THE STRUCTURE OF METHYLATED XANTHINES IN RELATION TO THEIR EFFECTS ON DNA SYNTHESIS AND CELL LETHALITY IN NITROGEN MUSTARD-TREATED CELLS

JOHN P. MURNANE, JOHN E. BYFIELD, CHAO-TUNG CHEN, and CHENG HSIA WANG, *Division of Radiation Oncology, University Hospital, University of California, San Diego Cancer Center, San Diego, California 92103*

ABSTRACT The variation in cellular response to alkylated xanthines possessing different side chains has been used to evaluate more fully the effect of caffeine on both survival and DNA synthesis in cells with DNA damage. A correlation is observed between the ability of these xanthines to reverse the inhibitory effects of nitrogen mustard damage on DNA synthesis and their ability to enhance nitrogen mustard lethality in human HT-29 cells. These findings are consistent with our theory that regulation of damaged replicon initiation protects against potentially lethal damage in the form of unrepaired DNA alkylations. Enhancement of nitrogen mustard lethality is observed to have a maximum limit, which can be reduced by highly toxic xanthine concentrations. The lethal effects of xanthines alone at higher concentrations are unrelated to the effects of caffeine specific to nitrogen mustard treated cells, and appear to be related to an immediate reduction in thymidine incorporation most likely caused by inhibition of other enzyme systems influencing DNA synthesis such as *de novo* and salvage pathways for purine biosynthesis.

INTRODUCTION

Caffeine has been demonstrated to have a variety of effects on eucaryotic cells, including inhibition of cyclic-nucleotide phosphodiesterases (1–4); inhibition of *de novo* and salvage pathways for purine biosynthesis (5, 6); inhibition of postreplication repair (7–9); inhibition of DNA elongation (10); increase in the number of replicon initiation sites (10–13); reversal of the inhibitory effects of DNA damage on DNA synthesis (9, 12–15); and increase in lethality due to DNA damage (16–19). The interrelationships among these phenomena remain unclear, and despite considerable research, numerous interpretations as to the primary site(s) of caffeine's influence persist (6, 9, 12, 15). In an effort to clarify this situation we have undertaken a pharmacological investigation into the relationship between alterations of caffeine's side chains and changes in the effect of these xanthines on specific cellular processes. Knowledge concerning the sensitization of cells to DNA damage is of particular importance owing to the possibility of utilizing any influence on survival as a selective advantage for normal cells in improving the effectiveness of cancer chemotherapy. Our studies

Dr. Murnane's current address is the Laboratory of Radiobiology, University of California, San Francisco, California 94143. Dr. Chen and Dr. Wang are affiliated, respectively, with the Institute of Chemistry, Academia Sinica; and the Department of Chemistry, National Taiwan Normal University, Nankang Taipei Taiwan.

have therefore centered around the human colon tumor cell line HT-29 (20), previously demonstrated to be resistant both in vivo (21) and in vitro (22) to chemotherapeutic drugs. Although reports of caffeine's effects on enhancing lethality in damaged human cells are somewhat contradictory (14, 23–25), we have demonstrated substantial synergistic lethal effects in HT-29 cells (12), despite the fact that similar to the findings of other studies with human cells (26–28), no effects are observed on postreplication repair. Since caffeine can be shown to reverse the inhibitory effects of DNA damage on replicon initiation after HN_2 damage (12), and because recent evidence suggests that DNA synthesis in the presence of damage is associated with lethality (29–31), it appears possible that inhibition of damaged replicon initiation may be an important survival mechanism. Consistent with this interpretation, the studies reported here indicate that most of the effects of caffeine can be attributed to influences on DNA metabolism. One influence occurring at lower concentrations is an increase in the number of replicon initiation sites (10–13), which promotes DNA synthesis in the presence of damage (12, 13) and correlates with the enhancement of lethality in combination with nitrogen mustard; although no mechanism is known for this process, the inhibition of cyclic-nucleotide phosphodiesterases by alkylating xanthines (1–4) does not appear to be involved (32). Other influences occurring at higher concentrations, such as interference with proper precursor production by inhibition of *de novo* and salvage pathways for purine biosynthesis (5, 6) result in inhibition of elongation (10) and appear related to cell death due to xanthine alone (this paper).

METHODS

Cell Culture

HT-29 human adenocarcinoma cells were a gift of Dr. Jorgen Fogh (20). Stock cells were maintained in antibiotic-free Eagle's Basal Medium (BME) (Grand Island Biological Co., Grand Island, N.Y.) with 10% fetal calf serum (FCS) (Gibco). Cultures were incubated at 37°C in a humidified incubator with a 5% CO_2 atmosphere in Falcon tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.).

Drugs

Nitrogen mustard (Merck, Sharp & Dohme Canada Ltd., Montreal, Quebec, Canada) stock solutions were prepared in phosphate buffered saline and stored at −20°C. Caffeine (1,3,7-trimethyl xanthine; CAF), theophylline (1,3-dimethyl xanthine; TP), theobromine 3, 7-dimethyl xanthine, TB), Paraxanthine (1,7-dimethyl xanthine; PARA), and 3-isobutyl-1-methyl xanthine (MIX) were all purchased from Sigma Chemical Co., St. Louis, Mo. Pentoxyphylline [1-(5-oxohexyl)-3,7-dimethyl xanthine; POF] was supplied by Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; and 1-*n*-butyl-3, 7-dimethyl xanthine (nB), 1-*n*-propyl-3,7-dimethyl xanthine (nP), 1-ethyl-3,7-dimethyl xanthine (Et), and potassium theobromine were all synthesized by the co-authors Dr. Chao-Tung Chen and Dr. Cheng-Hsia Wang. All xanthine preparations were dissolved directly in the growth medium and sterilized by filtration with a 0.2-micron Nalgene filtration unit (Nalge Co., Nalgene Labware Div., Rochester, N.Y.).

Colony Formation Assays

Exponentially-growing HT-29 cells were trypsinized, counted on a hemocytometer and 400 cells each plated onto 60-mm Lux tissue culture dishes (Lux Scientific Corp., Newbury Park, Calif.) in 2 ml of BME with 10% FCS (plating efficiency ~ 60%). After 1 h at 37°C in a humidified incubator, 1 ml of 0.3 mg/ml HN_2 (final concentration 0.1 μg/ml) in BME plus 10% FCS (prepared fresh) was added to the

appropriate plates. Cells were reincubated for 2 h at 37°C, after which time the medium in all plates was removed. 2 ml of BME plus 10% FCS containing the various concentrations of xanthines was then added to both HN_2-treated and untreated cells in triplicate. Cells were further incubated for 72 h, at which time the medium was removed and replaced with fresh BME plus 10% FCS without xanthines. Cells were then incubated for an additional 8 d with one more media change before rinsing with 0.9% NaCl, fixation with methanol, Giemsa staining, and counting visible colonies.

[^{3}H]Thymidine Incorporation Assays

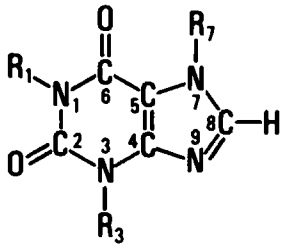
Exponentially-growing HT-29 cells were trypsinized, counted on a hemocytometer, and 2.5×10^5 cells plated onto 60-mm Lux tissue culture dishes in 2 ml of BME with 10% FCS. After 24 h, an additional 2 ml of BME plus 10% FCS containing [2-^{14}C]thymidine (52.4 mCi/mmol, 0.02 μCi/ml) was added (final concentration 0.01 μCi/ml). Cells were labeled in this medium for 24 h before its removal and replacement with either 2 ml of BME plus 10% FCS alone or containing 0.33 μg/ml HN_2. After 2 h this medium was removed and 2 ml of medium containing the [6-^{3}H] thymidine and various alkylated xanthines was added in duplicate, and the cells incubated for 2 h more. After removal of the labeling medium and rinsing with 2 ml of cold PBS containing 10^{-3} mM thymidine, 4 ml of this same buffer was finally added to each plate and the cells in each ($\sim 10^6$) were scraped off and transferred to tubes containing 0.4 ml of the 10% SDS. Tubes were heated at 70°C for 1 h, chilled, and 0.5 ml of 50% TCA added before refrigerating at 4°C overnight. Samples were filtered on GF/C glass fiber filters (Whatman Inc., Clifton, N.J.) using cold 5% TCA. For determination of radioactivity, 0.4 ml of 1 M HCl was added to each filter in scintillation vials, and the vials heated at 70°C for 1 h. After cooling, 2.5 ml of 0.4 N NaOH and 6 ml of Aquasol (New England Nuclear, Boston, Mass.) were added and the samples counted in gel phase. The ^{3}H/^{14}C ratio was then determined after first subtracting ^{14}C overlap from the ^{3}H channel.

RESULTS

Xanthine Structure and Cytotoxicity

Caffeine can specifically influence the survival of mammalian cells containing DNA damage (12, 14, 16–19, 24), and at higher concentrations, undamaged cells as well (23, 24). To understand more about the relationship between these two events, we have established a survival assay designed to compare simultaneously the effects of the various caffeine analogs shown in Fig. 1 on the survival of cells both with and without DNA damage. For this purpose, various concentrations of the xanthines are added for 72 h to tissue culture plates containing the newly plated HT-29 cells, with or without prior treatment with a minimally lethal dose of HN_2 (10% lethality). Influences due to variations in concentration and uptake among the individual xanthines can be ruled out since in each case the lethality curves with and without HN_2 serve as mutual controls, and any changes in one can be compared against the other.

For comparison, variations in lethality among the different xanthines will be classified according to their structure with respect to the three methylated positions of caffeine. Lethal effects due to caffeine alone in non-HN_2-treated HT-29 cells gradually begin to appear by 3 mM with a D_{37} at $\sim$ 6 mM (Fig. 2). Cytostatic effects are also observed, with decreasing colony size accompanying lethality. Caffeine's enhancement of lethality specifically in HN_2-treated cells, on the other hand, is seen as a sharp drop off in survival with a D_{37} of 0.5 mM, reaching a maximum by 3 mM. Fig. 2 demonstrates that changes in the side chain in position 1 can affect synergistic lethality in HN_2-treated cells, and lethality due to the xanthine alone. Increased toxicity with respect to caffeine in non-HN_2-treated cells is observed with nB (D_{37}:2 mM), and to a lesser extent nP, which shows increased levels of



Compound	Side Chains		
	R_1	R_3	R_7
CAF	$-CH_3$	$-CH_3$	$-CH_3$
TB	$-H$	$-CH_3$	$-CH_3$
nP	$-(CH_2)_2-CH_3$	$-CH_3$	$-CH_3$
nB	$-(CH_2)_3-CH_3$	$-CH_3$	$-CH_3$
POF	$-(CH_2)_4-\overset{O}{\overset{\|\|}{C}}-CH_3$	$-CH_3$	$-CH_3$
PARA	$-CH_3$	$-H$	$-CH_3$
MIX	$-CH_3$	$-CH_2-CH(CH_3)_2$	$-H$
TP	$-CH_3$	$-CH_3$	$-H$

FIGURE 1 Structure of the xanthine molecule and the various side chains present in the three alkylated positions of the caffeine analogs used in these experiments.

lethality at lower concentrations, whereas TB, POF (Fig. 2), and ethyltheobromine (data not shown) all show virtually no difference from caffeine. The potassium salt of theobromine was used in all experiments to achieve greater concentrations, since it was found to behave identically to theobromine. These changes in side chains in position 1 influence enhancement of HN_2 lethality in a much different fashion. nB and nP (Fig. 2) both demonstrate increased effectiveness in enhancing HN_2 lethality, and require only half the concentration (D_{37}:0.25 and 0.2) of caffeine (D_{37}:0.5) to observe this effect, whereas TB is less active and requires nearly twice the concentration (D_{37}:0.9) of caffeine. POF (Fig. 2) and ethyltheobromine (data not shown) again demonstrate no difference from caffeine. The survival data demonstrated in Figs. 2 and 3 are from a typical experiment. Although some minor variations are observed in the slope of survival curves from one experiment to the next, the relative differences among the various xanthines are constant and have been reproduced in numerous experiments. The triplicate values for the individual points show very little variation and have an average standard deviation of 10% of the values indicated in Figs. 2 and 3.

Regardless of the initial slope of the synergistic lethality curves observed with these compounds in Fig. 2, all appear to enhance lethality in HN_2-treated cells by approximately the same maximum extent (93–98%). This, therefore, appears to be the limit for enhancement of HN_2 lethality in these cells. Also clearly evident from Fig. 2 is the fact that these synergistic lethal effects can be reduced or eliminated at concentrations where substantial cytotoxic and cytostatic effects of the xanthines themselves are observed. For example, lethality due to the xanthines alone and loss of synergistic lethality are both observed with nB at 1–2 mM, nP at

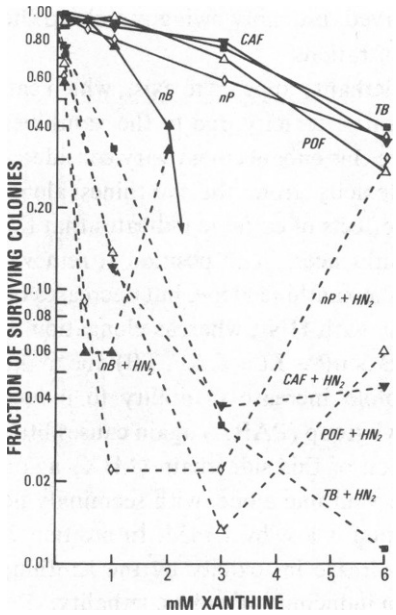


FIGURE 2

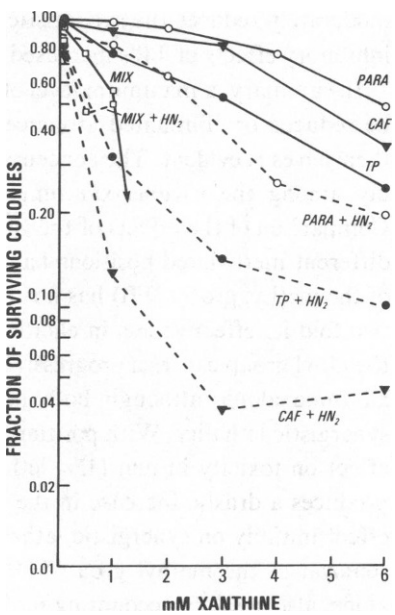


FIGURE 3

FIGURE 2 The side effects of substitution of various side chains in position 1 of caffeine on colony formation due to the xanthine alone or in combination with HN_2. CAF (▼), nB (▲), nP (◇), POF (△), and TB (■) were added to cells at various concentrations for 72 h with (dashed lines) and without (solid lines) a prior 2-h exposure to a 10% lethal dose of HN_2 (0.1 μg/ml).

FIGURE 3 The effects of substitution of various side chains in positions 3 and 7 of caffeine on colony formation due to the xanthine alone or in combination with HN_2. CAF (▼), MIX (□), PARA (○), and TP (●) were added to cells at various concentrations for 72 h both with (dashed lines) and without (solid lines) a prior 2-h exposure to a 10% lethal dose of HN_2 (0.1 μg/ml).

3–6 mM, and CAF or POF at 6 mM; but no such effect was observed with TB at the concentrations tested.

The data in Fig. 3 demonstrate that alteration of the position 3 side chain produces changes in survival curves in non-HN_2-treated cells nearly identical to those caused by similar changes in position 1. Removal of the methyl group (PARA), therefore, causes only a slight decrease in toxicity compared with caffeine, whereas elongation to a 4-carbon group (MIX) again causes a large increase. No such similarities are observed in the enhancement of HN_2 lethality (Fig. 3), as PARA demonstrates only minimal synergistic effects in HN_2-treated cells (D_{37}:4 mM), and MIX at most may equal caffeine's synergistic capabilities at lower concentrations although its highly toxic nature nearly eliminates this effect by 1 mM. Since MIX is known to be highly inhibitory to cyclic-nucleotide phosphodiesterases (2, 4), this is further evidence that these enzymes are not involved in the enhancement of DNA-damage-related lethality (32). Part of the high degree of toxicity seen with MIX alone in non-HN_2-treated cells may also be related to the absence of the methyl group in position 7, since TP (Fig. 3) shows a moderate increase in toxicity (D_{37}:4.5) with respect to caffeine. The absence of this methyl group also

moderately reduces the synergistic lethality (Fig. 3) observed, probably owing mainly to the inhibitory effects of TP's increased toxicity at lower concentrations.

In summary, a maximum level of enhancement of HN_2 lethality appears to exist, which can be reduced or eliminated at concentrations where substantial toxicity due to the xanthines themselves is evident. The concentrations required to evoke this enhancement vary considerably among the various xanthines tested, as does the toxicity from the xanthines alone. Comparison of the effects of the various analogs with the effects of caffeine indicates that the different methylated positions have separate functional influences. With position 1, removal of the methyl group (TB) has no effect on toxicity due to the xanthine alone, but decreases by two fold its effectiveness in eliciting a synergistic response with HN_2; whereas elongation of the alkyl group causes a progressive increase in toxicity (nB > nP > Et > CAF, TB) due to the xanthine alone, although both nB and nP show a twofold increase in ability to induce synergistic lethality. With position 3, removal of the methyl group (PARA) again causes little effect on toxicity in non-HN_2 lethality; whereas elongation of this side chain (MIX) again produces a drastic increase in the lethal properties of the xanthine alone, with seemingly no effect initially on synergistic lethality although this function is lost by 1 mM. In position 7, removal of the methyl group (TP) causes a moderate increase in toxicity by the xanthine alone, also possibly accounting for its reduced capability in inducing synergistic lethality.

Xanthine Structure and [^{3}H] Thymidine Incorporation

Previously published work from our laboratory (12) has suggested that increased lethality in HN_2-treated cells in the presence of caffeine may be related to a reversal of the inhibitory effects of DNA damage on DNA synthesis as a result of increased replicon initiation. To corroborate further this hypothesis and to establish any relationship between these effects and the influence on normal DNA/nucleotide metabolism, which occur at higher concentrations of caffeine, we have investigated the influence of the various alkylated xanthines on exogenous thymidine incorporation in both HN_2-damaged and undamaged cells. For this purpose [^{14}C]thymidine prelabeled cells were pulse-labeled with [^{3}H]thymidine for 2 h in the presence of various concentrations of the alkylated xanthines, with or without a prior 2-h dose of HN_2, hereby giving a 46% reduction in DNA synthesis and 70% lethality. Incorporation of [^{3}H]thymidine due to repair replication need not be considered in these experiments, since even a 15-fold greater level of HN_2 fails to induce significant levels of [^{3}H]thymidine uptake in human cells (33).

The use of [^{3}H]thymidine for measuring the effects of compounds on DNA synthesis is subject to many artifacts arising from possible influences on nucleotide uptake, thymidine kinase activity, or progression through other portions of the cell cycle. The inhibition of [^{3}H]thymidine incorporation at higher concentrations of the various xanthines alone in non-HN_2-treated cells cannot, therefore, be said to be directly due to inhibition of DNA synthesis in all cases, and is used mainly as a control for studies on HN_2-treated cells. The stimulation of [^{3}H]thymidine incorporation specific to HN_2-treated cells, however, can be demonstrated to be a result of increased DNA synthesis since (*a*) stimulation of incorporation is observed in HN_2-treated cells in many instances at concentrations of xanthines having no influence on control non-HN_2-treated cells; (*b*) increased incorporation in HN_2-treated cells is not observed proportionally in all size classes of newly synthesized DNA, and cannot therefore

be due to precursor availability (12); (*c*) similar studies using ionizing radiation (13) have demonstrated similar effects on DNA synthesis by caffeine (also observed by us) while demonstrating no effects on pool sizes; (*d*) synchronized cell studies both with sulphur mustard (34) and ionizing radiation (35) demonstrate that caffeine reverses the prolongation of S phase brought about by these agents, and (*e*) autoradiography fails to detect any change in the proportion of cells in S phase after caffeine treatment of HN_2-damaged cells, although reduced levels of labeling per cell are observed (data not shown).

Fig. 4 shows that while caffeine begins to inhibit [^{3}H]thymidine incorporation above 3 mM in non-HN_2-treated cells, concentrations of 1 mM and above actually promote incorporation in HN_2-damaged cells. Comparison of these data with the survival assays in Fig. 2 indicates decreased [^{3}H]thymidine incorporation in non-HN_2-treated cells at concentrations of caffeine demonstrating reduced cell survival, and increased incorporation in HN_2-damaged cells at concentrations demonstrating synergistic lethality. Other alkylating xanthines also show similar correlations, generally with predictable variations in [^{3}H]thymidine incorporation corresponding to changes in survival as shown in Figs. 2 and 3.

As with the survival curves, the data shown for [^{3}H]thymidine uptake in Figs. 4, 5, and 6 are taken from a typical experiment. Again, although minor variations in the curves are seen in repeat experiments, the relative differences seen with the various xanthines remain the same. Duplicate values for individual data points are very close, with an average standard deviation of 8% of the values indicated in Figs. 4, 5, and 6. In non-HN_2-treated cells, therefore, TB (Fig. 4) and PARA (Fig. 5) are very similar to caffeine in their inhibition of [^{3}H]thymidine incorporation except for a reproducible elevation at lower concentrations of TB; TP (Fig. 5) shows a moderate reproducible increase in inhibition of [^{3}H]thymidine incorporation compared with CAF, TB, or PARA; while nB (Fig. 4) and MIX (Fig. 5) both

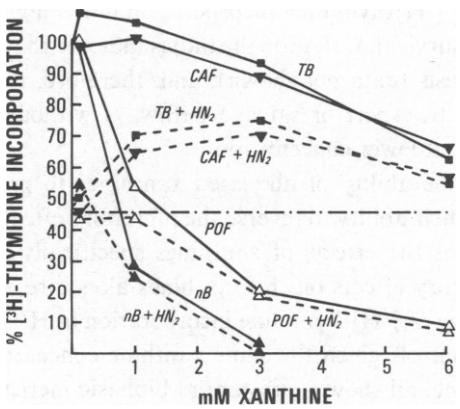


FIGURE 4 The effects of substitution of various side chains in position 1 of caffeine on [^{3}H]thymidine incorporation due to the xanthine alone or in combination with HN_2. CAF (▼), nB (▲), POF (△), and TB (□) were added to cells at various concentrations for 2 h in the presence of 0.5 μCi/ml [^{3}H]thymidine with (dashed lines) or without (solid lines) a previous 2-h exposure to a 70% lethal dose of HN_2 (0.33 μg/ml). This dose of HN_2 gave a 54% inhibition of DNA synthesis, represented by the broken line (—·—). Before these procedures, cells were labeled for 24 h with 0.01 μCi/ml of [^{14}C]thymidine, and results were calculated as the ratio of ^{3}H to ^{14}C in counts per minute.

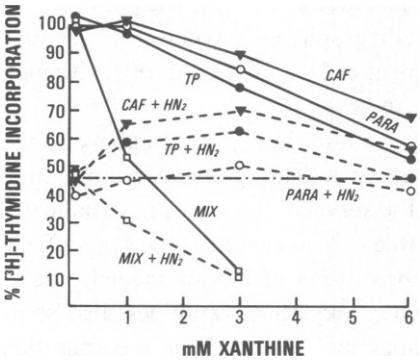


FIGURE 5

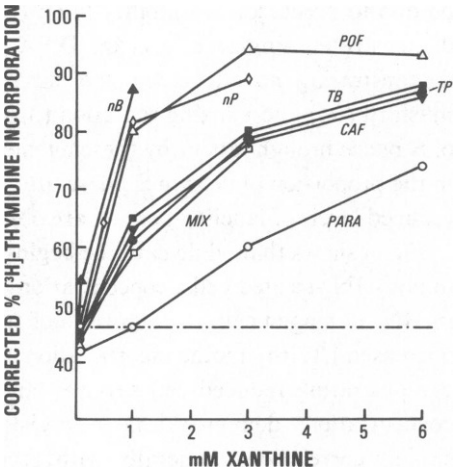


FIGURE 6

FIGURE 5 The effects of substitution of various side chains in positions 3 and 7 of caffeine on [^{3}H]thymidine incorporation due to the xanthine alone or in combination with HN_2. CAF (▼), MIX (□), PARA (○), and TP (●) were added to cells under conditions identical to those in Fig. 4, both with (dashed lines) and without (solid lines) prior treatment with HN_2.

FIGURE 6 The corrected values for stimulation of [^{3}H]thymidine incorporation in HN_2-treated cells by the various xanthines tested. Inhibitory effects of the xanthines alone, as shown in the non-HN_2-treated cells of Figs. 4 and 5 have been compensated for by directly proportional adjustments to values seen in HN_2-treated cells. Symbols are similar to those in Figs. 4 and 5.

give a sharp drop off in incorporation at relatively low concentrations. Closer inspection of these data indicate that nB, like POF (Fig. 4) and nP (similar to POF, data not shown), all give levels of inhibition of [^{3}H]thymidine incorporation much higher than would be expected from their effects on cell survival. Cell growth studies indicate that these results are unrelated to effects on DNA synthesis (data not shown), and, therefore, these compounds apparently inhibit either pyrimidine transport or salvage pathways without concurrently affecting *de novo* pyrimidine synthesis at lower concentrations.

In HN_2-treated cells, the ability of alkylated xanthines to promote synergistic lethality generally corresponds to their ability to reverse the inhibitory effects of HN_2 damage on DNA synthesis. Fig. 6 illustrates the effects of xanthines specifically on HN_2-treated cell DNA synthesis after the inhibitory effects on the xanthines alone are eliminated. This is done by determining the percentage of [^{3}H]thymidine incorporation in HN_2-treated cells in relation to their non-HN_2-treated controls given the same xanthine concentration (Figs. 4 and 5). TB, TP, and MIX, like caffeine, all show a substantial biphasic increase in HN_2-damaged DNA synthesis with increasing concentration, with a sharp rise by 3 mM and a gradual increase thereafter. Although a similar concentration dependence is observed in the appearance of synergistic lethality (Figs. 2 and 3), the greater variation in this activity among these xanthines indicates that any relationship between this process and stimulation of DNA synthesis in these cells must be subject to other influences. As discussed earlier, in the case of MIX and TP this is apparently a result of the toxicity of the xanthines alone; but no explanation is apparent for the reduced ability of TB to enhance HN_2 lethality compared with

caffeine. In position 1, removal of the methyl group (TB) has no effect on the xanthine influence on [^{3}H]thymidine incorporation in either HN_2 or non-HN_2-treated cells; whereas elongation of this side chain appears to enhance the ability of these compounds (nB, nP, POF) to stimulate incorporation in HN_2-treated cells. In position 3, removal of the methyl group (PARA) drastically reduces the ability of the xanthine to induce [^{3}H]thymidine incorporation in HN_2-treated cells, with little change on its inhibitory effects on incorporation in non-HN_2-treated cells. Elongation of this side chain (MIX) causes no change in the initial stimulation of [^{3}H]thymidine incorporation in HN_2-treated cells, although nearly eliminating this effect apparently due to the greatly increased inhibitory effects on incorporation due to this xanthine alone. Finally, in position 7, removal of the methyl group (TP) causes an increase in the inhibition of DNA synthesis in non-HN_2-treated cells at lower concentrations, but shows no apparent change in its capability to increase DNA synthesis in HN_2 treated cells.

DISCUSSION

A pharmacological investigation of the effects of various alkylated xanthines on exogenous thymidine incorporation and cell survival has allowed us to understand better the structure-function relationship of the methylated xanthine molecule as well as to determine the interrelationships among these cellular processes. Previous work from our laboratory suggests that increased DNA synthesis in HN_2-treated cells in the presence of caffeine is due to reversal of the damage-induced inhibition of replicon initiation (12). Owing to the evidence linking DNA synthesis in the presence of damage to increased lethality (29–31), we proposed that the enhancement of HN_2 lethality in these cells could be related directly to this process. This hypothesis would predict that inhibition of initiation of DNA synthesis in damaged replicons serves as a survival mechanism to prevent the expression of "potentially lethal" damage. The studies described in this paper are consistent with this proposal, since alkylated xanthines in general produce elevations in DNA synthesis in damaged DNA at concentrations corresponding to those required for synergistic lethality. This is most clearly demonstrated when comparing the results of nB, nP, PARA, and caffeine, where changes in the concentration required to induce synergistic lethality are reflected in the effectiveness of these compounds in elevating DNA synthesis in HN_2-damaged cells. Although some xanthines (TP, TB, POF) show somewhat less ability to enhance HN_2 lethality than would be expected from comparison of their effects on HN_2-damaged DNA synthesis with caffeine, other factors such as toxicity due to the xanthines themselves also appear to moderate this effect, and therefore some quantitative variation does not necessarily contradict this hypothesis. This reduction in the enhancement of HN_2 lethality at toxic concentrations of the xanthines themselves is most likely related to the ability of these compounds to prevent cells from passing through S phase (35, 36), which is required for synergistic lethality to occur (34).

In contrast, lethality due to the alkylated xanthine alone in undamaged cells appears related to an immediate inhibition of incorporation of exogenous thymidine, and is observed generally at higher concentrations. This reduction in incorporation due to caffeine has been related to inhibition of elongation (10) and inhibition of *de novo* and salvage pathways for purine biosynthesis (6). Although these same effects were used to explain the increased number of replicon initiation sites and increased lethality in cells containing DNA damage,

only relatively high concentrations of caffeine (10 mM and 7.5 mM, respectively) were primarily investigated, and little attempt was made to distinguish effects occurring at more relevant concentrations. Our research involving caffeine indicates that whereas alterations in replicon initiation (observed here as an elevation of DNA synthesis in HN_2-damaged cells) and synergistic lethality are highly evident at 1 mM and begin to level off as the concentration is increased to 3 mM, the inhibition of [^{3}H]thymidine incorporation in non-HN_2-treated cells does not begin until 3 mM. This is demonstrated in the case of PARA, where despite the fact that its inhibition of survival and exogenous thymidine incorporation in non-HN_2-treated cells is nearly identical to caffeine, much less of an effect is observed on stimulation of DNA synthesis in HN_2-treated cells.

We therefore propose that the cellular response to caffeine is the result of a variety of effects. At higher concentrations and associated with cell lethality due to the xanthine alone, the inhibitory effects of these compounds on nucleotide metabolism such as *de novo* and salvage pathways for purine biosynthesis (5, 6) are most likely responsible for the observed effects. This may be mediated by the inhibition of cyclic-nucleotide phosphodiesterases (1–4), since the concentrations of xanthines required to inhibit these enzymes (i.e., MIX > TP > TB, CAF) are similar to those producing toxic effects in non-HN_2-treated cells. Other enzymes involved in purine biosynthesis are also directly inhibited (5), and differences in lethality or inhibition of DNA synthesis due to the various xanthines themselves cannot always be assumed to be related to the same process. Regardless of this fact, no evidence linking inhibition of [^{3}H]thymidine incorporation to either synergistic lethality or increased replicon initiation has been found. Instead, we consider that alteration of chromatin structure is the most plausible mechanism for explaining caffeine's second influence, which increases the number of replicon initiation sites (12, 13). This conclusion is based upon the prediction that regulation of replicon initiation is controlled by the availability of initiation sites (37), and that perturbations of chromatin structure by certain types of DNA damage inhibit initiation of DNA synthesis in the vicinity (38). The same characteristics of caffeine that induce replicon initiation in normal cells would therefore also reverse the inhibitory effects of DNA damage on DNA synthesis.

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