belliferone glucuronidation (2-fold) was brought about by phenobarbital and TCDD. The conjugation of 2-aminophenol was elevated maximally (2-fold) by 3-methylcholanthrene. Hexachlorobiphenyls were the most potent PCBs to enhance monooxygenase and transferase activities; the effects of most PCBs resembled more those of phenobarbital than those of either 3-methylcholanthrene or TCDD. It was demonstrated that the elevation of drug-metabolizing enzyme activities by PCBs is determined by accumulation of the PCB in the target tissue. The activities of arylhydrocarbon hydroxylase and 7-ethoxycoumarin *O*-deethylase increased independently from each other since there was no correlation between the elevations of these monooxygenases. Similarly, 4-methylumbelliferone and 2-aminophenol transferases were found to be differentially induced. Interestingly, parallel induction of arylhydrocarbon hydroxylase and 2-aminophenol transferase activities, as well as of 7-ethoxycoumarin *O*-deethylase and 4-methylumbelliferone transferase activities, was found. The parallel inductions suggest coordinated regulation of the monooxygenase-transferase pairs.

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Enhancement of viral growth by the antitumor drug 4′-(9-acridinylamino) methanesulfon-*m*-anisidide (m-AMSA)*

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Patients undergoing extensive drug therapy for treatment of neoplastic disease often become more susceptible to viral infection due to the drug-associated immunosuppression. Patients treated with 4′-(9-acridinylamino) methanesulfon-*m*-anisidide (m-AMSA) are often ambulatory and exposed to viral infection. This report describes the drug augmentation of viral growth *in vitro*, independent of host immune-surveillance. The system consists of HeLa or Vero cells pretreated with m-AMSA and then infected with vaccinia virus or herpes simplex virus type I (HSV). This augmentation of viral growth produced by m-AMSA persists longer than the period of time required for cells to repair detectable DNA damage, suggesting that subtle and persistent alterations in cellular metabolism occur as a consequence of m-AMSA treatment.

m-AMSA is a cytotoxic agent currently undergoing phase III evaluation as a cancer chemotherapeutic agent [1–3]. The exact mechanism for the antitumor action of m-AMSA

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is unclear; however, several effects on cellular components have been reported. m-AMSA binds to DNA approximately one-tenth as tightly as the intercalating drugs adriamycin or proflavin [4, 5]. In cells treated with m-AMSA, chromosomal damage characteristic of intercalating agents occurs, i.e. (1) DNA or chromosomal breaks that are readily repaired [6, 7]; (2) incomplete chromosomal condensation [8]; and (3) stimulation of sister chromatid exchange and polyploidization [9]. m-AMSA fails to produce DNA damage in cells kept at 4° [10] or in purified DNA [6]. There are conflicting reports concerning the ability of m-AMSA to damage DNA in isolated nuclei [7, 11]; however, the evidence suggests that a cellular component other than DNA is probably necessary for damage to occur. This component may be a protein that has been reported to be associated with DNA in m-AMSA-treated cells [10]. m-AMSA also inhibits several cellular and viral DNA polymerases [8], but at concentrations that are in considerable excess of the LD_{50}; therefore, this direct interaction is not considered to be a primary cause of AMSA cytotoxicity.

Duplicate cultures of control cells and cells treated with m-AMSA (0.025 to 3.2 μg/ml) for 24 hr prior to its removal were subsequently infected with virus. One culture was used for determination of plaque-forming units (PFU) and enlarged plaques, and the other culture was used for determination of the number of infectious virus produced per culture. *Infectious virus yields* were determined by plaque assays of the latter cultures which had been frozen and thawed three times to release cell-associated virus. The ratio of the number of infectious virus produced per plaque-forming unit was determined using a multiplicity of infection of 0.001/cell. *Plaque assays* of the virus were performed by adding 0.25% human IgG to the culture medium 1 hr (2 hr for HSV) after adsorption. Two or three days later, neutral red, a dye that is taken up by living cells, was added. The next day the medium was decanted, the cells were dried, and the cell-free areas were measured and counted [12]. Enlarged plaques were up to 50% larger in diameter than controls but were defined as being at least 0.1 mm larger than the largest plaque observed in simultaneous control cultures. Control plaques were in a size range from 0.7 to 1.7 mm. Plaque size was measured using a Bausch and Lomb 7x measuring magnifier. *Recovery from m-AMSA treatment* was assessed by treating confluent monolayers with m-AMSA for 24 hr. The drug was removed, the monolayers were rinsed, and medium containing 2% fetal calf serum (FCS) was added. Plates were infected with virus and assayed for plaques as described above using these monolayers on days 1 through 10 after drug treatment.

HeLa cells, originally obtained from Flow Laboratories, Bethesda, MD, and Vero cells (African green monkey kidney), obtained from the American Type Culture Collection, Rockville, MD, were used in these experiments. Cells were maintained in continuous passage (or stored with glycerol in liquid nitrogen) in RPMI 1640 or McCoy's 5a medium plus 10–20% FCS and antibiotics (penicillin and streptomycin, 100 units/ml; amphotericin, 0.25 μg/ml). All culture experiments were conducted at 37° in an atmosphere of 95% room air and 5% CO_2 at 100% humidity. Vaccinia virus, originally obtained from the American Type Culture Collection, was passaged in HeLa cells. The KOS strain of HSV was provided by Dr. P. A. Schaffer (Sidney Farber Institute, Harvard Medical School). All experiments were conducted in RPMI media due to the lability of m-AMSA when exposed to L-cysteine—HCl [13].

m-AMSA was obtained from the National Cancer Institute, Bethesda, MD (NSC 141549). The drug was dissolved in ethyl alcohol and stored at −20°. m-AMSA was measured by fluorescence spectroscopy following conversion to its acridone derivative [14]. Cells were sonicated in 2 ml of distilled water, and 2 vol. of methanol was added per volume of suspension. Three milliliters of the suspension was then treated with 0.1 ml of the 1 M acetate buffer, pH 5.0, and 0.15 ml of mercaptoethanol. The mixture was heated at 80° for 1 hr in a tightly capped tube. The solution was cooled to room temperature and fluorescence was determined using an excitation wavelength of 390 nm and an emission wavelength of 415 nm on a Varian VSF-330 spectrofluorometer.*

Pretreatment of HeLa cells with m-AMSA for 24 hr allowed vaccinia virus to replicate to a greater extent in treated than in untreated cells (Fig. 1a). A dose-dependent increase in the number of enlarged plaques was observed up to the ED_{50} dose (as determined by inhibition of colony fomation) for HeLa cells (0.4 μg/ml). Below this dose, m-AMSA did not reduce the number of PFU; however, both the number of PFU and the number of enlarged plaques were reduced when the m-AMSA concentration was in excess of 0.4 μg/ml (data not shown). The increase in the number of enlarged plaques could occur due to an increased number of virus produced during the infection, an increase in the lytic ability of the virions, or a shortening of the viral replicative cycle. The graph in Fig. 1b indicates that m-AMSA pretreatment resulted in an increase in the number of PFU produced during the 96-hr incubation. However, the absolute number of virus produced per culture was not altered if the infection was allowed to proceed to completion (data not shown). Thus, the rate, rather than the extent, of infectious virus production was increased by m-AMSA under the conditions for the data illustrated in Fig. 1. It should be emphasized that m-AMSA was removed before adding the virus since vaccinia virus replication is inhibited by continuous exposure to m-AMSA (virus LD_{50} ~0.4 μg/ml). In experiments similar to that illustrated, augmentation of HSV growth was observed in Vero cells pretreated with m-AMSA, i.e. at 0.4 μg/ml there were 65% enlarged plaques. We confirmed the observation of Byrd [13] that HSV replication is not inhibited by continuous exposure to m-AMSA.

The augmentation of vaccinia virus growth produced by m-AMSA persists for several days following a 24-hr exposure of cells to 0.2 μg/ml (Table 1). The persistence of m-AMSA augmentation of vaccinia growth suggests that the drug, its metabolite(s), or an altered biological process is quite long-lived in the cells. As seen in Table 2, extensive uptake of m-AMSA occurs within 6 hr after drug addition. During the 24-hr exposure to m-AMSA, the media concentration of the drug was not altered; therefore, the intracellular and extracellular drug concentrations appear to have reached an equilibrium. Following removal of the drug at 24 hr, rapid and slow phases of m-AMSA release from the cells were observed. Measurable concentrations of m-AMSA persisted up to 4 days (96 hr in Table 2).

* This assay was developed at this institute by R. B. Hurlbert and D. R. Mills independently of the procedure of Gormley and Cysyk [P. E. Gormley and R. L. Cysyk, *Analyt. Biochem.* **96**, 504 (1979)]. In the presence of 66% methanol and mercaptoethanol under nitrogen and at pH 5, m-AMSA is thiolyzed quantitatively to the acridinyl mercaptide, with loss of the 4-amino methanesulfon-*m*-anisidide, in a few hours at room temperature. The adduct is readily degraded by mild heating in air to acridone. Alternatively, the overall conversion of m-AMSA to acridone is obtained in 45 min at 80° with none of the side products which are observable by reverse phase HPLC when m-AMSA is heated under alkaline conditions. The fluorescence of the product acridone is considerably greater at pH 5 than at alkaline pH, and the fluorescent yield from m-AMSA is at least 80–90% of the fluorescence from a molar equivalent of acridone. As applied here, the procedure detects primarily free m-AMSA, although certain other metabolites of m-AMSA may be thiolyzed by mercaptoethanol and/or degraded to acridone by heating.

Table 1. Enhancement of vaccinia virus growth in Vero cells*

Time (day) of virus inoculation	No. of plaques counted	% Enlarged plaques
1	348	16 ± 3
3	306	18 ± 3
5	336	15 ± 2
7	300	6 ± 3
10	270	3 ± 3

* Vero cells were treated on day 0 with m-AMSA (0.2 μg/ml). The drug was removed on day 1, and vaccinia virus was added on the days shown. The results given are mean values ± range for two separate experiments, each performed in triplicate.

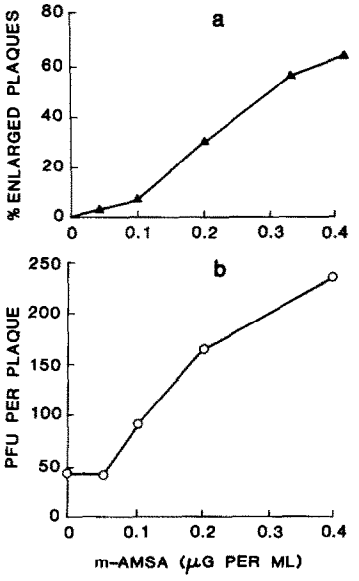

Fig. 1. Enhancement of vaccinia virus growth by m-AMSA. Stationary cultures of HeLa cells were pretreated for 24 hr with m-AMSA at the concentrations shown. The medium was removed, and the cells were inoculated with virus in fresh medium. Four days later, (a) plaque assays were harvested (top graph), and (b) simultaneous cultures were harvested and assayed to evaluate PFU produced per plaque (bottom graph). The results shown are mean values obtained from three experiments, each performed in duplicate.

Several antitumor agents have been reported to increase the capacity of cells to support viral growth, namely: 5-iododeoxyuridine [15]; methotrexate, 5-fluorouracil, mitomycin C [16]; and cyclophosphamide [17, 18]. However, except in the case of 5-iododeoxyuridine [15], the mechanisms by which increased viral growth occurred have not been studied in detail. Methotrexate, 5-fluorouracil, and mitomycin C have been shown to increase vaccinia virus production in cells of human origin by 100- to 1000-fold [16]. The optimum effect of these drugs occurs at a level that inhibits cell growth by 50%. Cyclophosphamide (an alkylating agent) markedly augmented vaccinia virus growth in Vero cells by increasing virus numbers [17, 18]. Furthermore, physical effects, such as DNA damage by ultraviolet light [12], will also increase the capacity of human cells to support HSV replication. In a previous study of the effect of m-AMSA on viral growth, Byrd [13] reported that m-AMSA inhibited vaccinia virus at non-cytotoxic concentrations; however, m-AMSA was found to have no antiviral effect on HSV, reovirus, or vesicular stomatitis virus. The work reported here adds m-AMSA to the list of antitumor agents that can increase viral growth *in vitro*.

Table 2. Uptake and disappearance of m-AMSA in HeLa cells*

Time after addition of m-AMSA (hr)	(ng/1 × 10^6 cells)
0 (blank)	0.9 ± 0.4
1	20 ± 6
6	48 ± 11
24	58 ± 9
Wash	
25	26 ± 5
30	16 ± 3
48	13 ± 2
72	11 ± 3
96	10 ± 3
120	6 ± 2

* HeLa cells in stationary culture were exposed to m-AMSA (0.4 μg/ml) for 24 hr, at which time medium was removed. At the times shown, the cells were rinsed and sonicated. m-AMSA was measured by fluorescence spectroscopy as described in the text. The results shown are mean values ± S.E. for three separate experiments.

Others have found that the efflux of m-AMSA from L1210 cells at concentrations used in these experiments was rapid and extensive over a 1-hr period [10, 19]. We have found a residual, though measurable, amount of m-AMSA in HeLa cells at least 24 hr and possibly up to 96 hr after removal of extracellular drug (Table 2). This residual drug may represent drug tightly bound to DNA and other cellular components. Since m-AMSA even at 96 hr exists in a form which yields an acridone derivative, it appears to maintain its structural integrity during this period; thus, long-term effects would not be totally unexpected. Although DNA strand breaks caused by m-AMSA apparently can be repaired in 1–6 hr after removal of the drug [6], the persistent augmentation of viral growth leads us to postulate that some other cellular alterations are not rapidly or efficiently repaired, resulting in more subtle and persistent metabolic changes.

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Relationships between propranolol plasma protein binding, glycoprotein concentration, and enzyme induction following phenobarbital administration in the dog*

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We have reported previously on the induction of the plasma protein binding of propranolol by phenobarbital, phenytoin, and Arochlor 1254 [1]. The mechanism of this increased binding was increased synthesis of α_1-acid glycoprotein (AAG). The known properties of these three substances as inducers of cytochrome P-450 suggested that the stimulation of AAG might be directly associated with induced drug metabolism. On the other hand, the induction of AAG could be an incidental finding. In the present work, we have performed a parallel experiment which measures the extent of antipyrine metabolism after cessation of phenobarbital administration. Antipyrine metabolism is known to depend on the level of hepatic cytochrome P-450 [2]. In this way, the time course of drug metabolism could be compared to the time course of AAG production and propranolol binding.

Most of the experimental details were presented in our earlier work [1]. Briefly, four male littermate beagle dogs were the experimental subjects. Phenobarbital was administered for 15 days at a dose of 180 mg/day, p.o. Antipyrine (15 mg/kg, i.v.) was dissolved in 5 ml of distilled water and filtered through a Millex-GS filter. Blood samples for antipyrine assay were obtained by syringe and placed in glass tubes containing heparin. The plasma was separated and frozen until assayed.

Plasma antipyrine was assayed by the method of Brodie *et al.* [3]. Blood samples were obtained prior to the antipyrine dose and at 30, 60, 90, and 120 min after injection. One antipyrine experiment was done before the animals were begun on phenobarbital and is designated as control. The occasion of the last phenobarbital dose was called day 0. Other antipyrine kinetic experiments were then performed on days 0, 5, 9, and 12.

For a flow-independent system the $T_{\frac{1}{2}}$ for a drug reflects the enzymatic competence for the process acting on it; the linear relationship is between λ, the elimination rate constant, and Q, the quantity of enzyme [4]. These λ values were used as the data reflecting the induction of antipyrine metabolism by phenobarbital. The percent induction was calculated as:

$$\frac{\lambda(t)\text{-}\lambda\ (\text{control})}{\lambda(0)\text{-}\lambda\ (\text{control})} \times 100 \qquad (1)$$

where $\lambda(t)$ is the observed rate constant on day 0, 5, 9, or 12 post-phenobarbital. A similar manipulation of the data previously obtained for AAG concentration and propranolol binding was performed [1].

The plasma antipyrine concentrations during various experimental conditions are shown in Fig. 1. The half-lives calculated between 30 and 120 min for antipyrine after intravenous doses in the four dogs for the five experimental periods are presented in Table 1. They show the anticipated progression with the shortest occurring immediately after the phenobarbital treatment and the longest during the control period.

The time course of the recovery of antipyrine metabolism from its induced state towards a control value can be examined in two ways. The λ values can be adjusted to the amount of enzyme which has been induced by subtracting the λ for the control period from the λ values during the phenobarbital decay phase. These data are presented in a semilogarithmic plot in Fig. 2. This shows an apparent

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