pattern for **4** and **5** was dominated by cleavage of the trimethoxybenzyl group to give as the base peak the ion, **6** (*m/e* 194), and an isoquinolinium ion at *m/e* 356 (**7**), and 274 (**8**). The mass spectrum of **5** does not allow differentiation between the 2-OMe and 3-OMe positions.

The THPB alkaloid (**4**) was evaluated for its beta adrenergic activity using guinea pig tracheal chains that were stimulated with 10^{-5} M histamine. The results revealed that **4** (pD_2 5.61) was much less potent than TMQ (pD_2 7.85) or isoproterenol (pD_2 7.42) as a beta agonist. This is consistent with binding studies for THBPs on beta adenylate cyclase [8], which showed that THBPs were generally much weaker than their THIQ precursors. It may be inferred that the protoberberine structure is less preferred for binding, and that the trimethoxybenzyl moiety must be conformationally mobile for optimal binding to the beta receptor.

This study has demonstrated that TMQ can be cyclized to a THPB (**4**) in mammalian systems in low yields (<3% of total isolated metabolites), and that **4** apparently does not contribute significantly to the overall beta adrenergic activity for TMQ. Because the pharmacological results for metabolite **4** were not promising, the identification of metabolites **4** and **5** after a single dosing of TMQ was not completed.

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Use of vaccinia, a DNA virus, to study the role of DNA incorporation in the mechanism of action of 6-thioguanine*

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Previous results from this laboratory, using human tumor cells in culture [1] or a *Bacillus subtilis* transformation system [2, 3], have supported the theory [4] of incorporation of 6-TG† into DNA as one mechanism responsible for the biological activity of 6-TG. Other proposed mechanisms of action for 6-TG and the related compound, 6-mercaptopurine, include: (1) inhibition of *de novo* purine biosynthesis [5–7], (2) sequential blockade of guanine nucleotide biosynthesis [8], and (3) incorporation into RNA causing subsequent effects on RNA maturation [9–11] or translation [12]. The effects of these antitumor and immunosuppressive agents have been reviewed [13].

In mammalian cell systems, it is often difficult to determine which biochemical events are causally related to 6-TG treatment because of marked drug cytotoxicity. A DNA virus, vaccinia, was used in this study as a model to investigate possible alterations in DNA function which occur as a consequence of 6-TG incorporation. This virus replicates in the host cell cytoplasm; therefore, effects of the drug on viral genome expression can occur independently of that of the host cell. A preliminary report of this work has been presented [14].

Methods

Cell and virus cultures. HeLa and Vero cells, originally obtained from Flow Laboratories (Bethesda, MD), were continuously passaged in RPMI 1640 or McCoy's 5a media (Gibco, Grand Island, NY) supplemented with FCS (K.C. Biologicals, Lenexa, KS) and antibiotics (0.25 μg/ml amphotericin and 100 units/ml of penicillin and streptomycin). Vaccinia virus (American Type Culture Collection, Rockville, MD) was grown and passaged in HeLa cells. Experiments were performed in a variety of flasks and

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† Abbreviations: 6-TG, 6-thioguanine; FCS, fetal calf serum; PBS, phosphate-buffered saline, 0.14 M NaCl plus 0.01 phosphate buffer, pH 7.4; and PFU, plaque forming units.

plates, the sizes of which are noted below. Vaccinia virus was assayed by plaque titration in Vero cells grown in 96-well microtiter dishes (No. 3596, Costar, Cambridge, MA) using medium with 2% FCS, antibiotics and 0.25% human serum globulin (Armor Pharmaceuticals, Kankakee, IL). After 2 days of incubation, neutral red was added to a concentration of 33 μg/ml. On the following day, the medium was removed and the cell sheets were dried at 80° to inactivate the virus.

Growth of vaccinia virus in 6-TG-treated cells. HeLa cell monolayers in 150 cm^2 flat or roller bottles (Costar) were infected with vaccinia virus at a multiplicity of infection of 1 PFU/cell. After 1 hr, the cells were rinsed twice with Hank's Balanced Salt Solution (Gibco) followed by the addition of RPMI 1640 or McCoy's 5a medium containing 5% FCS, antibiotics and, where indicated, 6-TG (Drug Synthesis and Development Branch, National Cancer Institute, Bethesda, MD). The cells were harvested 24 hr after infection, and the virions were purified by a modification of the method of Parkhurst and Heidelberger [15], as follows. The medium was decanted and the cell sheet was rinsed with 4 ml of PBS. The medium and PBS rinses were combined and centrifuged at 2000 rpm for 10 min, and the pellet was saved. The cell sheet was removed by repeated washing with three 2-ml rinses of lysing buffer (0.01 M Tris–HCl; 0.01 M EDTA; 1 mM KCl; pH 8.1). All rinses were combined with the original pellet, vortexed, and centrifuged. The supernatant fraction was saved and the pellet was subjected to three additional 1-ml rinses, with vortexing before each centrifugation. All supernatant fractions were combined and sonicated for 2 min using a Raytheon External Sonicator. Purified virus was obtained by centrifugation through 40% sucrose in 0.01 M Tris–HCl, pH 7.5, for 1 hr at 15,000 rpm in a SW-27 Rotor (Beckman Instruments, Irvine, CA). The pellet was resuspended in PBS, sonicated, layered onto a 40–60% sucrose gradient, and centrifuged for 1 hr at 15,000 rpm. The virus band was collected by pipette aspiration. Radiolabeled virus was prepared as above, except that 2 μCi/ml of [methyl-^{3}H]deoxythymidine (6 Ci/mmole; Schwarz/Mann, Orangeburg, NY) or [5-^{3}H]deoxycytidine (15 Ci/mmole; Moravek Biochemicals, Brea, CA) was added to the incubation medium. Similarly, [8-^{14}C]6-TG (55 μCi/μmole; Moravek Biochemicals) was used to determine 6-TG incorporation into the viral DNA.

Particle counts. Vaccinia virus particle counts [16] were provided by Liane Jordan in the laboratory of Dr. Heather Mayor (Baylor College of Medicine). The electron micrographs of 6-TG-treated and control viruses were examined to determine morphological characteristics.

Viral DNA induction. HeLa cell monolayers were prepared in 24-well dishes (No. 3024, Costar). The cells were infected with purified vaccinia virus grown in the presence or absence of 6-TG as described above. The cells were then washed, and [methyl-^{3}H]deoxythymidine (1 μCi/ml) was added. At various times following inoculation, the cells were rinsed three times with PBS, lysed, and centrifuged for 10 min at 2000 rpm. The pellet obtained was washed once with lysing buffer, and the supernatant fractions were combined. After addition of carrier DNA (50 μg/ml), the combined supernatant fractions were treated with 10% cold trichloroacetic acid. The precipitate formed was filtered through Whatman glass fiber filters (GF/A) and washed three times with 1-ml aliquots of cold 10% trichloroacetic acid followed by three washes with cold 80% ethanol. Radioactivity associated with the filters was then determined by liquid scintillation spectrometry.

Virion adsorption. Vaccinia virus was grown for 24 hr in HeLa cells in the presence or absence of 6-TG (30 μg/ml) as described above. [5-^{3}H]Deoxycytidine (1 μCi/ml) was present during the incubation to radiolabel the viral DNA. The virus particles were then purified as described above using two successive 40–60% sucrose gradient centrifugations. Prior to the second centrifugation, the preparations were incubated for 1 hr in the presence of DNAse I (Sigma Chemical Co., St. Louis, MO) to remove non-encapsidated DNA. The preparations were then diluted in PBS with or without 0.01 M $MgCl_2$ and $CaCl_2$. The virus particles were then incubated for various times with confluent monolayers of Vero cells on 13 mm glass coverslips (Fisher Scientific, Houston, TX). The coverslips were rinsed, and radioactivity in the rinses and on the coverslips was determined by liquid scintillation spectrometry.

Results and discussion

When vaccinia was grown in cells treated with 3 or 30 μg/ml of 6-TG, both the numbers of PFU and the infectivity of the virus particles formed were reduced in a dose-dependent manner (Table 1). The effect on infectivity was most pronounced at a dose of 30 μg/ml of 6-TG, i.e. the infectivity per particle was reduced from a control value of 0.076 to 0.014 PFU/particle. This reduction in the virulence of vaccinia particles was not observed when purified particles were incubated in the presence of high levels of 6-TG alone (data not shown). Thus, some aspect(s) of the viral replicative cycle and/or host cell metabolism is essential for the ability of the drug to reduce the PFU per particle. Although 6-TG (30 μg/ml) had delayed cytotoxicity in proliferating HeLa cells, it did not reduce the viability of HeLa cell monolayers (as determined by trypan blue exclusion) during a 24-hr incubation; therefore, host cell toxicity may not account for the results shown in Table 1.

To further understand the possible mechanism by which 6-TG treatment reduced the infectivity per virus particle, additional properties of the defective particles were evaluated. The particles separated on the 40–60% sucrose density gradient as a more heterogeneous band than control virus. The heterogeneous banding suggests that 6-TG may alter the physical properties of the viral DNA. Recent results ([17], unpublished data) indicate that 6-TG produces physical changes in mammalian cell DNA. Electron microscopic examination of the particles failed to reveal any discernible differences between the virus harvested from 6-TG-treated or control cells (data not shown). Also, virus grown in the presence of 6-TG and control virus were equally sensitive to incubation with DNAse or HeLa cell lysates for 24 hr; such incubation resulted in approximately a 1-log loss of infectivity in each case (data not shown). Thus, in several respects, physical properties of the virus particles obtained from 6-TG-treated cells were similar to those of control virus.

Vaccinia virus that was purified from cells treated with

Table 1. Reduction of vaccinia virus infectivity by 6-thioguanine*

Drug concn (μg/ml)	Total particles × 10^8	Total PFU × 10^8	PFU/Particle × 10^{-3}
0	10.2 ± 1.0	0.730 ± 0.030	76 ± 9
3	1.7 ± 0.4†	0.080 ± 0.025	50 ± 10
30	1.6 ± 0.2‡	0.019 ± 0.005	14 ± 4

* Vaccinia virus was grown in confluent monolayers of HeLa cells as described in Methods. Where indicated, 6-TG (3 or 30 μg/ml) was added at the time of virus inoculation. After 24 hr, virus particles were harvested and purified by sucrose gradient centrifugation. Particle counts were made by electron microscopy, and infectious virus was determined by plaque assay. Mean values ± S.E. are given for at least three separate experiments.

† Radioactivity associated with the purified virus was 12 ± 2% of control when [^{3}H]deoxythymidine was used.

‡ Radioactivity associated with the purified virus was 2.1 ± 2.9% of control when [^{3}H]deoxythymidine was used.

Table 2. Lack of effect of 6-thioguanine on the adsorption of vaccinia virus to HeLa cells*

Incubation time (min)	% adsorption of vaccinia virus			
	PBS only		PBS plus 0.01 M $MgCl_2$, 0.01 M $CaCl_2$	
	Control	6-TG	Control	6-TG
0	5	5	10 ± 2	13 ± 1
7.5	7	10	26 ± 4	33 ± 1
15	19 ± 5	20 ± 8	38 ± 1	32 ± 3
60	20 ± 8	28 ± 3	52 ± 1	53 ± 5

* Vaccinia virus was grown in HeLa cells in the presence or absence of 6-TG (30 μg/ml) for 24 hr. [^{3}H]Deoxycytidine was added to label the viral DNA as described in Methods. The virus was purified by two successive sucrose density gradient centrifugations. The ability of vaccinia virus to adsorb to HeLa cells was determined as described in Methods using PBS with and without 0.01 M $MgCl_2$ and $CaCl_2$. At least 5×10^4 virus particles were incubated in the presence of 2×10^5 HeLa cells attached to 13 mm glass coverslips. At the times indicated, the coverslips were removed and rinsed with PBS. Radioactivity was measured in the media, rinse and coverslips. Mean values ± S.E. are given for two to four determinations.

6-TG and the control virus adsorbed to untreated host cells at similar rates and to similar degrees (Table 2). Specifically, in medium containing $MgCl_2$ and $CaCl_2$, approximately 50% of the total radioactivity was adsorbed to the cells following a 60-min incubation period. The adsorption of vaccinia virus from both sources was enhanced by $MgCl_2$ and $CaCl_2$ [18].

For the above experiments, [^{3}H]deoxycytidine was used to radiolabel the viral DNA since 6-TG had been found to markedly inhibit the utilization of [^{3}H]thymidine (Table 1). Such inhibition was not apparent when [^{3}H]deoxycytidine was used to label the viral DNA. The mechanism by which 6-TG reduced the thymidine incorporation is not known; however, qualitatively similar effects of 6-TG on thymidine and deoxycytidine utilization for DNA synthesis have been observed by us in H.Ep.2, Chinese hamster ovary and HeLa cells (unpublished observations). Since the reduction in incorporation of [^{3}H]thymidine was more profound than the reduction in particles formed (and, presumably, the amount of DNA formed), it appears that 6-TG or one of its metabolites inhibited the salvage of deoxythymidine. Alternatively, 6-TG treatment may have altered the endogenous deoxyribonucleoside 5′-triphosphate pools so that the specific activities of the immediate precursors for DNA synthesis were changed, producing the results seen.

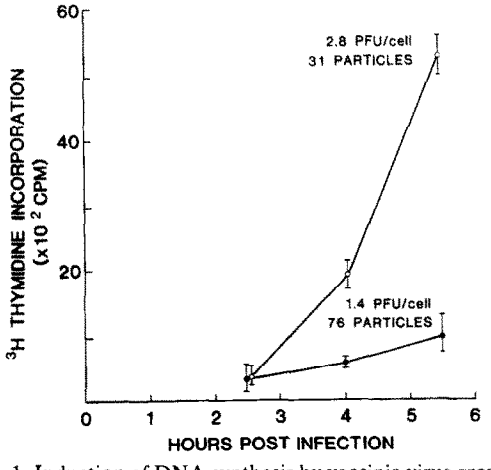

Fig. 1. Induction of DNA synthesis by vaccinia virus grown in the presence or absence of 6-TG. Vaccinia virus was allowed to replicate in control (○) HeLa cells or in cells treated with 30 μg/ml of 6-TG (●), as described in Methods. Virus was purified by sucrose density gradient centrifugation and added to untreated host (HeLa) cell monolayers. Induction of DNA synthesis was measured by determination of the incorporation of [^{3}H]deoxythymidine into the acid-insoluble, cytoplasmic radioactivity. The numbers associated with the curves indicate the number of plaque forming units (PFU) per cell and the number of particles added at time 0. The points represent the mean values with the S.E. indicated for three determinations.

Another early event in the replicative cycle of vaccinia virus involves the induction of viral DNA synthesis, observed 2–4 hr after infection when the multiplicity of infection is greater than 1 (Fig. 1). Particles grown and purified from cells treated with 6-TG (30 μg/ml) were defective in their ability to induce DNA synthesis. Although at least twice as many particles from the 6-TG-treated source were used (76 particles/cell) to inoculate the cells, induction of DNA synthesis was less than one-fifth that of control (31 particles/cell). Thus, growth of virus in the presence of 6-TG results in particles that are defective in their ability to induce DNA synthesis.

To determine the extent of 6-TG incorporation into the viral DNA, vaccinia virus was grown in cells treated with [8-^{14}C]6-TG (3 μg/ml; 55 μCi/μmole) as described above. The virions were purified by two successive sucrose density gradient centrifugations, with DNAse treatment after the first to remove non-encapsidated DNA. Following digestion of the DNA, the extent of radioactive 6-TG incorporation was determined by liquid chromatographic analysis of the permanganate oxidation product of 2′-deoxythioguanosine [19, 20]. The endogenous base, adenine, was determined by liquid chromatographic analysis of the fluorescent 1,N^6-etheno derivative of 2′-deoxyadenosine [21]. The amount of 6-TG incorporated was calculated to be about 2% of the total bases in the viral DNA. This is similar to the level of incorporation observed in Chinese hamster ovary cells at a dose of 6-TG which produces a 90% cell kill [20]; however, this amount of incorporated 6-TG is at least two orders of magnitude greater than that measured in *B. subtilis* DNA with reduced transforming activity [3].

In summary, following propagation in the presence or absence of 6-TG, vaccinia virus has been purified, and the abilities of the virions to adsorb to untreated host cells, induce DNA synthesis, and to replicate were evaluated. The results indicated that growth of vaccinia in 6-TG-treated cells results in progeny which adsorb normally to untreated host cells; however, the virions have a reduced capacity to induce DNA synthesis or to produce infectious particles. These effects of 6-TG on vaccinia virus may be a consequence of incorporation of the drug into the viral DNA.

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The influence of thyroid hormones and propylthioracil on salicylate hepatotoxicity in monolayer cell cultures*

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It has been shown in animal studies that propylthioracil (PTU) protects the liver from injury due to dietary deficiency [1, 2], carbon tetrachloride [3], alcohol [4] and acetaminophen [5, 6]. Based on the observation that normal thyroid function is necessary for complete expression of carbon tetrachloride injury [7, 8], the presumed mechanism of protection is inhibition of thyroid hormone synthesis. Further support for the concept that thyroid function modifies toxic liver injury is the observation that hyperthyroid animals have increased susceptibility to liver injury from anoxia [9], dietary deficiency [2, 10], chloroform [11], and infection [12, 13]. Recent studies have shown that PTU blocks the peripheral monodeiodination of thyroxine (T_4) to the more active triiodothyronine (T_3) [14–16]. This mechanism has been suggested as an explanation for the apparent protective effect of PTU [3].

Chronic alcohol administration in animals mimics the hypermetabolic state produced by excessive thyroid hormone [17]. PTU abolishes both this hypermetabolic state and the hepatic susceptibility to anoxic injury [4]. While development of a hypothyroid state may explain PTU suppression of liver injury, a more direct effect of PTU on the hepatocyte has not been excluded. Such an effect is suggested by evidence that alcohol-induced hepatic injury is suppressed by only 3 days of PTU treatment [4]. This does not appear to be consistent with thyroid hormone depletion in view of the prolonged effects of thyroid hormone [17] and the delayed onset of action of PTU in the treatment of hyperthyroidism.

This study was designed to determine the influences of PTU, T_4 and T_3 on salicylate-induced injury in hepatocyte monolayer cultures. By using such cultures, any effect of PTU would be independent of its effect on thyroid hormone synthesis. Furthermore, by studying T_3 alone any chemical effect of PTU would be independent of its affect on monodeiodination of T_4.

Methods and Materials

Hepatocyte monolayer cultures. Male Sprague–Dawley rats were subjected to subtotal hepatectomy under ether anesthesia and permitted to recover with free access to food and water. Four days later the regenerated livers were perfused using a modification of the method of Berry and Friend [18] and Bissell *et al.* [19]. The perfusate consisted of 0.01 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffered Hank's solution, pH 7.4, containing

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