Hydroxyquinolines Inhibit Ribonucleic Acid-Dependent Deoxyribonucleic Acid Polymerase and Inactivate Rous Sarcoma Virus and Herpes Simplex Virus

WOLFGANG ROHDE,¹ PETER MIKELENS, JEAN JACKSON, JANE BLACKMAN, JACK WHITCHER, AND WARREN LEVINSON*

Department of Microbiology and Proctor Foundation for Research in Ophthalmology, University of California, San Francisco, California 94143

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8-Hydroxyquinoline and several of its derivatives inactivate the transforming ability of Rous sarcoma virus and inhibit its ribonucleic acid-dependent deoxyribonucleic acid polymerase activity. The copper complex of these metal-binding ligands is as active as the free ligand. The activity of the 8-hydroxyquinolines is approximately 50-fold more effective than another group of metal-binding compounds that we have tested, the thiosemicarbazones. In contrast to the potency of the 8-hydroxyquinolines to inactivate Rous sarcoma virus, no intracellular inhibition of transformation could be demonstrated at a concentration that did not affect the growth and appearance of the cells. Cellular deoxyribonucleic acid synthesis was inhibited to a greater extent than was ribonucleic acid or protein synthesis. The phenomenon of "concentration quenching" was observed with high concentrations of drug, causing less inhibition of deoxyribonucleic acid synthesis than was observed with lower concentrations. Herpes simplex virus type 1 was inactivated also by the 8-hydroxyquinolines and their copper complexes. No intracellular inhibition of plaque formation was observed. Treatment with 8-hydroxyquinoline sulfate had no effect on the resolution of herpetic keratitis in rabbits. Some 8-hydroxyquinolines bind to deoxyribonucleic acid in the presence of copper, a phenomenon that may be important in their antiviral activity.

N-methyl isatin β -thiosemicarbazone (M-IBT) (Methisazone) inhibits the ribonucleic acid (RNA)-dependent deoxyribonucleic acid (DNA) polymerase of Rous sarcoma virus (RSV) and inactivates its malignant transforming ability (15). Copper is involved in the action of this drug since ethylenediaminetetraacetic acid prevents the antiviral effect (15), copper synergizes it (15), and a preformed, purified M-IBT-copper chelate complex is equally effective as is the free ligand (W. Kaska and W. Levinson, unpublished data). In addition, M-IBT-copper complexes, but not M-IBT alone, inhibit transfection by purified lambda phage DNA in *Escherichia coli* (16). Also, we have shown that the copper chelating agent orthophenanthroline inhibits the RNA-dependent DNA polymerase of RSV (21).

In view of this activity, we wished to determine whether other copper-binding ligands act in a similar manner. For this purpose we chose to investigate 8-hydroxyquinoline (8-HQ) and

some of its derivatives. 8-HQ is the only one of seven isomeric monohydroxyquinolines that has antibacterial activity and the only one that forms chelate complexes with divalent metal ions (4). The inhibitory activity of 5×10^{-6} M 8-HQ against *Staphylococcus aureus* was markedly enhanced by the presence of 4×10^{-5} M copper sulfate (20). Other cations were much less effective. 8-HQ possesses inhibitory activity in eukaryotic cells also since it inhibits RNA synthesis in yeast (9) and the copper (II) bis-chelate of the halogenated 5,7-derivative has antifungal activity (10). Recently, it was shown that 8-HQ inhibits the RNA-dependent DNA polymerase of avian myeloblastosis virus (6).

In this report, we show that 8-HQ and several of its derivatives inactivate the transforming ability of RSV and inhibit its RNA-dependent DNA polymerase. The nucleic acid of the virion may be the site of action since, in the presence of copper, these drugs bind to DNA. 8-HQ, its 5-chloro-7-iodo-derivative (Entervioform), and their copper complexes inactivate the infectivity of herpes simplex virus (HSV) type 1. Chicken embryo cell DNA synthesis is in-

¹ Present address: Institut für Virologie, Justus Liebig Universität, D-6300 Giessen, German Federal Republic.

hibited to a greater extent by 5-chloro-8-iodo-8-HQ than is RNA and protein synthesis.

MATERIALS AND METHODS

Virus and cells. The B-77 strain (group C) of RSV was propagated in chicken embryo cells as described in reference 7, and its transforming ability was assayed by focus formation as described in reference 17. The HSV type I was propagated and assayed in Vero cells (14).

RNA-dependent DNA polymerase assay. The enzyme assay used to evaluate these drugs has been described (15). Some experiments used the non-metal-chelating HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer rather than tris(hydroxymethyl)aminomethane (Tris) buffer, which chelates copper (11). No significant difference between the results using the two buffers was seen.

Nucleic acid-binding assay. The nucleic acid-binding assay has been described (18a).

Chemicals. The sources of the chemicals used in this study were: 5-chloro-7-iodo-8-HQ, 5,7-diiodo-8-HQ, 5,7-dichloro-8-HQ, 5-chloro-8-HQ, quinolines, and M-IBT from K and K Laboratories; 8-HQ-copper, 5-nitro-8-HQ, 7-iodo-5-sulfonic acid-8-HQ, 5-sulfonic acid-8-HQ, 8-mercaptoquinoline, 8-aminoquinoline, and 8-HQ sulfate salt from Sigma Chemical Co.; 8-HQ from Matheson, Coleman and Bell; 8-hydroxyquinoline from Aldrich; 8-nitroquinoline from Eastman Kodak; and 5-chloro-7-iodo-8-HQ-copper and M-IBT-copper from W. Kaska, Univ. of California-Santa Barbara.

RESULTS

Inhibition of RNA-dependent DNA polymerase of RSV. The inhibitory activity of 8-HQ and its copper complex is depicted in Fig. 1. It can be seen that the inhibitory dose₅₀ (ID₅₀) for both compounds is 2×10^{-7} M, that little if any inhibition occurs at 5×10^{-8} M, and that greater than 90% inhibition occurs with the copper salt at 5×10^{-7} M. The inhibitory activity of M-IBT, our standard reference compound, and its copper complex is shown in Fig. 2. The ID₅₀ for M-IBT is 1.5×10^{-5} M and that for the copper complex is 7×10^{-6} M. The inhibition by the hydroxyquinoline compounds is approximately 50-fold more effective than that by the thiosemicarbazones.

The kinetics of inactivation of enzyme activity by 10^{-5} M 8-HQ is illustrated in Fig. 3. Fifty percent inhibition occurs at approximately 9 min, and 90% occurs at 40 min.

The data in Table 1 describe the inhibitory activity of 8-HQ and several of its derivatives in the presence and absence of equimolar copper sulfate. The 5-nitro- and 5-chloro-substituted compounds are the most active. They are approximately four times better than is the parent compound. The 5-chloro-7-iodo, 5,7-dich-

FIG. 1. Inhibition of RNA-dependent DNA polymerase by 8-HQ and its copper complex. A total of 5 μ l of purified B-77 strain of RSV, suspended in 200 μ l of solution A (see below), was exposed to the drug by the addition of 2 μ l of solution and incubated at 37°C for 15 min. A total of 20 μ l of solution B (see below) was added, and the reaction mixture was incubated at 37°C for 60 min. Acid-precipitable counts were obtained by the addition of 0.5 ml of 0.01 M sodium pyrophosphate and 2 ml of 3.5% perchloric acid in 0.01 M sodium pyrophosphate. The precipitate was filtered onto glass fiber filters, dried, and counted in a liquid scintillation spectrometer. Solution A contained 10 mM phosphate buffer, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, and 25 μ M deoxyadenosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, and deoxycytidine 5'-triphosphate. Solution B contained 90 μ l of [³H]thymidine 5'-triphosphate (17 Ci/mmol), 45 μ l of 1% Nonidet P-40 and 5 μ l of 2-mercaptoethanol. The final concentrations of these reagents in the reaction mixture were 7.5 μ Ci/ml, 0.03%, and 0.3%, respectively. Stock solutions of drugs in 100% Me₂SO were used. No inhibition of enzyme activity was observed with 1% Me₂SO. Symbols: ○, 8-HQ; ●, 8-HQ-copper complex.

FIG. 2. Inhibition of RNA-dependent DNA polymerase by M-IBT and its copper complex. The enzyme assay was performed in the same manner as described in the legend to Fig. 1. Symbols: ○, M-IBT; ●, M-IBT-copper complex.

loro, and 5,7-diiodo are approximately as active as the parent 8-HQ. The 5-sulfonic acid and the 2-methyl-derivatives are much less active. 8-

FIG. 3. Kinetics of inhibition of enzyme activity by 8-HQ. The enzyme assay was performed in the same manner as described in the legend to Fig. 1, except that the samples were taken at 10-min intervals after the addition of the drug. Symbols: $\circ$, 1% Me_2SO ; $\bullet$, 10^{-5} M 8-HQ.

TABLE 1. Effect of quinoline derivatives on RNA-dependent DNA polymerase of RSV^a

Compound	ID ₅₀ (M)	
	No addition	+ CuSO_4 (1:1)
8-HQ	2×10^{-7}	2×10^{-7}
5-NO ₂ -8-HQ	5×10^{-8}	3×10^{-8}
5-Chloro-8-HQ	5×10^{-8}	6×10^{-8}
5-Chloro-7-iodo-8-HQ	2×10^{-7}	1×10^{-7}
5,7-Dichloro-8-HQ	9×10^{-8}	2×10^{-7}
5,7-Diiodo-8-HQ	9×10^{-8}	1×10^{-7}
2-CH ₃ -8-HQ	9×10^{-7}	4×10^{-7}
5-SO ₃ H-8-HQ	6×10^{-6}	5×10^{-6}
5-SO ₃ H-7-iodo-8-HQ	9×10^{-6}	2×10^{-6}
8-SH-quinoline	1×10^{-6}	2×10^{-7}
8-NH ₂ -quinoline	3×10^{-6}	9×10^{-7}
8-NO ₂ -quinoline	No ^b	No
Quinoline	No	No
M-IBT	1.5×10^{-5}	7×10^{-6}
CuSO_4	7×10^{-6}	

^a The enzyme assay was performed in the same manner as described in the legend to Fig. 1.

^b No, No ID₅₀ below 100 μM .

HQ is significantly more active than the 8-sulphydryl- and 8-amino-derivatives. The 8-nitro-derivative and quinoline itself have no activity at the highest concentration tested. The addition of copper sulfate to the reaction did not have an appreciable effect on the inhibition of enzyme activity, except in the case of the 8-mercapto-derivative, which was enhanced five-fold. Preformed, purified copper complexes of 8-HQ and its 5-chloro-7-iodo-derivative are as active as the free ligands alone (data not shown).

An experiment was performed to determine the rapidity with which 8-HQ inhibited an enzyme reaction that had already started. The data in Fig. 4 indicate that, when the 8-HQ-copper complex is added 10 min after synthesis has begun, incorporation of [³H]thymidine 5'-triphosphate is inhibited gradually. In contrast, the addition of the M-IBT-copper complex causes a prompt inhibition of [³H]thymidine 5'-triphosphate incorporation. The addition of 10% Me_2SO as a solvent control inhibited incorporation in a gradual but significant manner. It appears that the M-IBT-copper complex acts rapidly to inhibit elongation of the DNA, whereas the 8-HQ-copper complex does not.

Inactivation of RSV-transforming ability.

Several of the active compounds were evaluated for their ability to inactivate the transforming ability of RSV. The data in Table 2 demonstrate that these compounds are also effective inactivators of transformation by RSV. Both the free ligands and the copper complexes inactivated transforming ability by 99% at 4×10^{-8} M, and the copper complexes possessed 50% activity at 4×10^{-9} M. The hydroxyquinolines are vastly more active than is M-IBT. A comparison of this data with that in Table 1

FIG. 4. Kinetics of inhibition of enzyme activity after synthesis for 10 min. The enzyme assay was performed in the same manner as described in the legend to Fig. 1, except that HEPES buffer was used instead of Tris buffer, the drug was not added until 10 min after solution B was added, and samples were taken at 10-min intervals. Symbols: $\circ$, none; $\bullet$, 10% Me_2SO ; $\square$, 8-HQ-copper complex; $\blacksquare$, M-IBT-copper complex.

TABLE 2. Inactivation of RSV transformation by hydroxyquinolines^a

Compound	M	Inhibition (%)
8-OH-quinoline	4×10^{-8}	99
8-OH-quinoline-copper	4×10^{-8}	99
8-OH-quinoline-copper	4×10^{-9}	50
5-Chloro-7-iodo-8-OH-quinoline	4×10^{-8}	99
5-Chloro-7-iodo-8-OH-quinoline-copper	4×10^{-8}	99
5-Chloro-7-iodo-8-OH-quinoline-copper	4×10^{-9}	50
M-IBT	4×10^{-5}	99
M-IBT	4×10^{-6}	50
CuSO ₄	2×10^{-6}	50

^a The virus was exposed to the drug in the same manner as described in the legend to Fig. 1 and incubated at 37°C for 15 min, at which time a 5- μ l portion was taken and a 1:100 dilution was assayed for focus formation as described previously (17).

indicates that focus formation is significantly more sensitive to these drugs than is polymerase activity.

Inhibition of transformation by RSV. In view of the ability of the 5-chloro-7-iodo-derivative to inactivate transformation, we determined whether it could inhibit focus formation when the cells were exposed to the drugs after the 30-min adsorption and penetration period of viral infection. The drugs were included in the agar-growth medium overlay for the entire 6-day assay period. Variable results were obtained. A 50 and 75% inhibition were observed at 2 and 4 μ M, respectively, in some experiments but not in others. Cell growth was visibly decreased at 8 μ M.

Inhibition of cellular DNA, RNA, and protein synthesis by 5-chloro-7-iodo-HQ. In view of the finding that cell growth was visibly affected by 5-chloro-7-iodo-HQ at 8 μ M, experiments were performed to determine whether DNA, RNA, or protein synthesis was inhibited in chicken embryo cells. The data expressed in Fig. 5 indicate that, after 1 h of exposure to the drug at 20 μ M, DNA synthesis is inhibited 97%, RNA synthesis is inhibited 91%, and protein synthesis is inhibited 83%. At all drug concentrations tested, DNA synthesis was inhibited to the greatest extent. The effect of the drug at higher concentrations (40 and 80 μ M) is aberrant since these amounts inhibit less well than does 20 μ M. This relative loss of activity at higher concentrations is similar to the effect of this compound on the RNA-dependent DNA polymerase; viz., at 4×10^{-7} and 1×10^{-6} M the enzyme is 95% inhibited, but at 4×10^{-6} M virtually no inhibition was obtained. This un-

sual effect has been observed in other systems, and a possible explanation is described below.

Inactivation of HSV. The inactivation of RSV, an RNA tumor virus, prompted us to attempt to extend the antiviral spectrum to other classes of viruses. Table 3 shows that HSV type 1 is significantly inactivated by 8-HQ, the 5-chloro-7-iodo-derivative, and their preformed copper complexes. Using the compounds listed in Table 3, no inhibition of plaque formation was seen at any drug concentration (between 4 and 8 μ M) that did not visibly alter cell growth (data not shown).

In view of the marked inactivation of herpes simplex infectivity by 8-HQs, we tested the ability of the water-soluble salt 8-HQ sulfate to ameliorate the course of herpes keratitis in rab-

FIG. 5. Inhibition of cellular DNA, RNA, and protein synthesis by 5-chloro-7-iodo-8-HQ. A total of 1×10^6 chicken embryo cells was seeded onto 50-mm plastic culture dishes in 5 ml of medium 199 plus 4% calf serum and incubated at 37°C for 24 h. The medium was changed and the cells were exposed to the drug by the addition of 5- μ l solution. The cultures were incubated at 37°C for 1 h when either [³H]thymidine (0.2 μ Ci/ml; specific activity, 13 Ci/mmol), [³H]uridine (0.2 μ Ci/ml; specific activity, 23 Ci/mmol), or [³H]reconstituted protein hydrolysate (4 μ Ci/ml) was added. The cultures were incubated for a further 30 min at 37°C when the medium was discarded, and the cells were washed with Tris-buffered saline and solubilized with 1 ml of 1% sodium dodecyl sulfate in Tris-buffered saline with 10^{-3} M ethylenediaminetetraacetic acid. The acid-precipitable radioactivity was determined by adding 5 ml of 10% trichloroacetic acid at 4°C, filtering onto glass fiber filters, and counting in a liquid scintillation spectrometer. Symbols: $\circ$, DNA; $\square$, RNA; Δ , protein.

TABLE 3. Inactivation of HSV^a

Compound	M	Inhibition (%)
8-HQ	4×10^{-7}	75
8-HQ-copper	4×10^{-7}	80
5-Chloro-7-iodo-8-HQ	4×10^{-8}	90
5-Chloro-7-iodo-8-HQ-copper	4×10^{-8}	95

^a A 1- μ l amount of a solution of the compounds was added to 1 ml of a 1:200 dilution of HSV type 1 in phosphate-buffered saline. After incubation at 37°C for 15 min, a 1:100 dilution of the virus in medium 199 + 15% calf serum was made, and a 0.2-ml portion was assayed for plaque-forming ability.

bits (14). The infection was initiated by instilling 2 drops of a HSV type I (PH strain) suspension (titer, 2×10^7 plaque-forming units/ml) in both eyes of New Zealand white rabbits. After 72 h, when corneal lesions were visible by slit-lamp examination, the treatment of one eye of each rabbit with 0.1% (wt/vol) 8-HQ sulfate in 50% polyethylene glycol 4000 (2 drops every 2 h for 14 h) was begun and continued for 5 days. The control eye of each animal was treated with the suspending vehicle alone. The course of the disease was followed by daily slit-lamp examinations and by HSV infectivity assays three times a week. No effect of the drug on the course of the disease or on the amount of herpesvirus produced was observed.

Binding of hydroxyquinolines to DNA. We have demonstrated that M-IBT-copper complexes bind to DNA and RNA and have postulated that their antiviral effect may be due to their ability to bind to nucleic acid (18a). We have, therefore, determined whether the 8-HQs bind to DNA. The data in Table 4 indicate that 5-chloro-7-iodo-8-HQ (40 μ M) in the presence of CuSO₄ (10 μ M) is approximately as good as an M-IBT-copper complex in its ability to bind DNA. The 5-chloro-7-iodo-8-HQ (40 μ M) without added copper complex is significantly less active. The preformed complex of 5-chloro-7-iodo-8-HQ and copper is markedly more active than both the free ligand and the ligand to which exogenous CuSO₄ is added. This may be a function of the complex being in the correct ratio of ligand to metal (either 1:1 or 2:1) for DNA binding. 8-HQ, with or without added copper, possesses much less DNA-binding ability than its 5-chloro-7-iodo-derivative. The 8-HQ-copper complex is as active as 8-HQ to which exogenous copper is added.

DISCUSSION

The results presented in this paper extend the spectrum of metal-binding ligands that inhibit RNA-dependent DNA polymerase and in-

activate the transforming ability of RSV to include 8-HQ, several of its derivatives, 8-mercaptoquinoline, and 8-aminoquinoline. Other metal-binding ligands, which have the same activities, are M-IBT and other isatin β -thiosemicarbazones (15), ortho-phenanthroline (21), and isonicotinic acid hydrazide, which is the drug of choice for tuberculosis (W. Levinson, manuscript submitted for publication).

8-HQ is known to chelate copper (4), and the ability of the drug to inhibit *S. aureus* is enhanced by copper (20). However, no enhancement of the activity of 8-HQ to inhibit the RNA-dependent DNA polymerase was seen when equimolar copper was added. This could be due to the presence of sufficient copper or other first transition series metal either in the solutions or in the virion itself to form an active chelate complex. The finding of copper in the vaccinia virion lends support to the conjecture that the metal may be in the virus particle (13).

A study has been made of 8-HQ derivatives that chelate metals using a precipitation assay (5). Of the derivatives tested in Table 1 of this report, it was found that 8-HQ, its 5-NO₂, 5-chloro-5,7-dichloro-, 5-chloro-7-iodo-, and 2-CH₃-derivatives, and 8-mercaptoquinoline formed precipitates with copper and other metals. All of these strongly inhibit the RNA-dependent DNA polymerase. The two 5-sulfonic

TABLE 4. Binding of hydroxyquinolines to DNA^a

Compound	μ M	CuSO ₄ (μ M)	cpm bound (%)
None		0	3
M-IBT	50	10	100
5-Chloro-7-iodo-8-HQ	10	10	11
5-Chloro-7-iodo-8-HQ	20	10	63
5-Chloro-7-iodo-8-HQ	40	10	92
5-Chloro-7-iodo-8-HQ	40	0	29
5-Chloro-7-iodo-8-HQ-copper	0.6	0	8
5-Chloro-7-iodo-8-HQ-copper	1.2	0	52
5-Chloro-7-iodo-8-HQ-copper	2.4	0	79
8-HQ	200	0	1
8-HQ	100	50	10
8-HQ	200	500	30
8-HQ-copper	200	0	30

^a Reactions contained ³H-labeled HeLa cell DNA (275 ng) in 0.5 ml of 0.05 M phosphate buffer, pH 8.0. Appropriate volumes of drug solutions in 100% Me₂SO were then added. After incubation at 4°C for 5 min, the solutions were filtered through 0.45- μ m membrane filters (Millipore), dried, and counted in a Beckman liquid scintillation spectrometer. No filter retention was seen with 10 μ l of Me₂SO, the largest volume added.

acid derivatives were found to cause a color change (but no precipitate) only with ferrous and ferric ions; these compounds inhibit enzyme activity but are not as active as those that form definite precipitates. The 8-NH₂-quinoline does not cause a color change or precipitate with any metal, yet it is an enzyme inhibitor. The reason for this discrepancy is not clear. Quinoline itself does not change color or precipitate and does not inhibit the enzyme. The 5,7-diiodo-derivative was not tested in the precipitation assay.

Our initial interpretation of the correlation between the inactivation of RSV-transforming ability and the inhibition of its RNA-dependent DNA polymerase was one of cause and effect. However, since transforming ability is significantly more sensitive than is enzyme activity, we speculate that there may be an additional inactivating event of an unknown nature occurring.

The failure of these compounds to inhibit transformation of chicken cells after infection by RSV can be attributed to their lack of selective toxicity; e.g., the drugs significantly inhibit cellular processes as well. Another possibility is that virus-specific DNA synthesis is completed since the drug is added to the culture after the adsorption period. This explanation is unlikely since we have found recently that several thiosemicarbazone derivatives do inhibit transformation by RSV when added after the adsorption period.

The data in Table 4 demonstrate that the copper complexes of 8-HQ and its 5-chloro-7-iodo-derivative bind to DNA, significantly more so than do the free ligands. The binding of the drug to nucleic acid could be important in the inhibition of the RNA-dependent DNA polymerase, but the lack of correlation of the extent of binding to the activity of the compounds calls this supposition into question. For example, the ID₅₀ of 8-HQ and 5-chloro-7-iodo-HQ are the same (Table 2), yet their ability to retain DNA on the filter is markedly different (Table 4).

A second puzzling aspect of the DNA-binding data is the markedly enhanced ability of the preformed copper complex of 5-chloro-7-iodo-8-HQ compared with the free ligand to which equimolar copper was added. This raises the question of the relationship between the stoichiometry of the copper complex and its antiviral activity. It has been shown previously (3, 8) that the 2:1 and 1:1 ligand-metal complexes have different biological and chemical effects. It is possible, therefore, that the preformed complex has a different ratio from that formed

in the binding assay by mixing equimolar ligand and copper. In addition, these reports indicate that only the 1:1 complex possesses antibacterial and antifungal activity. These findings may explain the results shown in Fig. 5; e.g., as the concentration of ligand is increased, the inactive 2:1 complex is formed in greater amounts, which results in the observed loss of activity. This phenomenon has been given the term "concentration quenching" (2).

In addition to the possibility that these drugs act by binding to the viral nucleic acid, an alternative explanation for the ability of these drugs to inhibit the RNA-dependent DNA polymerase is that the drug binds to the enzyme. Since zinc is known to be present in the enzyme, it has been proposed that metal-binding ligands inhibit by binding to this atom (6, 21). The results presented in this report cause us to question this interpretation. Since we have shown that the preformed copper complex is equally as effective as the free ligand and it has been reported previously that the stability constant for the copper complex is 10³- to 10⁴-fold greater than for the zinc complex (1), it seems unlikely that the ligand in the copper complex form can bind to the zinc in the enzyme.

Lastly, it is interesting to note two recent findings regarding compounds similar to those described here. First, 4-nitroquinoline-1-oxide is a chemical carcinogen that is known to cause covalent bond breakage in DNA, increased integration of simian virus 40 DNA into cellular DNA, and increased transformation of CHO cells by simian virus 40 (12). Second, 5,8-dioxo-6-amino-7-chloroquinoline inhibits protein synthesis in *E. coli* apparently by preventing the charging of transfer RNA (19). In both cases an interaction of the drugs with nucleic acids either is or may be important in the mode of action.

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