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Butylanilinouracil: a selective inhibitor of HeLa cell DNA synthesis and HeLa cell DNA polymerase alpha

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ABSTRACT

A series of 6-anilinouracils, dGTP analogues which selectively inhibit specific bacterial DNA polymerases, were examined for their capacity to inhibit purified DNA polymerases from HeLa cells. The p-n-butyl derivative (BuAU) was found to inhibit DNA polymerase α with a K_i of approximately 60 μM. The inhibitory effect of BuAU was reversed specifically by dGTP and was observed only for DNA polymerase α; polymerases β and γ were not inhibited by drug at concentrations as high as 1 mM. BuAU also was inhibitory in vivo in HeLa cell culture; at 100 μM it reversibly inhibited cell division and selectively depressed DNA synthesis. The results of these studies indicate that BuAU is an inhibitor with considerable potential as a specific probe with which to dissect the structure of mammalian polymerase α and its putative role in cellular DNA replication.

INTRODUCTION

6-(Arylhydrazino)- and 6-(arylamino)-uracils, exemplified by 6-(p-hydroxyphenylhydrazino)uracil (H_2-HPUra), are selective inhibitors of DNA replication of Gram-positive bacteria (1). These inhibitors are dGTP analogs whose target in susceptible organisms is the replication-specific enzyme, DNA polymerase III (pol III) (2,3). The inhibitor mechanism, which has been elucidated with B.subtilis pol III (4,5), is summarized schematically below in Figure 1, using a 6-anilinouracil as a model drug. The mechanism involves: (i) the specific pairing of substituents of the uracil moiety with template cytosine, and (ii) the binding of the 6-aryl group and its substituents to the polymerase, sequestering the latter in a relatively stable protein:drug:DNA complex.

Although the active sites of all DNA polymerases might be expected to accommodate the uracil inhibitors as dGTP analogs, only a narrow spectrum of enzymes - the type III polymerases of Gram-positive bacteria - is susceptible to them (6). The Gram-positive pol III is susceptible because it possesses - at a critical location near the active site - a unique aryl site which strongly binds the 6-aryl moiety (7). We have hypothesized (7)

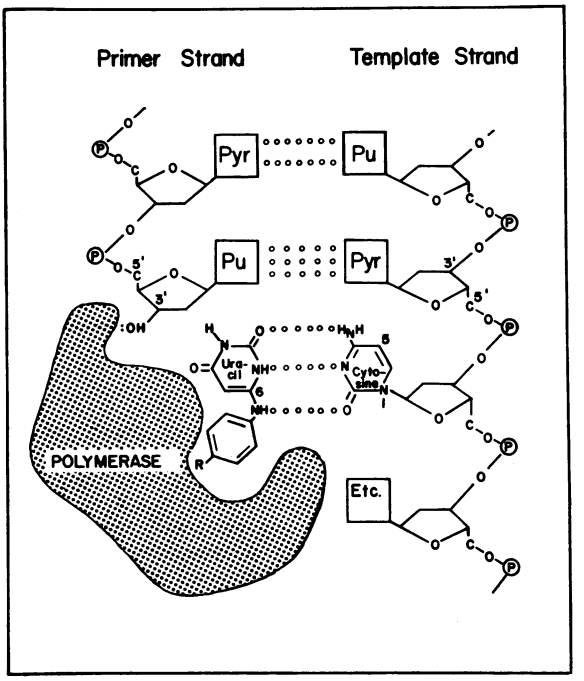

Figure 1 Mechanism of DNA Polymerase inhibition by 6-substituted aminouracils.

that DNA polymerases naturally resistant to the conventional, pol III-specific inhibitors are likely to contain binding sites accessible to chemically modified drug derivatives; to test this hypothesis we have initiated a systematic analysis of the effect of a large number of substituted 6-anilinouracils on the activity of several DNA polymerases of biologic interest. We report here the analysis of inhibitor effects on DNA polymerases derived from the human, HeLa cell line (8). Specifically, this paper reports the identification of 6-(p-n-butylanilino)uracil (BuAU) as a specific inhibitor of HeLa DNA polymerase α and its characterization *in vivo* as a selective inhibitor of HeLa cell division and DNA synthesis.

METHODS AND MATERIALS

Growth and Synchronization of Cells. HeLa S_3 cells were grown in suspension culture at a density of 3 to 6×10^5 cells/ml in F-13 medium containing 3.5% each of irradiated calf serum and irradiated fetal bovine

serum (9). The cultures were judged free from mycoplasma contamination via two methods of testing - the standard agar plate growth procedure under anaerobic conditions and fluorescent staining (Bioassay Systems Inc., Cambridge, MA). Cell counts were taken by means of a hemocytometer or a Royco cell counter. Cells were synchronized by the double hydroxurea block technique (10) using 0.5mM hydroxurea at each block and an initial cell density of 2 to $3x10^5$ cells/ml.

Materials. Radioactive materials were purchased from New England Nuclear. 6-(p-Alkylanilino)uracils (Wright and Brown, in press) were prepared by the method described by Brown et.al. (11). Aphidicolin was generously provided by Dr. B. Hesp, Imperial Chemical Industries Ltd.

Enzymes. HeLa cell DNA pol α and β were purified from synchronously dividing cells according to published procedures (9,12). B.subtilis pol III was purified by the antibody:agarose method of Barnes and Brown (6); B.subtilis pol II was a DEAE-cellulose side fraction produced in pol III purification (6). E.coli pol III was provided by Dr. Charles McHenry, Avian Myeloblastosis Virus (AMV) reverse transcriptase by Dr. Joseph Beard, and HeLa polymerase γ by Dr. Arthur Weissbach. E.coli pol I was purchased from P-L Laboratories. Slime mold DNA polymerase was prepared from an axenic strain (A-3) of Dictyostelium discoideum and was purified through the DNA-cellulose chromatography stage (Baril, E. and Pederson, T., in preparation).

DNA Polymerase Assay. All enzymes were assayed in the conditions employed by Clements et al. (4) for the assay of B.subtilis pol III, and unless specified otherwise, the assay employed [^{3}H]TTP and DNAase-activated calf thymus DNA as template-primer. The apparent inhibitor constants (K_i) for BuAU and related compounds were determined by the truncated, dGTP-deficient DNA polymerase assay described by Wright and Brown (13) in which [^{3}H]TMP incorporation is linear throughout the assay period.

DNA, RNA, and Protein Assay. DNA, RNA and protein of cold perchloric acid insoluble material were assayed as described previously (14).

RESULTS

The n-butyl group as a determinant of inhibitor potency. BuAU was one of several 6-(p-alkylanilino)uracils found to inhibit HeLa pol α, and of the six derivatives tested, it was the most potent, with an apparent inhibitor constant (K_i) of 50-60 μM. In the B.subtilis pol III system BuAU was the least potent of this series, with a K_i of ca. 5000 μM (Wright and Brown,

in press). The data of Figure 2 summarize the relationship between the size of the alkyl (R) group and log K_i for both HeLa pol α and B.subtilis pol III. Group size and log K_i are nearly linearly related for both enzymes, although the dependence was opposite; the binding site of pol α was clearly able to accommodate larger hydrophobic substituents than the binding site of pol III.

Mechanism of BuAU action. In surveying the drug susceptibility of selected DNA polymerases we have found several 6-substituted uracils which have inhibited enzymes atypically, in a manner not influenced by either dGTP or template composition. The inhibitory effect of BuAU on pol α was entirely conventional, although the maximal inhibition at high drug concentration was ca.80% (see DISCUSSION); its action was reversed by dGTP and was dependent on the presence of a template containing cytosine. The competitive relationship of dGTP and BuAU is shown in Fig. 3. The results clearly indicated that dGTP in sufficient concentration completely prevented the inhibitory action of the drug. The competitive effect was entirely specific for dGTP; experiments (results not shown) examining the effects of dATP, dCTP and dTTP indicated that alteration of the concentrations of these

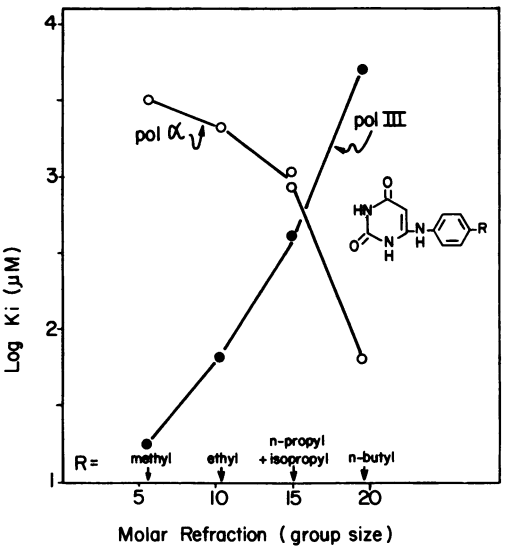

Figure 2 Relationship between the size of para alkyl substituents and inhibition potency for inhibition of HeLa pol α and B.subtilis pol III by 6-anilinouracils. Molar refraction values were obtained from the compilation of Hansch, et al. [(1973) J. Med. Chem. 16, 1207-1216]. K_i values were obtained by assay of the polymerases in the absence of dGTP (truncated assay).

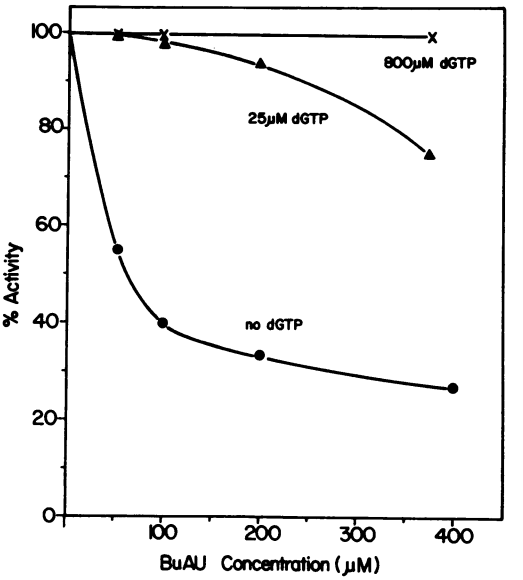

Figure 3 Reversal of BuAU action by dGTP. Pol α was assayed as described in the methods section in the presence of several concentrations of BuAU and in the absence and presence of dGTP at the indicated concentrations; 100% activity in the absence of dGTP and in the presence of 25 and 800 μM dGTP were represented by the incorporation of 10, 36 and 36 pmol of [^{3}H]TMP, respectively.

nucleotides had no effect on BuAU potency. Another experiment, designed to examine the influence of template on BuAU potency, compared the effect of drug in the presence of the usual calf thymus template:primer with its effect in the presence of poly (d)A:oligo (d)T_9 a template:primer which is well utilized by HeLa pol α (15). The results, which are not tabulated, indicated that BuAU at 400 μM, a concentration which inhibited TMP incorporation into calf thymus DNA by 80%, had absolutely no effect on its incorporation in poly (d)A-directed extension of oligo(d)T.

Enzyme specificity. In addition to HeLa pol α and B.subtilis pol III, BuAU was screened via the truncated assay (minus dGTP; see METHODS) against HeLa pol β and pol γ, E.coli pol III, AMV reverse transcriptase, B.subtilis pol II, E.coli pol I, and the α-type DNA polymerase from slime mold (Table I).

With the exception of E.coli pol III, these enzymes were immune to inhibition

TABLE I. ENZYME SPECIFICITY OF BuAU

ENZYME	pmoles [^{3}H-TMP] incorporated	
	Control	+mM BuAU
HeLa Cell:		
pol α	13.9	2.9
pol β	10.9	11.7
pol γ	1.6	1.7
Dictyostelium discoideum:		
pol A	12.7	13.1
AMV Reverse Transcriptase:	24.8	23.5
E.coli:		
pol I	5.7	5.0
pol III	9.0	0.3
B.subtilis		
pol II	8.9	8.2
pol III	7.9	6.2

All of the enzymes were assayed with activated DNA by the truncated, dGTP-deficient DNA polymerase assay (13). The assay conditions for HeLa cell DNA polymerases were as described by Novak and Baril (12). Dictyostelium discoideum DNA polymerase was assayed under similar conditions (12). E.coli pol I and III, B.subtilis pol II and III were assayed in the conditions employed by Clements, et.al. (4). AMV reverse transcriptase was assayed similarly, but with dATP and dCTP at 80 μM and TTP at 20 μM.

by BuAU at a concentration of 1 mM. (The inhibitory effect of BuAU on E.coli pol III was atypical and unaffected by the concentration of dGTP and, therefore, it was not investigated further.) Calf thymus pol β and rabbit intestinal epithelial cell pol γ have also been found to be unaffected by BuAU at 100 μM (Dr. Barbara Żmudzka, personal communication).

Effect of BuAU on cell division and morphology. The effects of BuAU on cell division are demonstrated by the experiment of Figure 4(A). The drug, in a range of concentration effective on pol α in vitro, strongly inhibited the increase of cell number in non-synchronous HeLa cell cultures. The inhibitory effect was clearly dependent on BuAU concentration. After 18 hours' exposure, inhibition was essentially complete at 100 μM. Increasing the concentration of BuAU to 200 μM reduced only slightly the lag in inhibition of division seen at 100 μM. BuAU at 400 μM, the maximal concentration attainable in the culture medium, effected a slight loss of cells which was apparent at 18 hours. Microscopic examination of the latter culture 24 hours after BuAU addition (results not shown) revealed a considerable number of enlarged and vacuolated cells, a change which was not noted in identical cultures exposed to BuAU at concentrations less then 200 μM. We have not investigated the possible reversal of these cytopathic

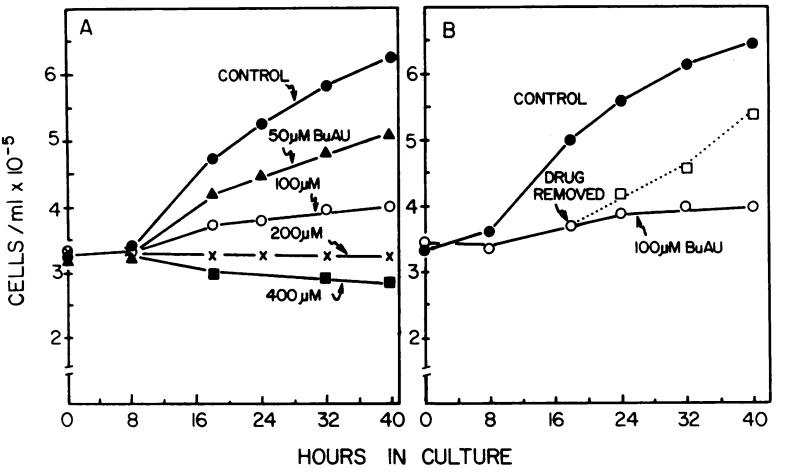

Figure 4 Reversible, BuAU-induced inhibition of HeLa cell division. (A) Identical cultures, seeded as described in methods, were exposed to drug diluent (control) or BuAU at the indicated concentrations, and at intervals each was assayed for cell number. (B) Cultures identical to those of 4(A) were incubated in the presence of drug diluent (control) or 100 μM BuAU. At 18 hours' incubation a portion of drug-treated cells was freed of BuAU by low speed centrifugation at 37°, resuspended in the same volume of fresh, warm medium, and re-incubated. Cell number was assayed as in (A).

changes by, for example, the addition of deoxyguanosine to the medium.

Reversibility of in vivo effect. The experiment of Figure 4(B) indicated that the effect of BuAU on cell division, under appropriate culture conditions, was reversible. When cells which were treated with 100 μM BuAU for 18 hours were washed and incubated with fresh medium, inhibition was relieved, and cell number increased at a rate commensurate with that observed for untreated cultures. Related experiments, the results of which are not shown, indicated that the capacity for reversal was inversely related to drug concentration. In cultures exposed for 18 hours to 200 μM BuAU, cell number increased little if at all in a 24 hour period following drug removal. In cultures exposed to 400 μM BuAU the removal of drug neither reversed nor perceptibly affected the loss of cells observed when the drug was present.

Effect of BuAU on the synthesis of cellular macromolecules. The cells used in these experiments were derived from cultures synchronized by exposure to double hydroxyurea block (see Methods section). Figure 5(A) summarizes the behavior of a released culture with respect to its capacity to incorporate [^{3}H]TdR in the absence and presence of BuAU. In the presence of BuAU at

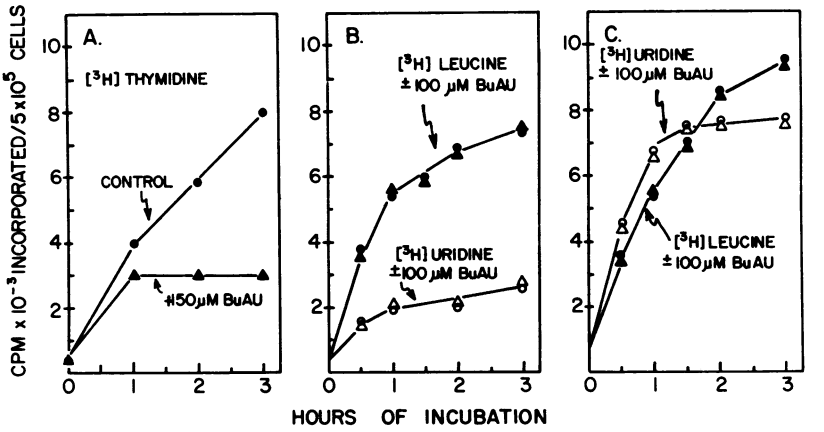

Figure 5 Effect of BuAU on the synthesis of macromolecules by randomly growing cells and cells synchronized by treatment with hydroxyurea. (A). DNA synthesis by synchronized cells. Cells were freed of hydroxyurea by low speed centrifugation at 37° by two washes with warm, fresh medium. The cells were divided into identical portions. One portion received drug diluent (control), and the other received BuAU at a concentration of 150 μM. Simultaneous with drug [^{3}H]TdR (sp. act., 56 Ci/mmol.) was added at a concentration of 1 μc/ml: incubation was continued, and at the indicated intervals, samples were removed for determination of incorporation of radioactivity into DNA as described in methods. (B). Protein and RNA synthesis in synchronized cells. Portions of the suspension of cells used in 5(A) were incubated in the presence of either [^{3}H] uridine (sp. act., 30 Ci/mmol; 1 μc/ml) or [^{3}H] leucine (sp. act., 64 Ci/mmol; 1 μc/ml) in the absence and presence of BuAU at 100 μM. Samples were removed for determination of radioactivity in RNA and protein. (C). Protein and RNA synthesis in non-synchronous growing cells. Cultures containing non-synchronized cells at a density of $3x10^5$ cells/ml were exposed to [^{3}H] uridine or [^{3}H] leucine and incubated with or without BuAU and analysed for incorporation of isotope into RNA and protein as in the experiment of 5(B).

50, 100, and 150 μ M, ^{3}H-TdR incorporation continued essentially unabated for 1 hour and then rapidly ceased at all drug concentrations. [^{3}H]TdR incorporated after 3 hrs in the presence of 50, 100 and 150μM BuAU was 49%, 43% and 36%, respectively, of that observed for the control culture.

As part of the experiment examining [^{3}H]TdR incorporation, identical cultures released from hydroxyurea block were assayed in the absence and presence of BuAU for their capacity to incorporate [^{3}H]leucine into protein and [^{3}H]uridine into RNA (Fig. 5(B)). BuAU at 100 μM, a concentration which profoundly inhibited [^{3}H]TdR incorporation, had no significant effect on incorporation of either labeled precursor. BuAU at

100 μM also had no discernable effect on leucine and uridine incorporation by rapidly growing, asynchronous cells (Fig. 5(C)) in which the processes of RNA and protein synthesis were considerably more vigorous and longer lived than those apparent in cells released from hydroxyurea block. These results were also consistent with results of experiments involving direct determination of DNA, RNA and protein in cultures of synchronized cells following the release from hydroxyurea block (data not shown). BuAU at 100μM had no significant effect on the increase of RNA and protein, whereas it had a clear inhibitory effect (over 90%) on the synthesis of DNA.

DISCUSSION

The identification of BuAU as a dGTP-specific inhibitor of HeLa pol α (Fig. 3), identical in mechanism to that of the pol III-specific 6-anilinouracils (Fig. 1), supports the hypothesis that a wide spectrum of DNA polymerases possess sites near their nucleotide binding sites which are susceptible to an appropriately constructed 6-aryl moiety. The results of comparison of the BuAU susceptibility with that of several other polymerases, in particular B.subtilis pol III (Fig. 2), also indicate that chemical manipulation of the aryl moiety of 6-anilinouracils can produce inhibitors with high selectivity for a particular polymerase - a selectivity which endows an inhibitor with considerable potential as a site directed specific probe of enzyme structure and function in vivo and in vitro.

We have not yet established the size and composition of the aryl substituent(s) required for optimal activity of the 6-anilino derivatives. We believe that the considerable tolerance of the aryl site of pol α (Fig. 2) for bulk should allow chemical tailoring and the production of a pol α inhibitor considerably more potent than BuAU; accordingly, we have initiated a wider study of structure-activity relationships involving the aryl moiety.

Purification of mammalian pol α yields several species of the enzyme which differ in apparent molecular weight, net charge (15) and behavior during DEAE-cellulose chromatography (16-17). Holmes et al. (16), using DEAE-cellulose, have demonstrated 3 major enzyme forms - form I - a complex, high molecular weight species - and two species generated by form I - form II and form III; the latter is a 150 kilodalton species considered to be the "core" form of pol α (16-17).

During the course of the above work with BuAU we prepared the three forms of pol α and compared them (experiments not described in RESULTS) with respect to their sensitivity to BuAU and aphidicolin, another pol α- specific

inhibitor of unknown mechanism (18-19). Although the three forms were equisensitive to aphidicolin (inhibition 80-85% at 2.5 μM), the sensitivity of each to BuAU differed significantly. Form III, the putative core form which was used in the experiment of Fig. 2 was clearly the most sensitive, inhibited 80% by 800 μM BuAU in the absence of dGTP. In the same conditions form II was entirely resistant to 800 μM BuAU and form I was only partially sensitive, inhibited by approximately 50%. We do not yet know why the form of pol α has such a profound effect on the susceptibility of the enzyme to BuAU. The behavior is likely a function of the conformation of the BuAU binding site - a conformation which must be affected profoundly by the state of aggregation of the enzyme and/or the reaction of its core with other chromatographically distinct "coproteins"; we are currently investigating both possibilities.

We have not yet established with certainty whether the susceptibility of HeLa pol α to BuAU represents a unique property of the HeLa enzyme per se or a characteristic of a broader spectrum of α class polymerases; the recent finding by Frenkel (Dr. Gerald Frenkel, personal communication) that pol α of KB cells is inhibited in a manner entirely comparable to that displayed by HeLa pol α suggests that BuAU sensitivity is at least characteristic of human alpha polymerase.

BuAU in vivo, in HeLa cell culture, inhibits reversibly (Fig. 4) cell division in a range of concentration effective in vitro on HeLa pol α . The data of the experiment examining incorporation of isotopes into RNA, protein, and DNA (Fig. 5) and experiments measuring RNA, DNA and protein directly (data not shown) strongly suggest that the effect of BuAU on cell division is derived primarily from selective inhibition of replicative DNA synthesis. The effect of BuAU on DNA synthesis, in view of: (1) the specificity of the drug for pol α in vitro and (2) the growing body of circumstantial evidence (9,20-23) that pol α is a replication-specific enzyme, suggests that pol α is the ultimate site of cytotoxic drug action in intact cells. The inhibitory effects of BuAU in vivo, particularly its effects at concentrations higher than 200μM, may not be related to a primary effect on DNA synthesis or specific inhibition of pol α; cytotoxicity could result from drug action at sites not yet apparent. The resolution of pol α as the putative target of BuAU in animal cells, like the resolution of pol III as the target of HPUra action in Gram-positive bacteria (3), will ultimately require the isolation of BuAU-resistant, mutant cell clones which specifically carry a BuAU-resistant pol α. Experiments designed to isolate such mutants are in progress.

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