Heterocyclic Thiosemicarbazones: Correlation Between Structure, Inhibition of Ribonucleotide Reductase, and Inhibition of DNA Viruses[1] (34528)

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(Introduced by F. M. Schabel, Jr.)

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Thiosemicarbazones were introduced as chemotherapeutic agents against tuberculosis by Domagk and his co-workers (1, 2). Hamre *et al.* found that certain compounds of this class inhibited poxviruses (3). Thompson *et al.* (4, 5) and Bauer and Sadler (6, 7) continued these studies which eventually led to the preparation of isatin thiosemicarbazone and its 1-alkyl derivatives. One member of this class of compounds, 1-methylisatin 3-thiosemicarbazone (methisazone), has been shown to be effective in reducing both morbidity and mortality of smallpox in unvaccinated humans previously exposed to active cases of the disease (8, 9).

Some years ago we observed that 2-formylpyridine thiosemicarbazone (picolinaldehyde thiosemicarbazone; pyridine-2-carboxaldehyde thiosemicarbazone) possessed antileukemic activity in mice (10). French and his associates pursued these initial observations and prepared a series of heterocyclic aldehyde thiosemicarbazones (11-15). Certain of these compounds exhibited anticancer activity, namely, those having a heteroaromatic ring system with the thiosemicarbazone moiety adjacent to the ring nitrogen atom, as in 2-formylpyridine and 1-formylisoquinoline thiosemicarbazones and hydroxy derivatives of these compounds (14, 15). Representatives of this class of compounds with these specific structural features inhibited the growth of several experimental neoplasms, inhibited the incorporation of thymidine-^{3}H into the DNA of sarcoma 180 ascites tumor cells, and inhibited the reduction of cytidine diphosphate to the deoxyribonucleotide (16, 17).

The observation that 6-formylpurine thiosemicarbazone, a compound which bears the structural features essential for anticancer activity, was an effective inhibitor of cytomegalovirus in cell culture (18) stimulated us to compare the effects of selected heterocyclic aldehyde thiosemicarbazones on the partially purified nucleotide reductase of human epidermoid carcinoma cells (H. Ep.-2) and on two representatives of the DNA-containing herpesvirus family, herpes simplex virus and human cytomegalovirus. The results of these observations form the basis of the present report.

Materials and Methods. Cytidine-2-^{14}C diphosphate was obtained from Schwarz Bioresearch, Inc.; deoxycytidine and ATP were obtained from P-L Biochemicals, Milwaukee, Wisconsin. Snake venom (*Crotalus adamanteus*), dithioerythritol, and Tris base were purchased from Sigma Chemical Co., St. Louis, Missouri. Alkaline phosphatase (*Escherichia coli*) was obtained from Worthington Biochemical Corp., Freehold, New Jersey. Other materials used in the preparation of the enzyme reaction mixture were reagent grade chemicals from standard sources.

The 2-, 3-, and 4-formylpyridine and isatin thiosemicarbazones were prepared in this laboratory. 1-Methylisatin 3-thiosemicarbazone and 6-formylpurine thiosemicarbazone (purine-6-carboxaldehyde thiosemicarbazone) were supplied by the Cancer Chemotherapy National Service Center, Bethesda, Maryland.

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Francisco, California, for the thiosemicarbazones of 5-hydroxy-2-formylpyridine, 1-formylisoquinoline, and 5-hydroxy-1-formylisoquinoline.

The viruses used in this study included herpes simplex (HSV), strain HF, (obtained from W. A. Rightsel, Parke, Davis and Co., Detroit, Michigan), and human cytomegalovirus (CMV), strain Casazza, provided by A. R. Casazza of the National Institutes of Health.

The studies with HSV were carried out in H.Ep.-2 cells (19). Human embryonic lung fibroblast (WI-38) (20) cells were used for the CMV studies. Eagle's (21) basal medium (BME) containing twice the concentration of vitamins and amino acids and supplemented with 5% fetal calf serum was used in all cell systems for the antiviral studies. For the enzyme studies H.Ep.-2 cells were grown in quantity in suspension culture by published procedures (22).

Procedures for antiviral studies. In the HSV experiments, growth medium was decanted from the cell monolayers grown in vinyl plastic panels (23), after which the virus and test chemical were added together to the cells, and the panels were sealed with cellophane tape. After an incubation at 37° for 4-5 days, the cells were examined microscopically for inhibition of virus-induced cytopathic effects (CPE).

The CMV studies were carried out in glass tubes and differed from the HSV experiments in that the test chemicals suspended in media were added to the virus-infected cells, from which the media had been decanted, on Days 2 and 4, as well as being added at the same time as the virus. Inhibition of CPE was determined on Day 6 or 7.

In all experiments, approximately 10 and 100 cell culture 50% infectious doses ($CCID_{50}$) of virus were used with five or more concentrations of test chemical, each concentration varying by one-half log dilution from the next. The highest concentration of compound used was calculated to be moderately cytotoxic to the cells. Antiviral activity was evaluated by use of the Virus Rating (VR) system (18, 24), which is a mathematical expression determined by consideration of the cytotoxicity and active concentrations of the test chemical, the virus level, and the degree of CPE inhibition. In our experience a VR of 1.0 or greater signifies marked antiviral activity, a VR of 0.5 – 1.0 indicates moderate antiviral activity, and <0.5 indicates questionable or no antiviral activity.

Enzyme preparation. Ribonucleotide reductase was prepared from a 20% suspension of H.Ep-2 cells in 0.05 *M* Tris-HCl buffer, pH 7.5, containing 0.01 *M* $MgCl_2$. The cells were disrupted at ice temperature using a Branson Sonifier run at maximum output. Three 15-sec pulses spaced 1 min apart were used. The broken cell suspension was centrifuged at 100,000*g* for 1 hr at 4°, and the supernatant solution was filtered through Schleicher and Schüll membrane filters (Preflex and Bac-T-Flex). The clear filtrate served as the starting point for ammonium sulfate fractionation of protein. Maximum reductase activity was obtained in the protein fraction precipitating between 25% and 40% saturation with ammonium sulfate at 4°. The 40% ammonium sulfate fraction was dissolved in 0.05 *M* Tris-HCl, pH 7.5, and dialyzed overnight against 0.01 *M* Tris–HCl, pH 7.5. Specific activity of typical enzyme preparations (mμmoles deoxyribonucleotide formed per mg protein per 30 min) were 16.1, 10.6, and 23.6. The specific activity of such a fraction was 40-fold that of the crude extract.

Assay for reductase activity. The reaction mixture used for enzyme assay in this work was that described by Moore (25). The composition and concentration (millimolar) of components of the reaction mixture was as follows: potassium phosphate buffer, pH 7, 8.3 m*M*; ATP, 4.4; magnesium acetate, 2.7; sodium fluoride, 8.3; ferrous chloride or ferrous ammonium sulfate, 0.06; dithioerythritol, 6.2; cytidine-2-^{14}C diphosphate, 0.4 (0.1 μCi). The final volume of the reaction mixture with H.Ep.-2 enzyme protein solution added was 0.2 ml. The mixture was incubated at 37° for 30 min and then heated to boiling for 2 min. Protein was removed by centrifugation. Snake venom (1 mg), alkaline phos-

phatase (0.2 unit), and deoxycytidine (50 μg) were added to the supernatant solution and incubated at 37° for 90 min. Such treatment converted all nucleotides to nucleosides. Deoxycytidine-2-^{14}C formed by reduction of cytidine-2-^{14}C diphosphate was separated from cytidine-2-^{14}C by descending paper chromatography. The solvent for developing the chromatograms was ethanol: 5 *M* ammonium acetate, pH 9.5: saturated sodium tetraborate: 0.5 *M* EDTA (11:1:4:0.02). Radioactivity on the chromatograms was detected by scanning in a Packard Strip Scanner or by exposing the chromatograms to X-ray film. In the latter instance quantitative measurement of radioactivity was accomplished by cutting out the radioactive spots and counting them in a Packard liquid scintillation spectrometer.

It was established that the reduction of cytidine diphosphate was linear with time for 60 min and that the rate of reduction was proportional to the amount of H.Ep.-2 enzyme protein added up to 0.7 mg. In the absence of added enzyme no radioactive deoxycytidine was formed.

Results. The thiosemicarbazones of 2-formylpyridine, 5-hydroxy-2-formylpyridine, 1-formylisoquinoline, 5-hydroxy-1-formylisoquinoline, and 6-formylpurine (purine-6-carboxaldehyde thiosemicarbazone) had moderate to marked activity against both viruses used in this study. Those compounds having VR values of 1.0 or greater inhibited all detectable virus-induced CPE at one or more concentrations, although in all the HSV studies a slight degree of cytotoxicity was apparent at the concentrations causing the CPE inhibition. This slight cytotoxicity was apparent as microscopically discernible cellular aberrations. The positive CMV activity observed using the WI-38 cells was at drug concentrations not visibly toxic to the cells. In most experiments the anti-CMV activity of the active test compounds was of greater degree than was the anti-HSV activity of the same materials.

The effect of time of addition of 5-hydroxy-2-formylpyridine thiosemicarbazone on its anti-CMV activity was determined in a separate experiment. Cells were exposed to 1000 $CCID_{50}$ of the virus, and 0, 4, 6, and 8 hr later the supernatant fluid was discarded and 100 μg/ml of the thiosemicarbazone was added. After 37° incubation for 2 and 4 days, the medium was replaced with fresh medium and drug; the degree of viral CPE was determined on Day 7 in a similar manner to the other experiments. Addition of the compounds inhibited all viral CPE at every time interval.

All of the compounds examined in which the thiosemicarbazone moiety was alpha to a nitrogen atom in the heterocyclic ring were inhibitors of ribonucleotide reductase activity (Table II). Thus, the thiosemicarbazone derivatives of 2-formylpyridine, 5-hydroxy-2-formylpyridine, 1-formylisoquinoline, 5-hydroxy-1-formylisoquinoline, and 6-formylpurine were inhibitors of the reduction of CDP to dCDP, whereas the thiosemicarbazones of 3- and 4-formylpyridine and of isatin and 1-methylisatin were without significant activity.

Discussion. A correlation was seen between antiviral activity and inhibition of ribonucleotide reductase by the thiosemicarbazones used in these studies (Tables I and II). Evidently, a considerable degree of structural specificity is required for inhibition of the reductase and for activity against HSV and CMV. The thiosemicarbazones which had the-CH=N-NH-C(=S)NH_2 moiety affixed to the heterocyclic ring system in the position alpha to the ring nitrogen were active inhibitors of reductase and of the DNA viruses. For example, the position isomers of 2-formylpyridine thiosemicarbazone, *i.e.*, the 3- and 4-formylpyridine derivatives, were inactive against enzyme and against virus. It is of interest that a similar specificity was observed in the antileukemic activity of these isomers (10, 13). These results imply that the antiviral activity of these compounds is due at least in part to inhibition of the ribonucleotide reductase. The observation that 5-hydroxy-2-formylpyridine thiosemicarbazone was active against CMV even after an 8-hr pre-exposure of cells to virus is consistent with this premise in that it showed that the inhibitor was not acting directly on the virus nor was it interfering with adsorption of

TABLE I. Summary of *in Vitro* Antiviral Activity of Heterocyclic Thiosemicarbazones.[a]

Thiosemicarbazone of:	Herpes simplex virus exp.		Cytomegalovirus exp.	
	Highest active concentration[b] (μg/ml)	Activity[c] (VR)	Highest active concentration[b] (μg/ml)	Activity[c] (VR)
2-Formylpyridine	3.2	0.9	320	2.0
3-Formylpyridine	320	0.4	<100	0
4-Formylpyridine	100	0.2	<320	0
5-Hydroxy-2-formylpyridine	100	1.1	320	1.3
1-Formylisoquinoline	<10	0.5	<32	0.6
5-Hydroxy-1-formylisoquinoline	10	0.8	<32	1.1
6-Formylpurine	<32	0.6	<100	1.4
Isatin-3-	<10	0	<320	0
1-Methylisatin-3-	<10	0.2	<320	0

[a] H.Ep.-2 cells were used in herpes simplex virus experiments, and WI-38 cells were used in cytomegalovirus experiments.

[b] Highest concentration of compound at which antiviral activity was discernible; < indicates that the drug was not entirely soluble in the experimental medium.

[c] Antiviral activity is expressed as Virus Rating (VR), in which >1.0 indicates marked antiviral activity, 0.5–1.0 indicates moderate antiviral activity, and <0.5 indicates questionable or no antiviral activity.

the virus on the cell surface, similar to observations reported by others using vaccinia virus (26). Thus, the inhibitor appeared to be interfering with an intracellular process essential for virus replication. The mechanism differs from the inhibition of vaccinia virus by 1-methylisatin 3-thiosemicarbazone since this compound was inactive against HSV and CMV and was a poor inhibitor of the reductase. Studies of the mechanism of action of the isatin thiosemicarbazones indicate that these derivatives may interfere with protein synthesis concerned with viral maturation (26). In the present study we are concerned with structurally different thiosemicarbazones and with a different mechanism of action.

To our knowledge, this is the first report of the antiviral activity of the majority of these thiosemicarbazones against HSV and CMV. It was noted that the anti-CMV activity was usually greater than activity of the same compounds against HSV; this inequality of antiviral activity was probably due to the procedures used in carrying out the experiments. Each compound was added to the cells only at the time of initial virus exposure in the HSV experiments, whereas in the CMV experiments the media containing each compound was removed and replaced with fresh media and compound every two days.

TABLE II. Inhibition of Ribonucleotide Reductase of H.Ep.-2 Cells by Heterocyclic Thiosemicarbazones.

Thiosemicarbazone of:	Concentration for 50% inhibition of reductase[a]	
	(μg/ml)	(Molar)
2-Formylpyridine	0.08	4.2×10^{-7}
3-Formylpyridine	360	2×10^{-3}
4-Formylpyridine	90	5×10^{-4}
5-Hydroxy-2-formylpyridine	0.78	4×10^{-6}
1-Formylisoquinoline	0.04	1.7×10^{-7}
5-Hydroxy-1-formylisoquinoline	0.06	2.3×10^{-7}
6-Formylpurine	0.07	3.2×10^{-7}
Isatin[b]	>22	$>1 \times 10^{-4}$
1-Methylisatin[b]	>2.3	$>1 \times 10^{-5}$

[a] Concentration for 50% inhibition was determined by graphical analysis of a plot of reductase activity at various concentrations of inhibitor.

[b] The thiosemicarbazone moiety is attached at the 3-position of the indole ring. These compounds were not inhibitory at the concentrations indicated.

The WI-38 cells appeared less sensitive than the H.Ep.-2 cells to the toxic effects of the chemicals used in these experiments. Since the WI-38 cells are derived from human lung embryo tissue and the H.Ep.-2 cells came from a human carcinoma, attempts were made to determine if these compounds also were inhibitory to ribonucleotide reductase in WI-38 cells. So far a sufficient quantity of these cells has not been available for such studies. Since a relatively close correlation was seen in activity against both HSV, CMV, and inhibition of reductase, it may be presumed that the enzyme inhibition seen in the H.Ep.-2 cells would be repeated in WI-38 cells.

The close correlation of enzyme inhibition with antiviral activity suggests that other inhibitors of ribonucleotide reductase may prove of interest as candidate inhibitors of DNA viruses of the herpesvirus group.

Berglund, Karlstrom, and Reichard (27) recently found that infection of *E. coli* with bacteriophage T4 resulted in the appearance of a new ribonucleotide reductase. This interesting observation prompted us to consider the possibility that virus-infected mammalian cells also might synthesize a new ribonucleotide reductase. If such were the case, then selective inhibitors of virus proliferation might be found among inhibitors of ribonucleotide reductase.

Summary. The thiosemicarbazones of 2-formylpyridine, 3-formylpyridine, 4-formylpyridine, 5-hydroxy-2-formylpyridine, 1-formylisoquinoline, 5-hydroxy-1-formylisoquinoline, 6-formylpurine, isatin, and 1-methylisatin were examined for activity against herpes simplex virus in H.Ep.-2 cells and human cytomegalovirus in WI-38 cells, and also for inhibition of ribonucleotide reductase activity in H.Ep.-2 cells. A correlation was seen between inhibition of reductase and antiviral activity, with those compounds having the -CH=N-NH-C(=S) -NH_2 moiety affixed to the heterocyclic ring system in the position alpha to the ring nitrogen being active. The suggestion is made that the activity of ribonucleotide reductase may be a limiting factor in the replication of certain members of the herpesvirus group.

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