



**Report Title: The Mechanism of Cell Growth Inhibition By Octopirox**

**Test Type:** Genotoxicity Study

**Conducting Laboratory and Location:** Sherbrooke University, Sherbrooke (Quebec, Canada)

**Test Substance(s):** G0539 – Octopirox in ethanol

**Species:** Mouse L1210 and normal human AG1565 fibroblasts

**Test Conditions:** L1210 mouse cells: Looked at effect of OP on dATP and dTTP concentrations and <sup>3</sup>H-thymidine incorporation. Cells treated for 2 hr.

AG1518 human fibroblasts: Looked at effects of OP on DNA synthesis and UV-induced repair

**Results:** Octopirox is a potent inhibitor of mouse L1210 cell growth and DNA replicative synthesis in both mouse L1210 cells and normal human fibroblasts, as measured by [<sup>3</sup>H] thymidine incorporation into DNA. This inhibition correlates well with decrease in dATP and dTTP concentrations. Octopirox has no direct effect on enzymes involved in DNA replication. Investigator states these data are consistent with hypothesis that the cytotoxic effects of OP result from the specific inhibition of ribonucleotide reductase.

**Study #:** Notebook G0539

**Report Date:** 4/27/92

## THE MECHANISM OF CELL GROWTH INHIBITION BY OCTOPIROX

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Laboratory Assistants: Bonnie Gowans  
Date Experiments Performed: 5/18/90 to 2/28/91  
Notebook: G0539  
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### SUMMARY

Octopirox is a potent inhibitor of mouse L1210 cell growth and DNA synthesis in both mouse L1210 and normal human fibroblasts (AG1518). The inhibition of DNA replicative synthesis correlates well with a reduction of deoxyadenosine triphosphate (dATP) concentrations while deoxythymidine triphosphate (dTTP) concentrations increase under the same conditions. These effects suggest that octopirox inhibits ribonucleotide reductase. Further experiments demonstrated that octopirox had no inhibiting effects on DNA replicative synthesis when permeabilized cells were supplied exogenous deoxynucleoside triphosphates, thereby bypassing ribonucleotide reductase. Thus, the data are consistent with the hypothesis that the cytotoxic effects of octopirox are due to the specific inhibition of ribonucleotide reductase.

### INTRODUCTION

The investigational experiments described below were funded by the Procter & Gamble Company and performed in the laboratory of Darel Hunting, Ph.D., Associate Professor, Sherbrooke University, Sherbrooke (Quebec), Canada. Octopirox, coded as G0539, was provided by Dr. Edward Thompson, Procter & Gamble, and was received May 18, 1990.

The purpose of these experiments was to determine the mechanism of cytotoxicity and inhibition of replicative DNA synthesis induced in mammalian cells in culture by octopirox. Because octopirox is a strong chelator of ferric iron and since other iron chelators have been shown to inhibit ribonucleotide reductase (Ganeshaguru et al., 1980), we hypothesize that octopirox inhibits DNA replicative synthesis by inhibiting this enzyme. If so, changes in deoxynucleotide triphosphate pools (dNTP) similar to those caused by iron chelators (Ganeshaguru et al., 1980; Barankiewicz and Cohen, 1987) and hydroxyurea (Snyder, 1984a; Snyder, 1984b) should also be induced by octopirox.

The effects of octopirox on other enzymes involved in replicative DNA synthesis was also examined as described by Hunting and Dresler, 1985.

### METHODS

**CELL CULTURE:** Human diploid fibroblasts (AG1518; Institute for Medical Research) were grown in monolayers in DMEM (GIBCO), supplemented with 5% fetal bovine and 5% newborn bovine serum. Mouse L1210 cells were grown in static suspension cultures in the medium described above.

**DEOXYRIBONUCLEOSIDE TRIPHOSPHATE DETERMINATIONS:** dATP and dTTP concentrations in neutralized perchloric acid extracts of cells were determined using a

modification of the DNA polymerase procedure (Hunting, D. and Henderson, J.F., 1981). The following controls were performed: background incorporation (i.e., in the absence of the limiting deoxyribonucleoside triphosphate) was measured; standards were added to cell extracts to determine if the assay was affected by the extracts; the linearity of the assay as a function of the amount of cell extract was measured; and finally, time courses were performed to determine the time of maximal incorporation. The dNTPS in the cell extracts will dilute the radioactive dNTPS used in the assay, thus reducing both the background and the assay values. These effects were corrected for using two simultaneous equations as described in the reference cited above.

**[<sup>3</sup>H]THYMIDINE INCORPORATION INTO DNA:** Cells were incubated with [<sup>3</sup>H]TdR, washed with PBS, harvested and resuspended in 1% SDS in water. The suspension was briefly sonicated, mixed with 10% TCA in 0.1 M Na<sub>4</sub>Pi, and left on ice 15 min. The DNA precipitate was collected on GF/C glass fiber filters and washed with 10% TCA, followed by 0.1 N HCl and finally, 95% ethanol. The filters were dried and the radioactivity was determined. In experiments with L1210 cells, incorporation was normalized to cell number. For AG1518 cells, the cells were incubated 24 h. with [<sup>14</sup>C]TdR and chased 24 h. in non-radioactive medium, to obtain uniform DNA labeling, prior to determining the rate of [<sup>3</sup>H]TdR incorporation. Thus, <sup>3</sup>H incorporation was normalized to the <sup>14</sup>C, which is proportional to DNA content.

**UV IRRADIATION:** The medium was removed from cultures of AG1518 cells and the cells were exposed to radiation from a G15T8 germicidal lamp with a flux of 2 W/m<sup>2</sup>.

**DNA STRAND BREAK DETERMINATIONS:** A modified version of the alkaline elution method of Kohn et al., 1976, was used, as described previously (Hunting, D. and Gowans, B., 1988). Internal standard cells were irradiated with ionizing radiation from a <sup>60</sup>Co source (gamma cell 200, Atomic Energy of Canada) such that elution rates for each filter could be normalized to the rate of elution of the DNA from the internal standard cells. Numbers of strand breaks are expressed as rad equivalents, by comparison with irradiated standard cells.

**PREPARATION OF PERMEABLE CELLS:** The cells were harvested and washed once in solution A (150 mM sucrose, 80 mM KCl, 35 mM HEPES, pH 7.4, 5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 5 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>). The cells were then resuspended in solution A containing 80 µg/ml lysolethicin and held on ice for 2 min. 20 µl of cell suspension was mixed with 50 µl of solution B (56 mM HEPES, pH 7.4, 40 mM sucrose, 128 mM KCl, 8 mM MgCl<sub>2</sub>, 13 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 1.8 mM CaCl<sub>2</sub>). 30 µl of solution C, containing nucleotides with or without inhibitors (octopirox, aphidicolin, or hydroxyurea) was added such that the final concentrations in the reaction mix were: 5 mM ATP, 3 µM [<sup>32</sup>P]dCTP, 20 µCi/ml, 50 µM each of dATP, dGTP, and dTTP, 35 mM HEPES, pH 7.4, 50 mM sucrose, 80 mM KCl, 5 mM MgCl<sub>2</sub>, 7.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM CaCl<sub>2</sub>. The permeable cells were incubated in the reaction mix 20 min. at 37°C, harvested by centrifugation and the radioactivity in acid-precipitable material was determined.

## RESULTS

Mouse L1210 cells were used in most of the experiments; however, certain experiments were repeated in AG1518 normal, human fibroblasts in order to determine if responses of the mouse tumor cell line were comparable to those of primary normal human cells.

The effect of octopirox on the growth of L1210 is shown in Fig. 1. Duplicate suspension cultures of L1210 cells were treated at 37°C with octopirox dissolved in 95% ethanol or with

ethanol alone, such that the final concentration of ethanol was 1% in all of the cultures. Cell densities were determined with a model ZBI Coulter Counter.

The effect of octopirox on dATP and dTTP concentrations and on  $^3\text{H}$  thymidine incorporation into DNA in mouse L1210 cells is shown in Fig. 2. Duplicate suspension cultures of L1210 cells were treated at 37°C for 2 h. with hydroxyurea (1mM) or with octopirox dissolved in 95% ethanol, or with ethanol alone, such that the final concentration of ethanol was 1% in all the cultures. The cells were then either centrifuged and extracted with PCA, for dATP and dTTP determinations (Methods), or were incubated a further 15 min., with [ $^3\text{H}$ ]thymidine (6.7  $\mu\text{Ci/ml}$ ), then centrifuged and extracted with PCA (Methods). Hydroxyurea is an inhibitor of ribonucleoside diphosphate reductase and served as a positive control. Following treatment with hydroxyurea, dATP, and dTTP concentrations were 9.1% and 143% of control, respectively and [ $^3\text{H}$ ]thymidine incorporation was 0.7% of control. All values were corrected for the small differences in cell number among the cultures. Tables 1 and 2 show octopirox has no effect on DNA replicative synthesis, even at concentrations more than 100-fold higher that required to inhibit cell growth. Aphidicolin, a direct inhibitor of DNA polymerases alpha and delta, was used as a positive control in these experiments.

The effect of octopirox on  $^3\text{H}$ -thymidine incorporation into DNA in growing normal human fibroblasts (AG1518) is shown in Fig. 3. Non-confluent monolayer cultures of cells were incubated 24 h. with [ $^{14}\text{C}$ ]thymidine (15 nCi/ml) and chased 24 h. in fresh medium. The cells were then incubated 2 h. with one of the following drugs: Octopirox, aphidicolin (2.2  $\mu\text{g/ml}$ ), hydroxyurea (10 mM), or ethanol (0.5%). The latter condition served as a control since the final concentration of ethanol in all the octopirox treated cell cultures was 0.5%. Aphidicolin and hydroxyurea are known to inhibit DNA synthesis and served as positive controls. These drugs reduced [ $^3\text{H}$ ]thymidine incorporation into DNA to 15% and 2.5% of control, respectively. [ $^3\text{H}$ ]thymidine incorporation was determined as described in Methods.

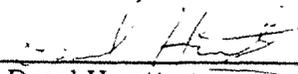
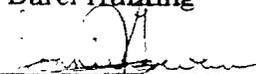
The effect of octopirox on UV-induced [ $^3\text{H}$ ]thymidine incorporation into DNA in confluent, non-replicating, normal human fibroblasts (AG1518) is shown in Fig. 4. Confluent cells, in which the DNA was labeled with [ $^{14}\text{C}$ ] during replicative synthesis, were used in order to suppress the incorporation of [ $^3\text{H}$ ]thymidine into replicative DNA, thus facilitating the measurement of repair synthesis. Cells were damaged with UV radiation from germicidal lamps (primarily 254 nm) and then incubated at 37°C in complete DMEM medium containing 2  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]thymidine for 1 hour. The cells were harvested, the DNA was precipitated with TCA and collected on glass fiber filters. Repair synthesis was expressed as the ratio of 3H/14C. Table 3 shows octopirox also causes DNA strand break accumulation following UV irradiation of human fibroblasts.

### CONCLUSIONS

Octopirox is a potent inhibitor of mouse L1210 cell growth (Fig. 1) and DNA replicative synthesis in both mouse L1210 cells (Fig. 2) and normal human fibroblasts (AG1518) (Fig. 3), as measured by [ $^3\text{H}$ ]thymidine incorporation into DNA. The inhibition of DNA replicative synthesis correlates well with a reduction in dATP concentrations, while dTTP concentrations increase under the same conditions (Fig. 2). These effects on dATP and dTTP concentrations are similar to those observed with hydroxyurea, a specific inhibitor of ribonucleotide reductase. Octopirox has no direct effect on enzymes involved in DNA replication, such as DNA polymerases alpha and delta, helicases, and topoisomerases, as indicated by its inability to inhibit these enzymes in permeable cells supplied exogenously with deoxynucleoside triphosphates, thus bypassing ribonucleotide reductase.

Inhibitors of ribonucleotide reductase, such as hydroxyurea, also inhibit UV-induced excision repair. As shown in Fig. 4, octopirox inhibits UV-induced repair synthesis. As shown in Table 3, octopirox causes DNA strand break accumulation following UV irradiation of human fibroblasts. This effect is characteristic of agents which block repair patch synthesis, such as aphidicolin and hydroxyurea.

In conclusion, the data are consistent with the hypothesis that the cytotoxic effects of octopirox result from the specific inhibition of ribonucleotide reductase. There is no evidence for any direct effects on other enzymes involved in DNA replication or repair.

	27/04/1992
Darel Hunting	Date
	27/04/1992
Bonnie Gowans	Date

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TABLE 1: EFFECT OF OCTOPIROX ON DNA REPLICATIVE SYNTHESIS IN PERMEABLE<sup>a</sup> HUMAN FIBROBLASTS (AG1518) IN WHICH THE DEOXYRIBONUCLEOSIDE TRIPHOSPHATES WERE SUPPLIED EXOGENOUSLY

EXPERIMENTAL CONDITIONS	REPLICATIVE SYNTHESIS (% OF CONTROL)		
	Experiment Number <sup>b</sup>		
	#1	#2	#3
No dNTPs	0	2.5	4.0
All 4 dNTPs	100	100	100
1 $\mu$ M Octopirox	92	103	96
10 $\mu$ M Octopirox	129	95	101
2.2 $\mu$ g/ml aphidicolin	9	7.1	3

<sup>a</sup> Cells were permeabilized by the lysolethycin method.

<sup>b</sup> In experiment #1, the concentrations of the deoxyribonucleoside triphosphates (dNTPs) were 3  $\mu$ M. In experiments #2 and 3, the concentrations of dATP, dGTP, dTTP were 50  $\mu$ M while that of [<sup>32</sup>P]dCTP was 3  $\mu$ M.

TABLE 2: EFFECT OF OCTOPIROX AT HIGH CONCENTRATIONS ON DNA SYNTHESIS IN PERMEABLE<sup>a</sup> HUMAN FIBROBLASTS (AG1518) IN WHICH THE DEOXYRIBONUCLEOSIDE TRIPHOSPHATES WERE SUPPLIED EXOGENOUSLY

EXPERIMENTAL CONDITIONS	REPLICATIVE SYNTHESIS (% OF CONTROL)	
	Experiment Number <sup>b</sup>	
	#1	#2
All 4 dNTPs	100	100
1 $\mu$ M Octopirox	90	106
10 $\mu$ M Octopirox	100	92
750 $\mu$ M Octopirox	97	100
1.1 mM Octopirox	n.d.	99
2.2 $\mu$ g/ml aphidicolin	12	11

<sup>a</sup> Cells were permeabilized by the lysolethicin method.

TABLE 3: EFFECT OF OCTOPIROX ON UV-INDUCED REPAIR WITH OR WITHOUT A PREINCUBATION

TREATMENT	DNA STRAND BREAKS* (RAD equivalent)	DNA STRAND BREAKS (% of control)
I. a. 20 J/m <sup>2</sup> UV; 2 h., then 15 min. with ETHANOL (0.25%) (control)	260	100
b. 20 J/m <sup>2</sup> UV; 2 h., then 15 min. with APHIDICOLIN	2115	813
c. 20 J/m <sup>2</sup> UV; 2 h., then 15 min. with OCTOPIROX (25 μm)	420	162
d. 20 J/m <sup>2</sup> UV; 2 h., then 15 min. with OCTOPIROX (250 μm)	760	292
II. a. 2 h. with ETHANOL; 20 J/m <sup>2</sup> UV; 10 min. with ETHANOL (0.25% control)	420	100
b. 2 h. with APHIDICOLIN; 20 J/m <sup>2</sup> UV; 10 min. with APHIDICOLIN	1915	456
c. 2 h. with OCTOPIROX (25 μm); 20 J/m <sup>2</sup> UV; 10 min. with OCTOPIROX (25 μm)	1100	262
d. 2 h. with OCTOPIROX (250 μm); 20 J/m <sup>2</sup> UV; 10 min. with OCTOPIROX (250 μm)	1100	262

\*DNA single-strand breaks were quantitated by alkaline elution using a 1000 rad internal standard.

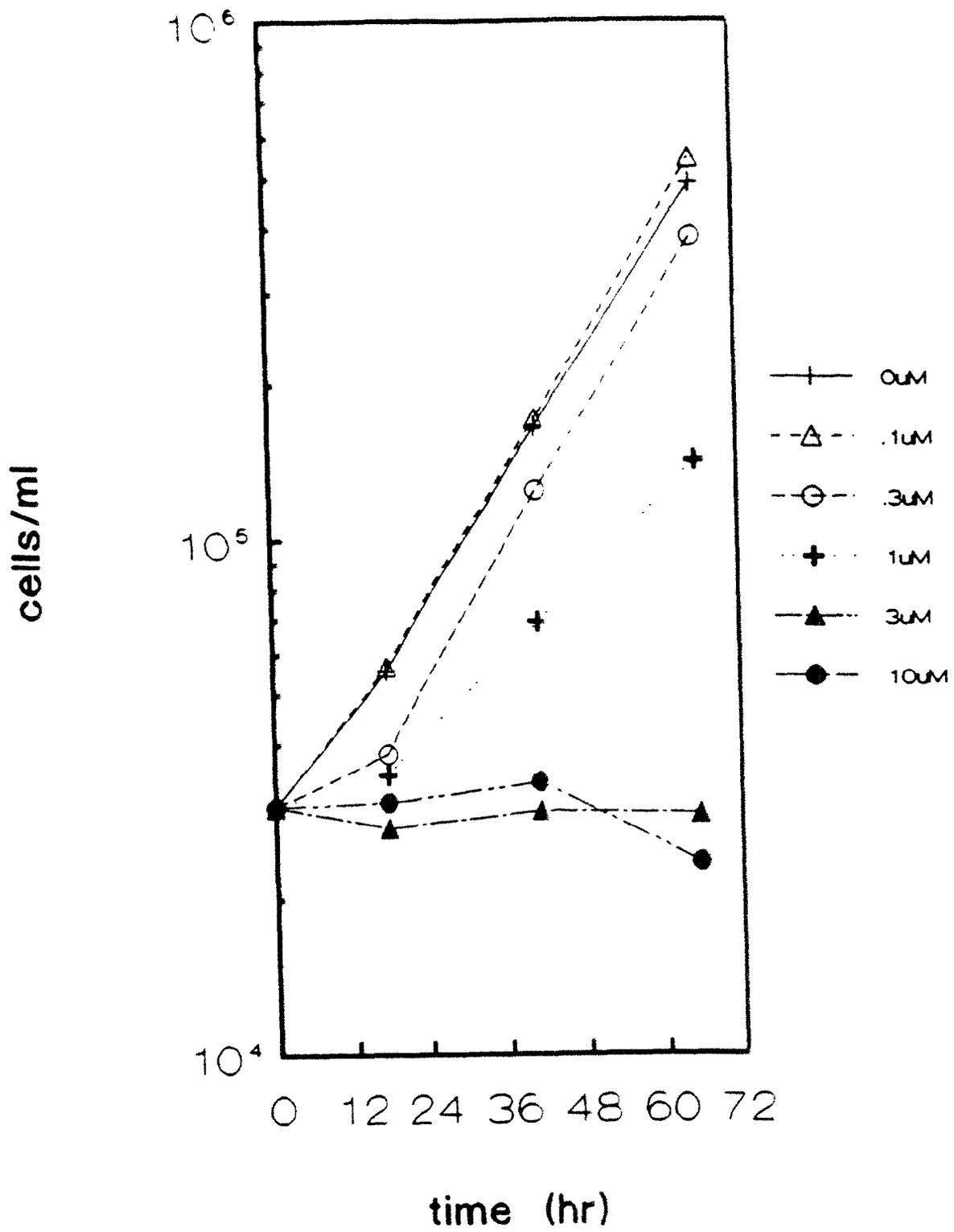


Figure 1. Effect of octopirox on the growth of mouse L1210 cells.

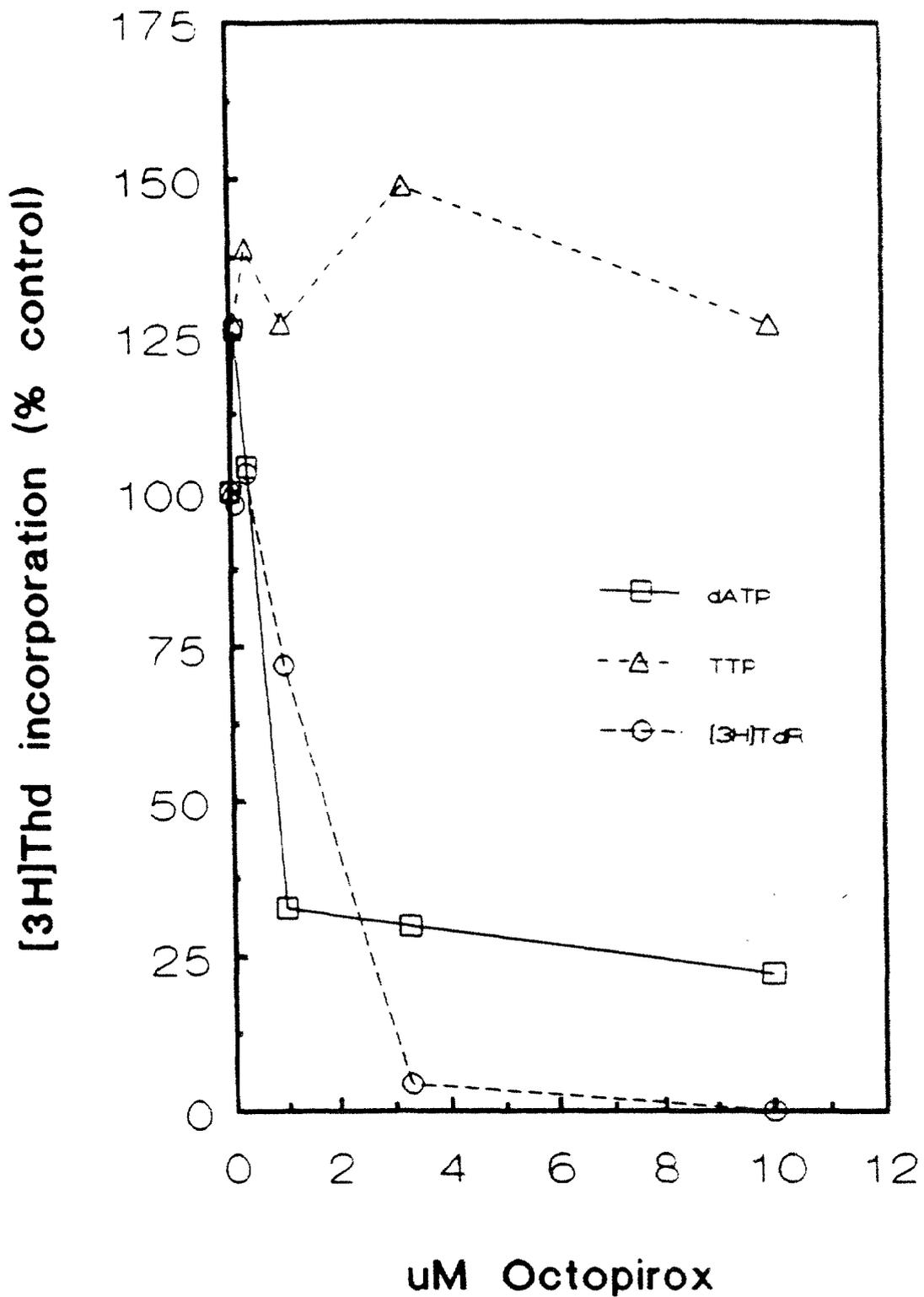
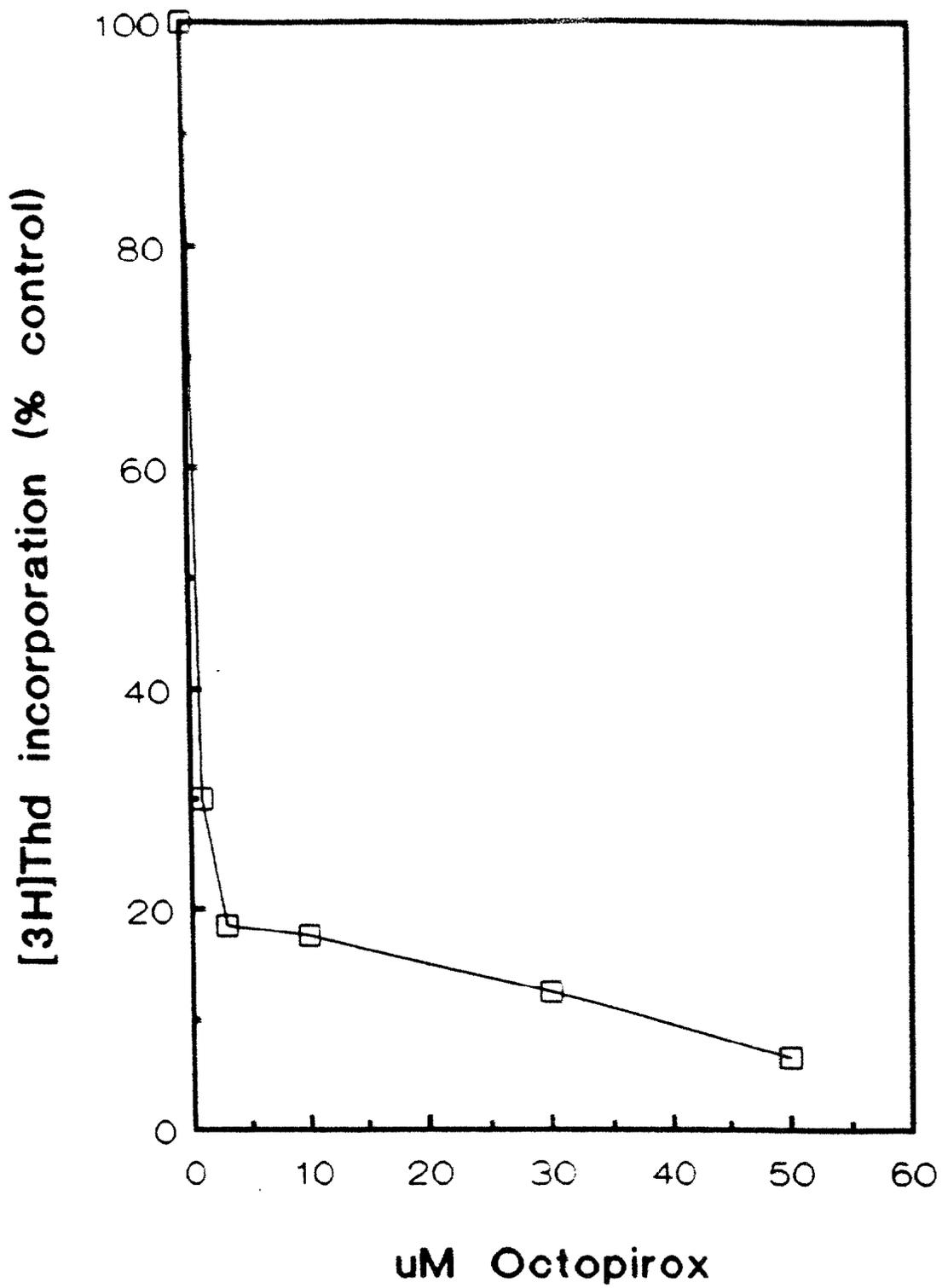


Figure 2. Effect of octopirox on dATP and dTTP concentrations and on [<sup>3</sup>H]thymidine incorporation into DNA in mouse L1210 cells.



**Figure 3.** Effect of octopirox on [ $^3\text{H}$ ]thymidine incorporation into DNA in growing normal human fibroblasts (AG1518).

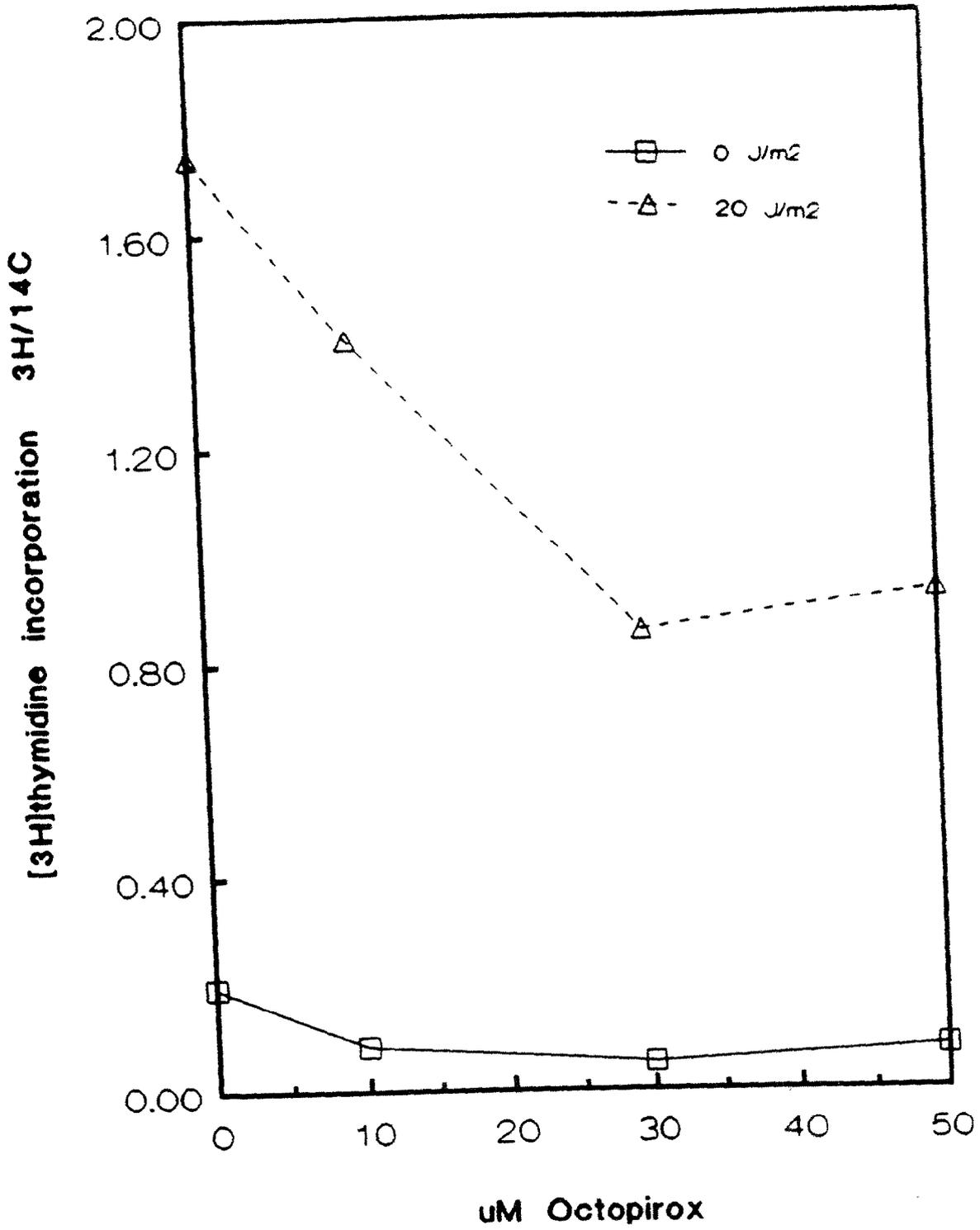


Figure 4. Effect of octopirox on UV-induced [<sup>3</sup>H]thymidine incorporation into DNA in confluent, non-replicating, normal human fibroblasts (AG1518).