Biochemical Pharmacology, Vol. 34, No. 20, pp. 3677–3682, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

DISSIMILAR ACTIONS OF 6-MERCAPTOPURINE AND 6-THIOGUANINE IN CHINESE HAMSTER OVARY CELLS*

JONATHAN MAYBAUM,†,‡ LAURA A. HINK,‡ W. MAURICE ROETHEL‡ and H. GEORGE MANDEL§

§Department of Pharmacology, The George Washington University Medical Center, Washington, DC 20037; and ‡Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A.

(*Received* 15 *June* 1983; *accepted* 25 *March* 1985)

Abstract—The actions of 6-thioguanine (TG) and 6-mercaptopurine (MP) were compared in Chinese hamster ovary (CHO) cells. Several differences were noted between these two agents. TG caused a greater maximal loss of clonogenicity, leaving about one log fewer survivors than did MP, although the cells killed by MP appeared to succumb much more rapidly than those killed by TG. MP-treated populations experienced a G_1 or G_1/S arrest which was quickly reversed upon drug removal, while TG-treated cells were arrested in late S/G_2, after some delay. Although TG induced a gross chromosome deformation [unilateral chromatid damage, as described earlier in Maybaum and Mandel, *Cancer Res.* **43**, 3852 (1983)] MP caused little or no such deformation. Addition of 4-amino-5-imidazolecarboxamide (AIC) to MP treatments antagonized MP-induced loss of clonogenicity, while AIC caused a dose-dependent potentiation of TG-induced loss of clonogenicity. The interaction between TG and AIC does not seem to represent an increase in either purine starvation or incorporation of TG into DNA, suggesting that a third mechanism is involved. We suggest that this additional mechanism may possibly be related to the induction of differentiation by TG that has been reported in other systems.

In general, MP‖ and TG behave similarly in most biological systems examined, although TG is usually more potent than MP [1]. The antitumor spectra of the two drugs are almost indistinguishable, and cells resistant to one drug are usually cross-resistant to the other. No major differences in toxicity in either higher animals or humans have been recognized, although differences in disposition of the thiopurines may account for occasional selective organ toxicity. Both thiopurines rely on hypoxanthine-guanine phosphoribosyl transferase for conversion to their mononucleotides, which are required for pharmacological activity, and thereafter both analogs follow the pathways for IMP and GMP respectively. Also, both thiopurines prevent accumulation of *N*-formyl glycinamide ribonucleotide and *de novo* purine synthesis [2, 3] and interfere with purine interconversion [4–6].

There are, however, some differences between the drugs which may be more than quantitative. Combination of MP and TG prolonged survival time markedly beyond that produced by any dose of either drug alone, suggesting that the two drugs exert their antitumor effects by different mechanisms [7]. Both MP and TG form S-methylated derivatives, such as 6-methylthioinosinic acid and 6-methylthioguanylic acid, respectively but, in contrast to the methylated MP derivative, that for TG does not contribute significantly to the drug's inhibition of *de novo* purine synthesis [3].

While most evidence indicates that TG cytotoxicity *in vitro* is a result of its incorporation into DNA [8, 9], the basis of MP cytotoxicity is less clear, as both nucleic acid incorporation and purine deprivation have been implicated in the action of that drug [9]. We have demonstrated recently that in the CHO cell line TG induces a specific chromosome deformation (unilateral chromatid damage, UCD) and that the appearance of this damage is closely associated with TG-induced cytotoxicity [10, 11]. The present work was undertaken to determine if MP also induces UCD and, more generally, to ascertain whether the cytotoxic actions of TG and MP in CHO cells are mediated through common mechanisms.

MATERIALS AND METHODS

Wild type CHO cells (a gift from Mary Ann Wormstead, University of California, San Francisco) were grown as monolayers in alpha minimum essential medium (GIBCO, Grand Island, NY) supplemented with 10% calf serum (GIBCO) and 5% fetal calf serum (Biofluids Inc., Rockville, MD) in a humidified 5% CO_2 atmosphere, at 37°. Thioguanine was recrystallized from boiling water and stored in the dark. Procedures for determination of colony forming ability, flow cytometry, and induction of

* This work has been presented in part [*Proc. Am. Ass. Cancer Res.* **24**, 294 (1983)]. Supported by PHS Grant 5T-32-CA09223 awarded by the National Cancer Institute and by a research grant from the Children's Leukemia Foundation of Michigan.

† To whom requests for reprints should be sent.

‖ Abbreviations: MP, 6-mercaptopurine; TG, 6-thioguanine; CHO, Chinese hamster ovary; UCD, unilateral chromatid damage; PCC, prematurely condensed chromosome; HPLC, high performance liquid chromatography; AIC, 4-amino-5-imidazolecarboxamide; MMPR, 6-methylmercaptopurine ribonucleoside; and PRPP, 5-phosphoribosylpyrophosphate.

premature chromosome condensation have been described recently [11]. This reference also illustrates the scheme used for assigning deformation scores to G_2-PCCs.

Experiments in which dye-excluding ability was determined were designed to encompass all cells in the flask, including those which had detached from the monolayer. Drugs were added at t = 0 and cells were washed and given fresh, drug-free medium at t = 16 hr. At the indicated times, flasks were scraped to suspend attached cells and this suspension was then centrifuged. After decanting the supernatant fraction, a few drops of trypan blue solution (0.4% in 0.9% NaCl) were added to the cell button for 2 min, at which time the cells were assayed for dye exclusion with a hemocytometer.

For determination of endogenous nucleotide pools the following procedure was used. Cells were treated with drug for 3 hr, colllected by scraping, washed once with 0.9% NaCl–0.1 M phosphate buffer, pH 7.4, and lysed by addition of 250 μl of 0.4 N $HClO_4$. After removing acid-precipitable material by centrifugation, the extract was neutralized with tri-*N*-octylamine/freon [12] and analyzed by HPLC. A portion of the extract was injected onto a 10 μm Altex strong anion exchange column and eluted isocratically with 0.4 M NaH_2PO_4, pH 3.6, at 3 ml/min. Peaks were detected by u.v. absorbance at 254 nm and quantified by integration.

Incorporation of TG into DNA was quantitated either by the HPLC procedure of Tidd and Dedhar [13] or by measurement of absorbance at 350 nm of a solution of purified DNA isolated from TG-treated cells [14]. In the cases where both of these methods were applied to replicate samples, we obtained equivalent results. The intracellular content of acid-soluble TG-containing molecules was measured as described by Ishiguro and Sartorelli [15].

RESULTS

To define appropriate conditions for comparison of TG and MP, we determined the loss of colony forming ability caused by exposure to various concentrations of the drugs for 16 hr (Fig. 1). Sixteen hours was chosen as the length of drug treatment so that all cells in an asynchronous population would be able to undergo one round of DNA synthesis in the presence of the drug, and also to be consistent with our previous studies on TG cytotoxicity [10, 11]. As Fig. 1 shows, TG can produce up to 98–99% cell kill, with 4 μM being the minimum concentration needed to reach this level. MP was considerably less cytotoxic, with 60–80% cell kill being the maximum effect observed using concentrations up to 500 μM. In most experiments, 50 μM MP caused a near-maximal loss of clonogenicity, and this concentration was therefore defined as being comparable, though not equitoxic, to 4 μM TG (i.e. the minimum dose needed to achieve maximal cell kill). In some cases, 50 μM MP was also compared to 0.4 μM TG, since these concentrations each reduce cloning efficiency by about 50%.

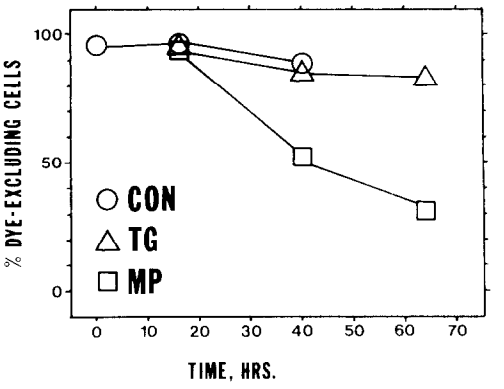

Fig. 2. Kinetics of thiopurine-induced loss of dye exclusion. Either 4 μM TG or 50 μM MP was added at t = 0 hr and removed at t = 16 hr. One of duplicate experiments with similar results is shown.

The rapidity with which thiopurine-treated cells lost membrane integrity was assayed by trypan-blue exclusion (Fig. 2). Cells exposed to 4 μM TG for 16 hr had no loss of dye-excluding ability through the first 64 hr of the experiment. On the other hand, about 50% of those given 50 μM MP failed to exclude dye at t = 40 hr.

The rapid loss of dye excluding ability caused by MP made analysis of chromosome deformation a difficult task, since the process of cell fusion is sensitive to the membrane integrity of the cells involved. Therefore, it is likely that those cells which did fuse would be from the portion of the population which

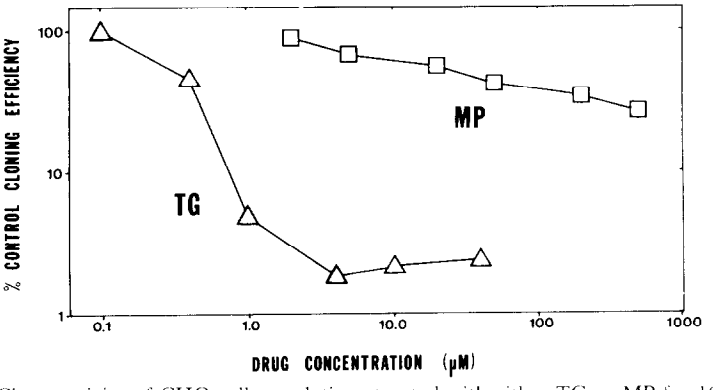

Fig. 1. Clonogenicity of CHO cell populations treated with either TG or MP for 16 hr.

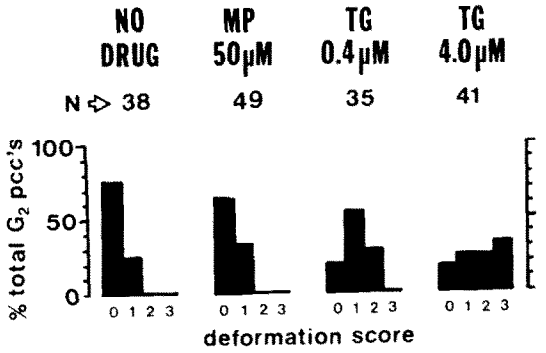

Fig. 3. Specific chromosome deformation (unilateral chromatid damage) caused by thiopurines. CHO cells were treated with drug for 16 hr followed by 28-hr incubation in drug-free medium, at which time premature chromosome condensation was induced. Deformation scores were assigned as in Ref. 11. One of duplicate experiments having similar results is shown for each treatment. N = number of G_2 PCCs scored for each treatment group.

excluded dye at t = 40 hr. Figure 3 shows that PCCs formed from the MP-treated group had only a minor degree of deformation present, if at all. This is consistent with the idea that these cells represent the surviving half of the population. The induction of deformation by TG is much more severe and has been discussed in detail previously [11].

Effects of MP and TG on cell cycle progression were determined by flow cytometry (Fig. 4). The two drugs behaved dissimilarly, with TG causing little or no inhibition of progression until 16–20 hr, at which time the accumulation of cells with a late S or G_2 content of cells could be observed. MP treatment induced a pileup of cells in G_1 or G_1/early S which was detectable at 8 hr and was pronounced at 16 hr. Upon removal of the drug at t = 16 hr the population proceeded through S phase with some degree of synchrony.

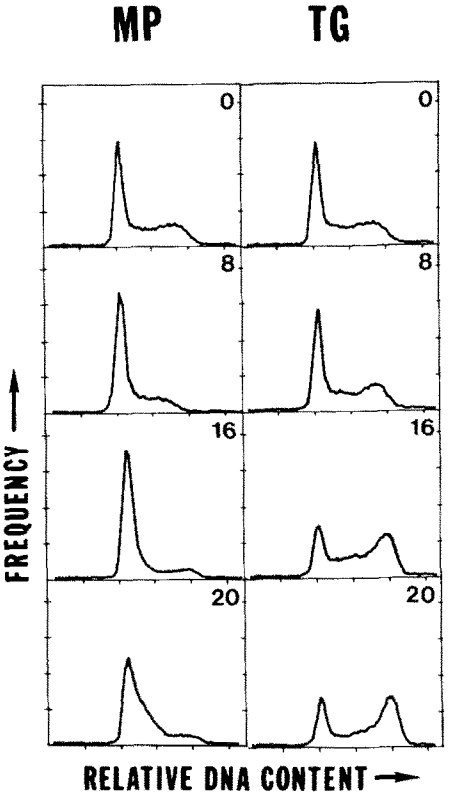

Fig. 4. Cell cycle kinetics (as revealed by flow cytometry) of CHO cells exposed to 4 μM TG or 50 μM MP for 16 hr, at which time drug was removed, cells were washed, and the incubation was continued in drug-free medium. Number of hours elapsed since beginning of drug exposure is indicated in each box.

The role of purine starvation in mediating TG- and MP-induced effects was probed by the addition of AIC to drug treatments. Loss of viability caused by MP was partially prevented by 200 μM AIC and

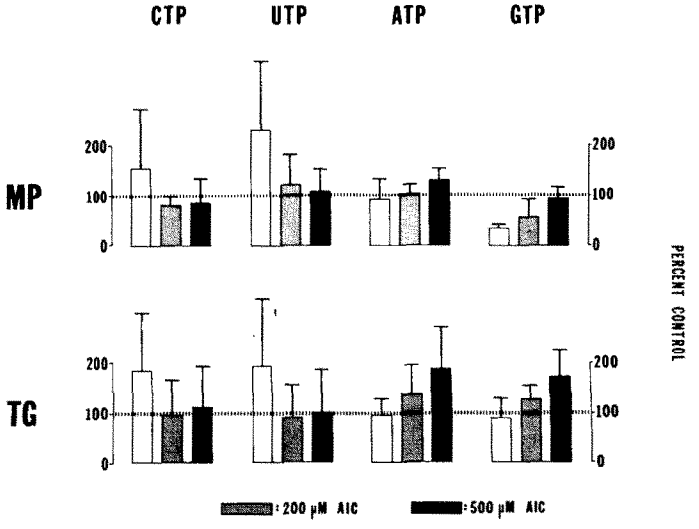

Fig. 5. Changes in intracellular ribonucleoside triphosphates induced by 3 hr of treatment with 40 μM TG or 50 μM MP, ± AIC. Key: (□) thiopurine alone; (▨) thiopurine + 200 μM AIC; and (■) thiopurine + 500 μM AIC. Dotted line represents control levels. Each value is the mean of three experiments ± S.D.

Table 1. Effects of AIC on thiopurine-induced loss of clonogenicity*

Treatment	Concn (μ)	Colony forming ability (% control) AIC (μM) 0	200	500
TG	0	100	101	105
	4	1.4 ± 0.1	0.9 ± 0.1	>50
	10	2.6 ± 0.1	0.5 ± 0.1	>50
	40	3.6 ± 0.9	0.3 ± 0.1	4.2 ± 0.8
MP	50	45 ± 4	59 ± 4	95 ± 7
	200	37 ± 1	76 ± 4	88 ± 4

* Cells were treated concurrently with AIC and TG or MP for 16 hr, as for Fig. 1.

more completely prevented by 500 μM AIC (Table 1). No conditions were found by which AIC potentiated cell kill by MP. In contrast, the effect of AIC on TG-induced cell kill was dose dependent. Over the range of TG concentrations shown, 200 μM AIC potentiated loss of viability while 500 μM AIC was protective. At slightly lower TG concentrations (0.4 to 1.0 μM), 200 μM AIC partially prevented cell kill (data not shown).

Effects of AIC on TG metabolism are given in Table 2. Addition of 200 μM AIC to either 4 or 40 μM TG resulted in diminished incorporation of the drug into DNA, an effect which was more pronounced at 500 μM AIC. The intracellular content of TG-containing, acid-soluble species was also reduced by 200 μM AIC, roughly in parallel with the incorporation data.

Changes in pyrimidine ribonucleotide pools caused by MP and TG were virtually identical (Fig. 5). Each drug apparently induced elevations of UTP and CTP which were negated by either concentration of AIC, although there was considerable variation in the values obtained. Purine ribonucleotides responded somewhat differently to the two drugs. The concentration of ATP was not changed by either drug alone, nor by MP plus 200 μM AIC, while TG plus either concentration of AIC produced an expanded ATP pool. The concentration of GTP was depressed severely by MP but not changed by TG. Addition of AIC to either drug increased GTP levels in a dose-dependent manner.

DISCUSSION

Although the thiopurines TG and MP are among the most extensively studied chemotherapeutic agents known, it has not been possible to consistently assign a single mechanism of cytotoxicity to them. This is due in large part to the fact that, like many other agents, MP and TG can produce multiple biochemical lesions which are interactive and therefore difficult to study individually. Each of the actions of thiopurines (inhibition of *de novo* purine biosynthesis, inhibition of purine nucleotide interconversion and incorporation of TG into nucleic acids) has been proposed as contributing to thiopurine cytotoxicity, and it is clear that modulation of one of them may affect the others. For instance, inhibiting purine synthesis may enhance incorporation of thiopurines by lowering the pool of a competing natural purine nucleotide. On the other hand, inhibition of IMP dehydrogenase might decrease the conversion of 6-thio-IMP to 6-thio-GMP and therefore attenuate incorporation of TG nucleotides into nucleic acids.

In addition to this multiplicity of biochemical actions, it has also been shown that in some cases thiopurine cytotoxicity may be manifested either in a delayed manner or immediately upon drug exposure [16, 17]. This raises the possibility that the major cytotoxic mechanism of either MP or TG may vary with conditions of drug exposure, or from one cell line to another, and that under some circumstances the two drugs might kill cells by different mechanisms [18]. For example, in L5178Y cells treated with MP, inhibition of thiopurine incorporation into DNA following treatment with high-dose thymidine [18] or mycophenolic acid [8], which inhibit DNA synthesis, reduces the cytotoxic action of MP. Furthermore, in this cell line, the similar extents of thiopurine incorporation into DNA resulting from equitoxic treatments with TG and MP [18] indicate that both drugs act by interfering with DNA synthesis. In contrast, in CHO cells there is relatively little DNA incorporation of MP compared to TG, and these cells are not protected from MP toxicity by DNA synthesis inhibitors [18]. Thus, in CHO cells the mechanisms of cytotoxicity of these agents are different. Since we have shown recently that the delayed cytotoxicity of TG in CHO cells is strongly associated with a specific and unique chromosome deformation (UCD) [11], we wished to determine whether this

Table 2. Effects of AIC on TG metabolism in CHO cells*

	TG incorporation into DNA (% replacement of G by TG) AIC (μM) 0	200	500	TG-containing molecules in acid-soluble fraction (pmoles/10^6 cells) AIC (μM) 0	200
4 μM TG	1.5 (0.2)	0.27 (0.11)	0.05 (0.05)	331 ± 26	64 ± 12
40 μM TG	1.3 ± 0.4	0.41 ± 0.22	0.09 ± 0.01	576 ± 45	87 ± 2

* Exponentially growing CHO cells were exposed to TG with or without AIC for 16 hr, at which time cells were harvested and analyzed for TG incorporation into DNA or, in separate experiments, total intracellular TG-containing, acid-soluble compounds. Data shown represent either the mean of duplicate experiments followed by the range of the two observations (in parentheses) or the mean of triplicate observations ± S.D.

deformation is also produced by MP in this line and whether it plays a role in MP-induced cytotoxicity.

Our first objective was to define appropriate treatment conditions under which to compare the two drugs. In preliminary experiments it became apparent that there was a difference of about one log in the maximal cell kill achievable by 16-hr exposure of CHO cells to the thiopurines, with TG-treated populations maintaining about 1–2% survivors versus 20–30% for MP (Fig. 1). Tidd and Paterson [8] previously made a similar observation in L5178Y cells, although the absolute levels of cell kill in their study were much higher than those seen here. Paradoxically, there was a slight, but significant, decrease in TG-induced cytotoxicity as the drug concentration exceeded 4 μM. This effect has been noted previously [19, 20] and may be due to antagonism of DNA synthesis (and therefore thiopurine incorporation into DNA) by purine starvation [19].

To determine whether the toxicities of MP and TG were immediate or delayed effects, we examined the dye excluding ability of TG- or MP-treated cells. Figure 2 indicates a major discrepancy in the actions of MP and TG on the ability of cells to exclude trypan blue. Whereas the population exposed to TG had few long-term survivors, there was no detectable loss of dye exclusion through the first 64 hr of the experiment. In contrast, the MP-treated population had about 50% of its cells failing to exclude dye within 40–60 hr, which corresponds closely to the total loss of clonogenicity caused by this treatment. Although trypan blue exclusion is not a reliable indicator of long-term survival, it is reasonable to assume that those cells which fail to exclude dye are not viable, and therefore we conclude that the great majority of MP-induced cytotoxicity in this situation is through a relatively immediate process.

Previous studies have shown that *delayed* MP cytotoxicity is related to drug incorporation into nucleic acids [8, 9], and is not due to purine nucleotide depletion [21]. However, it is also known that MMPR, when anabolized to the nucleotide level, can induce cell death [22] and this agent is thought to act solely by purine synthesis inhibition. It is therefore possible that in the present case 50 μM MP kills cells by purine nucleotide depletion. Flow cytometric analysis illustrates that MP (50 μM, LD_{50}) had a qualitatively different effect on cell cycle progression than did TG (4 μM, LD_{98}) (Fig. 4). The G_1 or G_1/S accumulation shown to occur following MP treatment would be expected of cells whose DNA synthesis was inhibited, as is the progression of a wave of cells into S-phase upon relief of the block by drug removal (Fig. 4, t = 20 hr).

To further examine the role of purine synthesis inhibition, we measured the effect on clonogenicity of adding AIC to MP or TG treatments. Since aminoimidazole carboxamide-5′-phosphate (formed from AIC and PRPP) occurs in the *de novo* purine pathway after the steps inhibited by thiopurines, AIC has classically been used to circumvent thiopurine-induced purine synthesis inhibition [23]. The results in Table 1 illustrate that nearly complete protection from MP cytotoxicity could be attained by co-incubation with 500 μM AIC. This protection is consistent with previous data [9, 18] and parallels the elevation of GTP to control levels (Fig. 5), further suggesting a role for purine synthesis inhibition in MP cytotoxicity. On the other hand, the effects of AIC on TG cytotoxicity depend on the concentrations of both TG and AIC. From the data in Fig. 5 it is clear that TG has little or no effect on purine pools although an elevation of pyrimidine pools occurred, indicating that some perturbation of nucleotide or nucleic acid metabolism had taken place. When 200 μM AIC was present, all four ribonucleoside triphosphates were at or near control levels, suggesting that the metabolic disturbances caused by 40 μM TG had been relieved. Since this change was accompanied by increased cell kill, we speculated that the combination of 40 μM TG and 200 μM AIC might permit some fraction of cells to synthesize DNA (and therefore incorporate TG) more efficiently than in the presence of 40 μM TG alone. Also, since AIC inhibits guanase [24], the addition of this compound might antagonize catabolism of TG, resulting in greater anabolism of the drug. The results in Table 2 contradict these hypotheses, however, as they demonstrate that both the incorporation of TG into DNA and the accumulation of acid-soluble intracellular TG derivatives were antagonized by 200 μM AIC. These data suggest that the loss of clonogenicity resulting from adding 200 μM AIC to 40 μM TG is not easily attributable to either of the mechanisms that have traditionally been implicated in thiopurine action (i.e. purine starvation or DNA incorporation). A third possibility is suggested by recent reports that TG can induce differentiation in HL60 cells and that this effect can be amplified by concurrent treatment with various nucleosides [15, 25, 26]. We are continuing to study the interaction between AIC and TG in CHO cells in order to discern whether differentiation or some related phenomenon may be responsible for the synergistic loss of clonogenicity observed here.

In summary, the data presented here are consistent with previous observations that in CHO cells the cytotoxic mechanisms of TG and MP are different. We have provided additional evidence that in this cell line MP caused a rapidly reversible G_1 or G_1/S block (consistent with nucleotide deprivation), that MP did not induce the unilateral chromatid damage associated with TG incorporation into DNA, and that cytotoxicity caused by MP was manifested in an immediate, rather than a delayed manner. These properties are in contrast to the cytotoxicity induced in CHO cells by TG, which has been shown previously to be associated with delayed G_2 arrest and unilateral chromatid damage, probably resulting from incorporation of the drug into DNA. Furthermore, we have described a novel synergism between TG and the purine precursor AIC which cannot be explained on the basis of either purine depletion or DNA incorporation, and which we suggest may be related to the TG-induced differentiation described by other investigators. This effect, which is dependent on the concentration of both TG and AIC, may have therapeutic utility if it can be shown that there is a sufficient difference between the concentrations of AIC needed to potentiate TG action in tumor versus host tissue.

Acknowledgements—We wish to thank Dr. Oliver Alabaster, Ms. Connie Ernst and Ms. Kathy Clagett for their excellent assistance in obtaining flow cytometric data.

REFERENCES

1. A. R. P. Paterson and D. M. Tidd, in *Handbook of Experimental Pharmacology* (Eds. A. C. Sartorelli and D. G. Johns), Vol. 38, pp. 383–403. Springer, Berlin (1975).
2. G. A. LePage and M. Jones, *Cancer Res.* **21**, 642 (1961).
3. P. W. Allan and L. L. Bennett, *Biochem. Pharmac.* **20**, 847 (1970).
4. R. P. Miech, R. E. Parks, Jr., J. H. Anderson, Jr. and A. C. Sartorelli, *Biochem. Pharmac.* **16**, 2222 (1967).
5. A. Hampton, *J. biol. Chem.* **238**, 3068 (1963).
6. R. P. Miech, R. York and R. E. Parks, Jr., *Molec. Pharmac.* **5**, 30 (1969).
7. J. F. Henderson and I. G. Junga, *Biochem. Pharmac.* **5**, 167 (1960).
8. D. M. Tidd and A. R. P. Paterson, *Cancer Res.* **34**, 738 (1974).
9. J. A. Nelson, J. W. Carpenter, L. M. Rose and D. J. Adamson, *Cancer Res.* **35**, 2872 (1975).
10. J. Maybaum and H. G. Mandel, *Expl Cell Res.* **135**, 465 (1981).
11. J. Maybaum and H. G. Mandel, *Cancer Res.* **43**, 3852 (1983).
12. J. X. Khym, *Clin. Chem.* **21**, 1245 (1975).
13. D. M. Tidd and S. Dedhar, *J. Chromat.* **145**, 237 (1978).
14. S. Yoshida, M. Yamada, S. Masaki and M. Saneyoshi, *Cancer Res.* **39**, 3955 (1979).
15. K. Ishiguro and A. C. Sartorelli, *Cancer Res.* **45**, 91 (1985).
16. L. L. Wotring and J. L. Roti Roti, *Cancer Res.* **40**, 1458 (1980).
17. K. Horakova, J. Navarova and A. R. P. Paterson, *Biochim. biophys. Acta* **366**, 333 (1974).
18. S. Drake, R. L. Burns and J. A. Nelson, *Chem. Biol. Interact.* **41**, 105 (1982).
19. N. T. Christie, S. Drake, R. E. Meyn and J. A. Nelson, *Cancer Res.* **44**, 3665 (1984).
20. S. Matsumura, T. Hoshino, M. Weizsaecker and D. F. Deen, *Cancer Treat. Rep.* **67**, 475 (1983).
21. D. M. Tidd and A. R. P. Paterson, *Cancer Res.* **34**, 733 (1974).
22. C. T. Warnick and A. R. P. Paterson, *Cancer Res.* **33**, 1711 (1973).
23. M. T. Hakala and C. A. Nichol, *Biochim. biophys. Acta* **80**, 665 (1964).
24. H. G. Mandel, J. L. Way and P. K. Smith, *Biochim. biophys. Acta* **23**, 402 (1957).
25. E. L. Schwartz, O. C. Blair and A. C. Sartorelli, *Cancer Res.* **44**, 3907 (1984).
26. K. Ishiguro, E. L. Schwartz and A. C. Sartorelli, *J. cell. Physiol.* **121**, 383 (1984).