results of anti-polymerase antibody experiments, therefore, must be regarded as ambiguous. These experiments do raise the possibility, however, that polymerases α and δ may both be involved in DNA replication.

An alternate explanation for the data presented here is the possibility that replication and UV-

induced repair synthesis in human cells are entirely mediated by DNA polymerase α and that, in the cells, the polymerase behaves differently than it does as an isolated enzyme. There is precedent for such an alteration in the characteristics of a DNA polymerase. The K_m for dTTP of bateriophage T4 DNA polymerase changes dramatically, for example, when it associates with accessory proteins to form a replication complex [36]. Thus, it is possible that the BuPh-dGTP sensitivities of DNA replication and repair synthesis differ from that of polymerase α as a result of the cellular environment of the enzyme and not because of the involvement of a different DNA polymerase.

Finally, we should consider the possibility that DNA polymerases α and δ are not totally distinct, but may be derived, either by proteolysis or by some other mechanism, from a common primary gene product. Wahl *et al.* [19] found two forms of polymerase δ in calf thymus, one of which, δ_1, had the properties of DNA polymerase α with a loosely-associated nuclease, while the other, δ_2, had the properties of polymerase α with a tightly-associated or covalently-attached nuclease. These results suggest that isolated forms of DNA polymerases α and δ may be derived from a single native cellular polymerase which mediates replication and UV-induced repair synthesis *in vivo*. This possibility would explain the fact that these processes have some characteristics (e.g. anti-polymerase α antibody sensitivity) which are similar to DNA polymerase α and some characteristics (e.g. BuPh-dGTP and 2′,3′-dideoxythymidine-5′-triphosphate sensitivities) which are similar to DNA polymerase δ, but have inhibition curves (see Fig. 1) which suggest the involvement of a single DNA polymerase. The data presented here do not enable us to identify definitely the DNA polymerase involved in mammalian DNA replication, but they do indicate the desirability of reevaluating the concept that DNA polymerase α alone serves as the replicative polymerase in mammalian cells.

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