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EFFECTS OF DDMP AND DDEP ON THE DEOXYRIBONUCLEOSIDE TRIPHOSPHATE CONCENTRATIONS IN HUMAN CELLS

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Abstract—Both DDMP and DDEP were found to lower the free deoxythymidine triphosphate concentration and raise the free deoxyadenosine triphosphate concentrations and uptake of tritiated thymidine into DNA in normal human phytohaemagglutinin-stimulated lymphocytes. With both drugs, these effects could be detected after as little as 15 min incubation and with DDMP at concentrations as low as 10^{-8} M and the effects could be reversed by the reduced folates 5-methyltetrahydrofolate or 5-formyltetrahydrofolate. The effects of these drugs closely paralleled those of methotrexate and show that both drugs are powerful inhibitors of dihydrofolate reductase in normal human cells.

DDMP (2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine) and DDEP (2,4-diamino-5-(3',4'-dichlorophenyl)-6-ethylpyrimidine are two inhibitors of dihydrofolate reductase which have been shown to inhibit growth of tumours in animals and in tissue culture, some of which were resistant to methotrexate [1–5]. Recent studies have shown that it is possible to quantitate the effects of dihydrofolate reductase inhibitors on DNA synthesis in human cells by measuring the effects of the drugs on the free concentrations of deoxythymidine triphosphate (dTTP) and deoxyadenosine triphosphate (dATP) [6–8]. The present study compares the effects of DDMP and DDEP with those of methotrexate on uptake of labelled thymidine and on the dTTP and dATP pools in normal human phytohaemagglutinin-transformed lymphocytes.

Chemicals. Reagent chemicals were supplied by British Drug Houses and by Sigma Chemicals. dATP and dTTP were purchased from Sigma Chemicals and *Micrococcus luteus* DNA polymerase and polyd(A-T) were purchased from Miles Laboratories. [^{3}H]dATP (14 Ci/m-mole), [^{3}H]TTP (23.7 Ci/m-mole) and [^{3}H]methyl thymidine (5 Ci/m-mole) were purchased from the Radiochemical Centre, Amersham, England. Medium TC199, single strength, and phytohaemagglutinin (PHA) were purchased from Wellcome Reagents and Ficoll and Triosil from Parmacia Fine Chemicals, and Nyegaard respectively. DDMP and DDEP were a generous gift from the Wellcome Foundation Limited.

Cell culture. Venous blood was taken into heparin from normal adult volunteers. The methods used for collecting human lymphocytes and conditions for lymphocyte culture have been described before [8, 9]. 3×10^6 lymphocytes were cultured in 3.0 ml volumes consisting of 20% autologous serum in single strength TC199 medium with 100 μl PHA for 72 hr at 37° in 7.5% CO_2.

To study the effect of DDMP and DDEP, the drugs were added to 10 μl saline (with 5% alcohol) to pooled cultures and incubated for specific time intervals at 37°. An equal number of cells which received an equivalent volume of saline (with 5% alcohol) served as controls. [^{3}H]methylthymidine (^{3}H-TdR) incorporation into DNA was studied by adding 1 μCi (0.2 μmole) in 100 μl saline to triplicate cultures each containing 3×10^6 PHA-transformed cells and incubating for 1 hr at 37°. The cells were then washed twice in cold phosphate-buffered saline, pH 7.4, and allowed to stand for 10 min at 4° in 2.0 ml cold, 0.5 M perchloric acid. After centrifugation, the pellet was hydrolysed in 0.5 ml, 0.5 M perchloric acid at 85° for 20 min. The suspension was spun and 100-μl aliquots of the supernatant were counted for radioactivity after the addition of 10 ml liquid scintillation fluid.

dATP and dTTP extraction and assay. Following drug incubation, the cells were spun at 4° and washed twice in cold phosphate-buffered saline, pH 7.4. Cold, 60% methanol, (1.0 ml) was added to the cell pellet, and the suspension was stored at −20° overnight to extract the nucleotides [10]. The supernatant after centrifugation was collected, freeze-dried and the dried extract was resuspended in 350 μl water and stored at −20° until assayed.

The assays of dATP and dTTP have been described [11, 12]. Poly-d (A-T) (0.05 units) was used as template with 0.3 units DNA polymerase. Assays were incubated in triplicate at 37° for 35 min. The results are expressed as pmole/10^6 cells or as per cent of control cultures with no added drugs.

Effects of DDMP and DDEP on dTTP and dATP pools and [^{3}H]thymidine incorporation into DNA

(a) *DDMP* The effects of incubating DDMP 10^{-5} M with normal 72-hr PHA-stimulated lymphocytes for 1 hr on the dATP and dTTP concentrations and [^{3}H]TdR incorporation into DNA are shown in Fig. 1. DDMP cause a rise in dATP concentration in 11 of 12 experiments, to a maximum of 53 per cent of control level. A decrease in the dTTP concentration occurred in all 12 experiments and varied from 30 to 79 per cent of control levels. The mean

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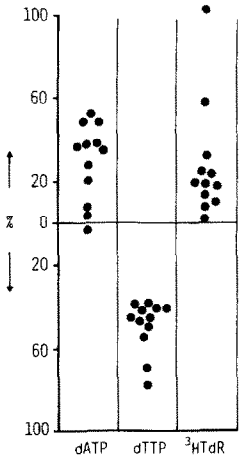

Fig. 1. Effect of DDMP (10^{-5}M) at 1 hr on the dATP and dTTP concentrations and [^{3}H]TdR incorporation into DNA in normal human 72-hr PHA-stimulated lymphocytes. Results are given as percent rise or fall compared with control cells. The dATP concentration (pmole/10^6 cells) ranged from 1.7 to 5.4 (mean 3.5) and in drug-treated from 2.8 to 7.3 (mean 4.4) while the dTTP concentration (pmole/10^6 cells) in controls ranged from 7.0 to 17.5 (mean 10.9) and in drug-treated from 2.3 to 9.0 (mean 5.4).

dTTP concentration at 1 hr with 10^{-5}M DDMP was 50 per cent of the mean dTTP concentration in control cultures and the mean dATP concentration was 125 percent of mean control dATP concentration.

An increase in [^{3}H]TdR incorporation into DNA at 1 hr with 10^{-5}M DDMP occurred in all experiments, the mean increase being 131 per cent of control levels (Fig. 1).

(b) *DDEP* Figure 2 shows the effect of 10^{-5}M DDEP for 1 hr on the dATP and dTTP concentrations and on [^{3}H]TdR incorporation into DNA, in normal 72-hr, PHA-stimulated lymphocytes. There were consistent decreases in the dTTP concentration and increases in [^{3}H]TdR incorporation into DNA.

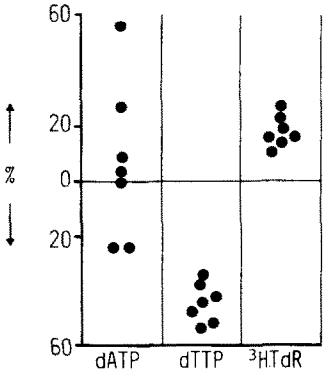

Fig. 2. Effect of DDEP (10^{-5}M) at 1 hr on the dATP and dTTP concentrations and [^{3}H]TdR incorporation into DNA in normal human 72-hr PHA-stimulated lymphocytes. Results are given as per cent rise or fall compared with control cells. The dATP concentration (pmole/10^6 cells) in controls ranged from 1.7 to 3.3 (mean 2.5) and in drug-treated from 2.0 to 3.9 (mean 2.8) while the dTTP concentration (pmole/10^6 cells) in controls ranged from 8.9 to 11.5 (mean 13.0) and in drug-treated from 4.5 to 9.0 (mean 6.1).

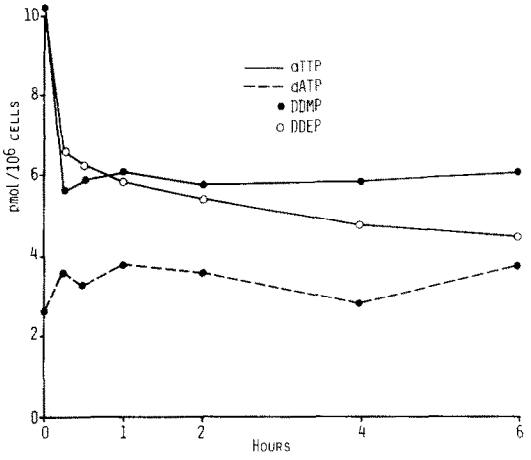

Fig. 3. Time course of action of DDMP (10^{-5}M) on the dATP and dTTP concentrations in normal human 72-hr PHA-stimulated lymphocytes and of DDEP (10^{-5}M) on dTTP concentration.

Although there was on overall mean increase in the dATP concentration of 12 per cent, this increase was not consistent. The mean decrease in dTTP concentration was 47 per cent with a mean increase in [^{3}H]TdR incorporation of 14 per cent of control values.

Effects of time and concentration on the actions of DDMP and DDEP. DDMP 10^{-5}M produced unbalanced dATP and dTTP concentrations in as short a period as 15 min (Fig. 3). After this initial change in dATP and dTTP concentrations, further incubation up to 6 hr with DDMP did not markedly alter their concentrations. DDEP 10^{-5}M also caused a fall in dTTP concentration after as little as 15 min incubation and there was a further decrease in the dTTP concentration from 35 per cent of control value at 15 min to 56 per cent at 6 hr (Fig. 3).

The effects of DDMP and methotrexate on dTTP and dATP concentrations and on [^{3}H]TdR incorporation into DNA are compared in Fig. 4. With both drugs, a rise in [^{3}H]TdR uptake could be detected

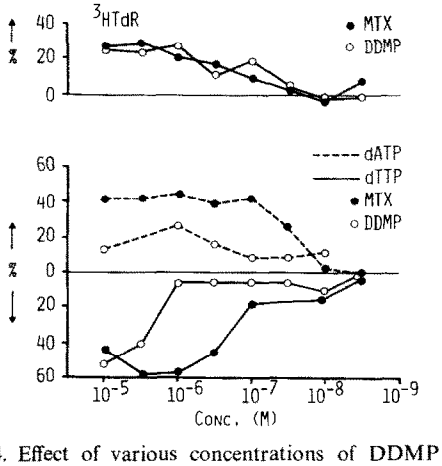

Fig. 4. Effect of various concentrations of DDMP and methotrexate on the dATP and dTTP concentrations and on [^{3}H]TdR incorporation into DNA in normal human 72-hr PHA-stimulated lymphocytes. Results are given as per cent rise or fall compared with control cells.

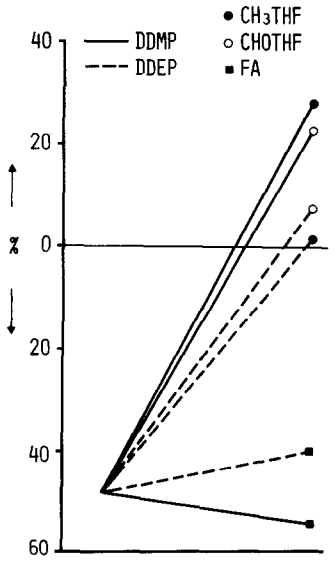

Fig. 5. The effect of 5-methyltetrahydrofolate (CH_3THF), 5-formyltetrahydrofolate (CHOTHF) and folic acid (FA) at 10^{-3} M on dTTP concentrations of DDMP (10^{-5} M) or DDEP (10^{-5} M) treated normal human 72-hr PHA-stimulated lymphocytes. DDMP or DDEP incubations were carried out for 1 hr and the folate compounds were added 15 min before DDMP or DDEP.

at 0.5×10^{-7}M while a rise in dATP and fall in dTTP cold be detected at 10^{-8}M. The effects of methotrexate on the pools were, however, more marked over the concentration range 10^{-5}M to 10^{-7}M.

Effect of folate protection. Figure 5 shows the rescue effect in normal 72-hr PHA-lymphocytes, in terms of reversal of the decrease in dTTP concentrations, after incubation of DDMP 10^{-5}M or DDEP 10^{-5}M with either formyltetrahydrofolate (CHO THF) methyltetrahydrofolate (CH_3 THF) or pteroylglutamic acid (FA), all at 10^{-3}M. The reduced folates reversed the decrease in dTTP concentrations caused by either DDMP and DDEP, whereas FA had no effect. There was no obvious difference in the efficiency of rescue between the formyl and methyl reduced folates.

The results of these studies show that DDMP and DDEP have similar actions to methotrexate, pyrimethamine and to a much lesser degree trimethoprim in lowering the dTTP concentration, raising the dATP concentration and increasing tritiated thymidine uptake in normal human PHA-transformed lymphocytes [8]. The results have shown that the DDMP effect is, like that of methotrexate, manifest after as little as 15 min incubation. This observation is consistent with the finding of Hill *et al*[5] that entry of DDMP into L5178Y cells occurs rapidly with early association of the drug (between 1 and 5 min), a plateau intracellular concentration being reached after 15–30 min incubation.

In view of the generally weaker inhibition of dihydrofolate reductase extracted from neoplastic cells by DDMP than by methotrexate[4], it was surprising to find that, assessed by all three parameters of fall in dTTP, rise in dATP and increased in [^{3}H]thymidine uptake, DDMP appeared to be as active as methotrexate over a wide range of concentrations from 10^{-8}M to 10^{-3}M. As expected both fully reduced folates, 5-formyltetrahydrofolate (folinic acid) and 5-methyltetrahydrofolate reversed all three effects of both DDMP and DDEP whereas folic acid had no effect.

Taken together with the observations that DDMP may be active against methotrexate resistant tumour cells [4, 5] and that uptake of DDMP may occur into methotrexate impermeable/resistant L5178Y lymphoblasts sufficient to kill these cells[5], the present results suggest that DDMP (and DDEP) have potential value as antitumour agents in man and, indeed, clinical trials with DDMP to assess its usefulness are now in progress [13]. However, since the studies here show that both DDMP and DDEP are powerful inhibitors of dihydrofolate reductase in normal human cells, toxic effects of these drugs, similar to those with methotrexate are to be expected.

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