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ACTION OF ANTISCHISTOSOMAL DRUGS, HYCANTHONE AND ITS ANALOG IA-4 *N*-OXIDE, ON VIRAL INTERFERON INDUCTION

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Two antischistosomal drugs, hycanthone and its indazole analog IA-4 N-oxide, of which the former is a potent mutagen, differed markedly in their ability to depress interferon induction by influenza virus in rhesus monkey kidney (LLC-MK$_2$) cell cultures. At the concentration of 10 μg/ml, hycanthone reduced interferon yields as much as 73%; the same quantity of analog had no adverse effect on interferon induction. Pretreatment of cell cultures with the analog negated the inhibitory activity of hycanthone on viral interferon induction; however, this did not occur when the sequence was reversed. Interferon-mediated antiviral cellular resistance was not affected when cell cultures were pretreated with either hycanthone or IA-4 N-oxide. A possible association may exist between the mutagenicity of antischistosomal drugs and their ability to affect interferon synthesis.

INTRODUCTION

Although hycanthone is regarded as one of the best antischistosomal drugs, experimental studies of its mutagenic activity have shown that it is a potent mutagen in *Salmonella*, bacteriophage T4, *Saccharomyces*, *Neurospora*, and *Drosophila* organisms (Hartman and Hulbert, 1975; Bueding and Batzinger, 1977; Ong, 1978) and teratogen in mice and rabbits (Moore, 1972; Sieber et al., 1974), and has the ability to induce transformations in Rauscher murine leukemia virus-infected rat embryo cells (Hetrick and Kos, 1973) and to induce hepatic neoplastic lesions in partially hepatectomized mice (Tsuda et al., 1979). These undesirable properties have been markedly reduced by modifying the structure of the hycanthone molecule without altering its antischistosomal action. The indazole analog of hycanthone, IA-4 *N*-oxide, has only a fraction (0.6%) of the mutagenic potency, is significantly lower in acute toxicity, and is

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equipotent in schistosomacidal activity compared to hycanthone (Hulbert et al., 1974; Bueding, 1975; Batzinger and Bueding, 1977).

The effect of hycanthone on DNA, RNA, and protein synthesis has been documented and reviewed (Ong, 1978). However, little is known about the activity of this mutagen or IA-4 *N*-oxide on cellular control mechanisms involved in derepression of genes for interferon messenger RNA or, alternatively, on depletion of repressor content in cells leading to interferon synthesis (Stewart, 1979). Extensive studies by DeMaeyer and DeMaeyer-Guignard (1967) demonstrated that carcinogens (polycyclic aromatic hydrocarbons) decreased interferon formation in rat and C_3H mouse embryo fibroblasts and in L cell cultures, but structurally related noncarcinogens had no effect.

In view of the similar basic structure but difference in toxicity and mutagenic activity between hycanthone and IA-4 *N*-oxide, we were interested in determining and comparing the effects of these antischistosomal drugs on a cellular defense mechanism, the interferon system. This report describes the response of mammalian cell cultures treated with hycanthone or IA-4 *N*-oxide on viral infection induction and interferon-mediated antiviral cell resistance.

MATERIALS AND METHODS

Viruses

The A/PR/8/34 influenza and parainfluenza (Sendai) virus strains were obtained from the American Type Culture Collection (ATCC), Rockville, Md. Stocks of each virus strain were prepared from embryonated chicken eggs (Hahon et al., 1973). Influenza and Sendai virus pools contained 5.0×10^7 and 1.0×10^9 cell-infecting units (CIU) of virus per milliliter, respectively, when assayed by the immunofluorescent cell-counting procedure (Hahon et al., 1973).

Cell Cultures

Rhesus monkey kidney (LLC-MK_2) and Chang human conjunctiva (clone 1-5c-4) cell lines obtained from ATCC were used for induction and assay of interferon, respectively. Cell lines were propagated in plastic culture flasks (75 cm^2) with Eagle minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), both purchased from M. A. Bioproducts (Walkersville, Md.), and were maintained with MEM plus 0.5% FBS.

Chemicals

Hycanthone was donated by Dr. Ernest Bueding, Johns Hopkins University, Baltimore, Md. The indazole analog of hycanthone, IA-4 *N*-oxide, was donated by Dr. Leslie M. Werbel, Warner-Lambert/Parke-

Davis, Ann Arbor, Mich. Aqueous aseptic solutions of these chemicals were prepared by dissolving them in small amounts of dimethyl sulfoxide and filtering the solutions through a Nalgene (0.2 μm) filter unit (Nalge Corp., Rochester, N.Y.).

Interferon Induction

The following procedure was employed to study the effects of chemicals on viral interferon induction. Appropriate quantities of the chemicals (1–100 μg) in 10 ml maintenance medium were added to plastic flasks (75 cm^2) containing approximately 3×10^7 LLC-MK_2 cells in monolayer, which were then incubated at 35°C for 24 h. Maintenance medium was decanted, and 2 ml influenza virus (ultraviolet-irradiated for 45 s at a distance of 76.2 mm and wavelength of 253.7 nm) was added onto cell monolayers and incubated at 35°C for 2 h. The multiplicity of infection (ratio of input virus to cells) was approximately 1.0. Inoculum was removed and 10 ml maintenance medium was added to each flask, which was then incubated at 35°C for 24 h. Supernatant fluid was decanted, centrifuged at 100,000 × *g* for 1 h, dialyzed against HCl-KCl buffer (pH 2.0) at 4°C for 24 h, and then dialyzed against two changes of phosphate-buffered saline (pH 7.1) at 4°C for 24 h. Fluids were passed through 0.22-μm Millex filters (Millipore Corp., Boston, Mass.) to obtain sterile preparations. Samples were stored at −80°C until assayed for interferon activity. Controls, consisting of cell monolayers that were not pretreated with chemicals, were handled exactly as described above. Preparations exhibiting virus inhibitory activity had the properties ascribed to viral interferons (Lockart, 1973).

Interferon Assay

Samples were assayed for interferon in duplicate, using an immunofluorescent cell-counting procedure that has been described in detail (Hahon et al., 1975). Interferon-treated clone 1-5c-4 cell monolayers were challenged with 1×10^4 CIU of Sendai virus, and infected cells were visualized by direct fluorescent-antibody staining. The reciprocal of the interferon dilution that reduced the infected cell count to 50% of the control value—that is, the $ICDD_{50}$ (50% infected cell-depressing dilution)—served as the measure of interferon activity. With this assay system, 0.8 international reference human (69/19) interferon unit assayed as 1 U.

RESULTS

Because cell viability is a requisite for studies of viral interferon induction, the survival of LLC-MK_2 cell monolayers (3.0×10^7 cells) incubated for 24 h with different concentrations of hycanthone and IA-4 *N*-oxide was determined. Results (Table 1) show that hycanthone was more cytocidal than IA-4 *N*-oxide. Whereas either chemical at 10 μg/ml

TABLE 1. Effect of Hycanthone and IA-4 *N*-Oxide on Viability of LLC-MK_2 Cells

Quantity of chemical (μg/ml)	Surviving cells (%)[a]	
	Hycanthone	IA-4 *N*-oxide
50.0	43.1 ± 0.4[b]	71.6 ± 8.8
20.0	78.8 ± 2.2	103.7 ± 3.2
10.0	91.7 ± 5.4	100.0 ± 0.5
5.0	95.4 ± 0.4	100.1 ± 0.4
1.0	100.7 ± 4.6	100.3 ± 1.4
0 (control)	100.0	100.0

[a] Computed from two determinations as 100 X the number of living cells (trypan blue dye exclusion) in treated cell monolayers divided by the number of living control cells (1.2×10^7).
[b] Values are means ± SE.

minimally affected cell viability, hycanthone at 20 μg/ml or more was progressively more detrimental to cell viability than comparable concentrations of IA-4 *N*-oxide.

Various concentrations of both hycanthone and IA-4 *N*-oxide were added to LLC-MK_2 cell monolayers to compare the effects of the chemicals on interferon induction by influenza virus. Results (Table 2) reveal that increased concentrations of hycanthone, ranging from 0.1 to 10 μg/ml, progressively reduced viral interferon yields as much as 73% compared to that of controls. Comparable concentrations of IA-4 *N*-oxide had no adverse effect on interferon production.

TABLE 2. Effect of Hycanthone and IA-4 *N*-Oxide on Interferon Induction by Influenza Virus in LLC-MK_2 Cell Monolayers

Quantity of chemical (μg/ml)	Inhibition of interferon (%)[a]	
	Hycanthone	IA-4 *N*-oxide
10.0	73.5 ±0.3[b]	0.0 ± 0.0
5.0	38.2 ± 5.7	4.3 ± 1.4
1.0	19.5 ± 7.0	1.2 ± 0.9
0.1	9.2 ± 12.6	0.6 ± 0.6
0 (control)	0.0	0.0

[a] Computed from two experiments as 100% minus the interferon titer per milliliter from treated cell cultures divided by the control interferon titer of 175 or 80 (reciprocal of 50% infected cell-depressing dilution).
[b] Values are means ± SE.

To determine whether hycanthone or IA-4 *N*-oxide could alter interferon-mediated antiviral cellular resistance, clone 1-5c-4 cell monolayers were pretreated for 24 h with the test chemicals, and then an interferon preparation of known potency was assayed in the usual manner with Sendai virus as the challenge agent. The mean interferon titers of two determinations in cells treated with hycanthone or IA-4 *N*-oxide were 172 and 170 $ICDD_{50}$, respectively. These titers were comparable to that of 175 $ICDD_{50}$ attained in untreated (control) cell cultures. The findings indicate that the ability of interferon to confer antiviral protection was not affected by prior treatment of cells with either of these chemicals.

To eliminate the possibility that reduced interferon yields may be related to inactivation of interferon by hycanthone, a known interferon preparation was mixed with 10 μg/ml hycanthone and incubated at 35°C for 24 h. A similar procedure was carried out with IA-4 *N*-oxide and maintenance medium (control). No significant difference in interferon titers was found between these preparations and the control preparation; which indicates that the chemical had no deleterious effect on interferon per se.

That the depression of viral interferon production may be the consequence of an impediment of primary virus-cell interactions by hycanthone, resulting in decreased virus infectivity, was investigated by using influenza virus inoculum that contained hycanthone (10 μg/ml), IA-4 *N*-oxide (10 μg/ml), or maintenance medium (control) to infect cell monolayers. The equivalent virus infectivity titers attained with all three inocula (2.2×10^7, 3.0×10^7, and 3.0×10^7 CIU/ml) indicate that early virus-cell interactions (i.e., virus attachment and penetration) were not significantly affected by the presence of the chemicals.

Table 3 shows the results of an experiment to determine whether

TABLE 3. Blocking of Inhibitory Effect of Hycanthone on Viral Interferon Induction by Pretreatment of LLC-MK_2 Cell Monolayers with IA-4 *N*-Oxide

Pretreatment of cell monolayer[a]	$ICDD_{50}$/ml[b]	Interferon inhibition (%)
5.0 μg/ml IA-4 *N*-oxide (24 h) + 10.0 μg/ml hycanthone (24 h)	118	0
1.0 μg/ml IA-4 *N*-oxide (24 h) + 10.0 μg/ml hycanthone (24 h)	43	64.6
5.0 μg/ml IA-4 *N*-oxide (24 h)	120	0
10.0 μg/ml hycanthone (24 h) + 10.0 μg/ml IA-4 *N*-oxide (24 h)	26	78.0
10.0 μg/ml hycanthone (24 h)	25	78.9
Maintenance medium (control)	118	0

[a] Designated amounts of chemicals in 10 ml maintenance medium were used to pretreat cell monolayers at 35°C before addition of ultraviolet-irradiated influenza virus (inducing agent).

[b] Reciprocal of 50% infected cell-depressing dilution per milliliter.

similar macromolecular compounds or receptor sites were involved in the interaction of hycanthone or IA-4 *N*-oxide with cells, leading to the induction of interferon. When cell cultures were pretreated with 5 or 1 μg/ml IA-4 *N*-oxide and then with 10 μg/ml hycanthone, the adverse effect of hycanthone on viral interferon induction was nullified. At 5 μg/ml the analog eliminated hycanthone, and at 1 μg/ml it reduced the effect to 35%. The analog, however, could not limit the inhibitory action of hycanthone when cell cultures were pretreated with hycanthone. It appears that both chemical agents interact with similar cell macromolecules or sites involved in interferon induction.

DISCUSSION

The findings of this study demonstrate that two structurally related antischistosomal drugs, hycanthone and IA-4 *N*-oxide, of which the former is a potent mutagen, differ significantly in their ability to affect the induction of interferon by influenza virus in mammalian cell cultures. Hycanthone was not only more toxic to cell cultures but depressed interferon production. Whereas 10 μg/ml hycanthone reduced interferon yields as much as 73%, the same concentration of IA-4 *N*-oxide had no adverse effect on interferon synthesis. In general, our findings agree with those of earlier studies of the inhibition of interferon synthesis by mutagenic and carcinogenic hydrocarbons and the lack of effect of structurally related noncarcinogenic compounds on the interferon system (DeMaeyer and DeMaeyer-Guignard, 1967). In contrast to the earlier studies, where the phenomenon was noted in murine but not in primate cell cultures, we observed the inhibitory effect of hycanthone on interferon production in primate cell cultures. The refractoriness of monkey cells to the carcinogenicity of polycyclic aromatic hydrocarbons may account for this.

Our experiments eliminated the possibility that depression of interferon production may be accounted for by inactivation of interferon or inhibition of early virus-cell interactions by hycanthone. Depending on the test system examined and the concentration used, hycanthone may inhibit either DNA, RNA, or protein synthesis (Ong, 1978). The main effect of hycanthone on the synthesis of macromolecules is probably on transcription and DNA replication rather than translation. Although the mechanism of hycanthone intervention in viral induction of interferon is not known, it appears that the drug acts on cell regulatory functions rather than on virus infectivity or replicative processes.

Interferon-mediated antiviral cellular resistance was not affected when cell cultures were pretreated with hycanthone or IA-4 *N*-oxide. This has also been noted with other mutagenic and carcinogenic agents that inhibit viral induction of interferon (Hahon and Eckert, 1976; Hahon et al., 1979). It implies that the antiviral state established by interferon treat-

ment, which may involve such processes as binding of interferon molecules to cell membrane sites or transcription or translation inhibition of viral messages in interferon-treated cells (Stewart, 1979), was not affected by the antischistosomal drugs.

That cells preincubated with structurally related noncarcinogenic hydrocarbons became resistant to the interferon-inhibitory effect of the carcinogens (DeMaeyer and DeMayer-Guignard, 1967) was also noted in our study of hycanthone and IA-4 *N*-oxide. Pretreatment of cell cultures with the analog negated the adverse effect of hycanthone on interferon synthesis; however, this did not occur when the sequence was reversed. Although the mechanism for this phenomenon remains to be elucidated, it is possible that IA-4 *N*-oxide either impaired the penetration of hycanthone into the cell by blocking critical receptor sites or prevented hycanthone from interacting with macromolecules within the cell that are involved in the viral interferon induction process.

By altering the chemical structure of mutagenic schistosomal drugs, eliminating the enteric bacteria responsible for mutagenic activation, or pretreating the host with antioxidants, the mutagenicity of antischistosomal chemicals may be nullified without loss of their useful pharmacological properties (Bueding and Batzinger, 1978). Evidence from numerous studies with indazole analogs of hycanthone, other schistosomicidal drugs, and structurally related compounds supports the concept that the property of mutagenicity is independent of antischistosomal activity (Bueding, 1975; Bueding and Batzinger, 1977; Ong, 1978). From our studies, it appears that the action of hycanthone and IA-4 *N*-oxide on interferon induction is also unrelated to antischistomal activity. Although we studied a limited number of schistosomicidal drugs, our evidence suggests a relationship between mutagenicity and depression of interferon synthesis.

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