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ANTITUMOR EFFECTS OF *BIS*-THIOSEMICARBAZONES—INHIBITION OF DEOXYRIBONUCLEIC ACID SYNTHESIS *IN VITRO**

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Abstract—A group of *bis*-thiosemicarbazones was evaluated for potential antitumor activity, using the L1210 murine leukemia in cell culture. Drug levels required to inhibit DNA synthesis by 50 per cent, under standard conditions, were determined. The most potent of the agents examined had the structure $X[CH_2CR_1{=}NNHCSNHR_2]_2$ where X = C or S and $R_1 = R_2 = CH_3$. Optimal activity was also obtained with R_1 = H and $R_2 = CH_3$ only when X = S. The most potent derivatives inhibited DNA synthesis by 50 per cent within 10 min at 10^{-6} M levels (ID_{50}). Metal chelates of several compounds tested were extremely potent inhibitors of DNA synthesis ($ID_{50} = 10^{-7}$ M or less). Insolubility in water and short duration of action *in vivo* may limit effectiveness of the *bis*-thiosemicarbazones.

Studies on antitumor effects of thiosemicarbazones have been summarized by Stock [1]. The best known such compounds, the *bis*-thiosemicarbazone of an α-ketoaldehyde, 2-keto-3-ethoxybutyraldehyde (KTS), has been extensively studied [2–9]. KTS inhibits growth of several experimental animal tumors. The zinc and copper chelates are more potent than the parent compound [4, 5, 7], and inhibit several enzymes involved in nucleic acid metabolism [8, 9]. A series of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones has also been examined [10–12]. These compounds are potent inhibitors of ribonucleotide reductase [13–16], presumably via chelation with an iron atom required by the enzyme.

The present series of compounds arose during an investigation of potential anti-neoplastic agents in Dublin [17–19]. These agents differ from the α-ketoaldehyde structure of KTS; the most potent compounds reported here are *bis*-thiosemicarbazones of 1,5-dicarbonyl compounds. A screening of 33 compounds, together with several metal chelates and heterocyclic salts derived from the *bis*-thiosemicarbazones, is described here. Structure–activity relations obtained may prove useful in selection of compounds for further study.

MATERIALS AND METHODS

Organic synthesis. Synthesis of these *bis*-thiosemicarbazones has been described [18, 19]. All batches of compounds tested were pure by criteria of infrared and ultraviolet spectra, and all had satisfactory elemental analyses. All were insoluble in water and dissolved readily in dimethylformamide. Common structural features are shown in Fig. 1 (see below).

Treatment of certain *bis*-thiosemicarbazones with HCl led to formation of water-soluble heterocyclic salts. Reactions of four compounds (see Fig. 2) led to formation of derivatives which were examined here. Synthetic procedures are described in Ref. 20. The parent compounds were potent inhibitors of DNA synthesis (see Tables 1 and 2). Water solubility of the heterocyclic salts might be a pharmacologic advantage; hence the biologic studies are described here.

Chelates of compounds shown in Table 4 were prepared by addition of an equimolar amount of aqueous cupric acetate to *bis*-thiosemicarbazone dissolved in dimethylformamide. The products crystallized readily from ethyl acetate, and analysis showed that, in each, two hydrogen atoms of the *bis*-thiosemicarbazone had been replaced by one copper. The structures are being investigated.

Cell culture systems. L1210 murine leukemia cells were maintained in culture using stoppered flasks.

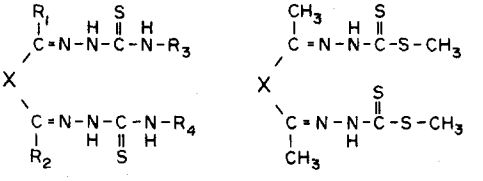

Fig. 1. Structure of the *bis*-thiosemicarbazones tested. The left configuration shows the structure of all the drugs except two. The latter are described by the right formula.

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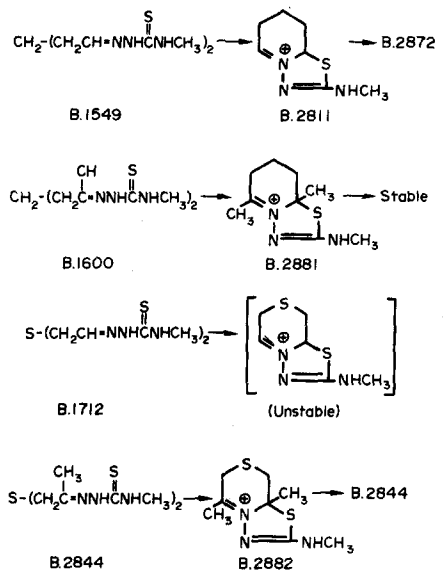


Fig. 2. Products obtained upon acidification of four *bis*-thiosemicarbazones. The first reaction results from addition of HCl. One unstable product was formed, others could be isolated. The second reaction results from water hydrolysis of the first product.

MEM-Eagle's medium (Spinner modification) was supplemented with 10% fetal calf serum, penicillin and streptomycin. All media were purchased from Grand Island Biological Co., Grand Island, N.Y. Studies were done using exponentially growing cells suspended 20 min before addition of drug in fresh medium at 37°. One-ml portions of these suspensions contained 6–7 mg of cells (wet wt). Drug was dissolved in dimethylformamide* so that 5-μl portions were added to each ml of cell suspension. Ten min later (longer if specified), 0·02 μCi of labeled compound was added in a volume of 5 μl. Final levels were 100 μM of thymidine-2-^{14}C, 100 μM of leucine-U-^{14}C or 10 μM of uridine-2-^{14}C. Ten min after addition of radioactive substances, the cell suspensions were chilled and the cells were collected by centrifugation. Incorporation of label into material insoluble in 0·3 M $HClO_4$ was measured by liquid scintillation counting [21, 22]. In other studies, effects of drugs on conversion of labeled thymidine into nucleotide pools were examined [23].

A few studies were carried out *in vivo*. Animals bearing advanced L1210 leukemia were inoculated with suspensions of test compounds made up in 1% carboxymethyl cellulose + 1% Tween 80. At intervals after such injection, cells were removed, suspended in growth medium, and incorporation of labeled thymidine into DNA was measured. Control samples were taken just before injection of drug. All inoculations were at the 100 mg/kg level.

Experiments involving *Escherichia coli* were done using cells grown in "minimal" medium [24]. Drug was dissolved in dimethylformamide and the experimental procedure followed the plan used with L1210 cells.

* Heterocyclic salts were dissolved in water; concentration was adjusted so that 5-μl portions of such solutions were added to 1-ml cell suspensions.

RESULTS

Inhibition of DNA synthesis by L1210 *cells in culture.* Incorporation of labeled precursors into nucleic acid and protein proceeded at a linear rate, following a brief lag. Reproducible results were obtained when cells were first incubated at 37° for 20 min before any additions. A log dose-response to drug was generally found: the extent of inhibition of macromolecular synthesis was proportional to the log of the drug dose. Moreover, such inhibition increased progressively with the time of exposure of cells to a compound. Typical results for one *bis*-thiosemicarbazone are shown in Table 1. For this agent, and all other drugs examined here, we found DNA synthesis to be inhibited most markedly, with RNA and protein synthesis inhibited to lesser extents.

When a group of 33 *bis*-thiosemicarbazones was tested for capacity to inhibit incorporation of labeled thymidine into DNA, the results summarized in Table 2 were obtained. Drug levels required to inhibit incorporation of labeled thymidine into DNA by 50 per cent are reported.

Relevant structural features of compounds tested are shown in Fig. 1 (left portion). This formula holds for all compounds tested except for two (first column, Table 2) for which the terminal $NHR_{3,4}$ was replaced by SCH_3. At the —X— position various configurations of carbon, sulfur and oxygen were placed. The R_1 and

Table 1. Effect of *bis*-thiosemicarbazone B.1600 against macromolecule synthesis by L1210 cells

First incubation* (min)	Rate of precursor incorporation (% of control) Leucine	Thymidine	Uridine
0	92	26	85
10	80	13	71
20	69	5	54

* Time of exposure of cells to drug (5 μg/ml) before addition of radioactive precursor. Ten min after such addition, incorporation of label into acid-insoluble material was measured. Data represent the average of four determinations which agreed by $\pm$10 per cent.

Table 2. Effect of *bis*-thiosemicarbazones on DNA synthesis by L1210 cells*

X	R_1	R_2	R_3 and R_4 = SCH_3	H	CH_3†	C_2H_5	C_3H_7	C_2H_4OH
CH_2CH_2	H	H			50			
CH_2CH_2	Me	Me			80			
$CH_2CH_2CH_2$	H	H			30†	15	15	
CH_2OCH_2	H	H			350			
CH_2SCH_2	H	H		22	2†	2		
CH_2SOCH_2	H	H			70			
$CH_2SO_2CH_2$	H	H			450			
$CH_2CH_2CH_2$	H	Me		30	5			
$CH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}HCH_2$	H	Me		12				
$CH_2CH_2CH_2$	Me	Me	10	25	0·5†	2	2	
$CH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}HCH_3$	Me	Me		12	0·5			
$CH_2CH_2CH_2$	Et	Me		20	1	0·5		
CH_2SCH_2	Me	Me	10	2·5	0·5†	0·5	2·5	10
$CH_2CH_2CH_2CH_2$	H	H			150			
CH_2SSCH_2	H	H			15			
CH_2SSCH_2	Me	Me			2			

* Structural formulas of drugs are shown in Fig. 1. The left configuration describes all drugs except for those in column 4 (SCH_3), for which a terminal-SCH_3 replaces NHR_3 and NHR_4 (see Fig. 1, right). The table shows levels, in μg/ml, of each compound required to inhibit DNA synthesis by 50 per cent under standard conditions.

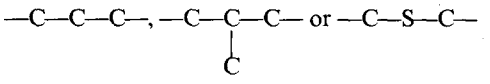
† Four drugs from this column were selected for further study. Reading from top to bottom, they are identified as follows: B.1207, B.1549, B.1712, B.1600 and B.2844.

R_2 groups were H (hydrogen), or Me (methyl) or Et (ethyl). At R_3 and R_4, H, CH_3, C_2H_5, $CH_2CH_2CH_3$ or CH_2CH_2OH groups were substituted.

Structure–activity relations derived from Table 2. (1) A three-carbon chain at X (Fig. 1 left) was more effective than C_2 or C_4.

(2) The —C—S—S—C— or —C—S—C— configurations resulted in a more potent drug than all carbon analogs. The —C—O—C— structure, or structures with O substituted on S, showed greatly reduced activity.

(3) Alkyl groups at the R positions enhanced activity. With methyl groups at all four of the R positions, either



configurations at X provided optimal inhibitory activity. With H substituents at R_1 and R_2, the —C—C—C— group at X was less effective than the other two structures.

Table 3. Comparison of *bis*-thiosemicarbazones, bicyclic salts and hydrolysis products as inhibitors of DNA synthesis*

Parent compound†	ID_{50} (μg/ml)	Bicyclic salt‡	ID_{50} (μg/ml)	Hydrolysis product§	ID_{50} (μg/ml)
B.1549	30	B.2811	10	B.2872	100
B.1600	0·5	B.2881	>100	‖	‖
B.1712	2	Unstable¶	‖	‖	‖
B.2844	0·5	B.2882	20	B.2844**	0·5

* Structures are shown in Fig. 2. The ID_{50} levels inhibit DNA synthesis by 50 per cent under standard conditions.

† Parent compound from Table 2.

‡ Product formed upon acidification.

§ Product formed from bicyclic salt by water hydrolysis.

‖ No hydrolysis product formed (see text).

¶ No stable product isolated.

** B.2882 disproportionates (see text).

Table 4. Inhibition of DNA synthesis by metal chelates

Parent compound*	ID_{50}†	Metal‡	ID_{50} of chelate†
B.1549	11	Cu	0·56
B.1600	1·6	Cu	0·54
		Ni	1·2
B.1712	3·4	Cu	0·56
B.2844	1·6	Cu	0·52

* Structure of parent compounds is shown in Fig. 2.

† The ID_{50} units are micromoles of drug per l. These levels result in inhibition of DNA synthesis by 50 per cent under standard conditions.

‡ Supplied as the chloride salt.

(4) Substitution of ethyl for methyl at R_1–R_2 did not significantly alter activity. Propyl or larger groups at R_3 and R_4 were less effective than methyl or ethyl.

(5) Replacement of the terminal NHR groups with SCH_3 (Fig. 1, right) reduced but did not abolish activity. Substitution of methyl groups on nitrogen at the N_2 positions (not shown here) abolished activity.

Studies on heterocyclic salts. Treatment of certain *bis*-thiosemicarbazones with HCl led to formation of water-soluble bicyclic salts. Results of treatment of four compounds with acid are shown in Fig. 2. The parent compounds showed high activity in the anti-DNA screen (Table 2). Procedures are described in Ref. 20.

Cyclic salts were formed from B.1549, B.1600 and B.2844. The postulated initial product formed upon acidification of a solution of B.1712 was unstable and could not be isolated. Structures are assigned based on elemental analyses, the known [25] cyclization of thiosemicarbazones to thiazolidines in strong acid, and the availability of another carbonyl group for attachment by an imino group to form the bicyclic structure. The NMR spectra of these salts are consistent with the assigned structures.

The second reaction shown in Fig. 2 results from water hydrolysis of the bicyclic salt. In water solution, B.2811 slowly forms a stable product, B.2871 ($C_{12}H_{20}N_5S_2$). This is, empirically, a dimer of the base corresponding to B.2811 minus a C_2H_2N fragment. B.2881 is stable and no hydrolysis product is formed. Hydrolysis of B.2882 results in a disproportionation reaction whereby two molecules react to form one molecule of starting material (B. 2844) plus an unidentified compound.

The products shown in Fig. 2 are all water soluble and might, therefore, be more useful therapeutic agents than the insoluble parent compounds. All products shown in Fig. 2, except B.2811, were less potent inhibitors of DNA synthesis than the parent compounds (Table 3).

* This study was kindly carried out by Dr. Joseph Cory, Department of Medical Microbiology and Chemistry, University of South Florida at Tampa.

Inhibition of DNA synthesis by chelates. Copper, nickel or cobalt chelates were prepared from a few *bis*-thiosemicarbazones listed in Table 2; the resulting structure contained one metal atom per drug molecule. The precise structure of these chelates is not yet certain, but all were crystalline. The data available show all chelates tested to be more potent inhibitors of DNA synthesis than the parent compounds (Table 4). On a molar basis, the effect is striking, as shown in Table 4. Copper ion is a weak inhibitor of DNA synthesis, the ID_{50} level being 1·6 mM.

Other studies using mammalian cells. Compounds from Table 2 which inhibited DNA synthesis at 0·5–2·0 μg/ml levels were tested for possible effects on uptake of thymidine and biotransformation of the nucleotide into TTP. Drug levels which inhibited DNA synthesis by >90 per cent within 10 min had no effect on incorporation of exogenously supplied thymidine into intracellular TTP as described in Ref. 23. The action of these drugs is therefore assumed to involve inhibition of incorporation of TTP into DNA.

The α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones are inhibitors of the enzyme, ribonucleotide reductase [13–15]. Inhibition of this enzyme could readily result in inhibition of incorporation of TTP into DNA. All four *bis*-thiosemicarbazones shown in Fig. 2 (B.1600, B.1712, B.2844 and B.1549) did not inhibit a mammalian ribonucleotide reductase preparation [26].*

Experiments with Escherichia coli. The four *bis*-thiosemicarbazones shown in Fig. 2 were tested against exponentially growing *E. coli.* Much higher levels of drug were required to inhibit DNA synthesis by 50 per cent as compared with the L1210 system (Table 5). The most effective compound in the bacterial system was B.1549, the least effective of the group against L1210.

Studies in vivo. Preliminary results of experiments carried out *in vivo* show that the most potent inhibitors listed in Table 2, i.e. B.1600 and B.2844, caused only a transient inhibition of DNA synthesis *in vivo.* Incorporation of labeled thymidine into DNA was inhibited by 40–50 per cent after administration of 100 mg/kg of drug, but this persisted for only a few hr. The low solubility of drug in water may contribute to this result, as discussed below.

Table 5. Effect of *bis*-thiosemicarbazones on nucleic acid and protein synthesis by *E. coli**

Drug	Level (μg/ml)	Rate of synthesis (% of control) DNA	RNA	Protein
B.1600	250	85	90	100
B.2844	250	100	100	100
B.1712	250	80	85	90
B.1549	250	50	55	45

* Figures represent relative rates of incorporation of labeled thymidine into DNA, uridine into RNA and leucine into protein, comparing drug-treated with control cultures.

DISCUSSION

The study described here was designed to evaluate a series of *bis*-thiosemicarbazones as potential antineoplastic agents. Since these drugs exerted their primary effect against synthesis of DNA, our major screening effort was designed to elucidate structure–activity relations using inhibition of DNA synthesis in culture as an end point. The low solubility of these compounds could be an important determinant of drug action *in vivo*; therefore a series of derived heterocyclic salts was also examined.

Structure–activity relations show that optimal activity is obtained using the *bis*-thiosemicarbazone of a 1,5-diketone. The presence of a central sulfur atom affords no advantage if four methyl groups are substituted at the R positions (Fig. 1); if, for pharmacologic considerations, fewer methyl substituents are needed, the central S atom would be required for highest activity.

Metal chelates of a few compounds were examined. All were more potent inhibitors of DNA synthesis than parent compounds. This finding therefore corresponds to the similar data obtained using another *bis*-thiosemicarbazone, KTS [4, 5, 7]. Addition of metal may improve inward transport of drug into the cell, or uptake of metal may be the primary toxic phenomenon. Further study is being directed toward the solution of this problem.

The mode of action of these drugs is unknown. Uptake of thymidine into nucleotide pools was not inhibited. In studies not reported here, we found no effects of these agents on DNA polymerase preparations obtained from *E. coli* or L1210 cells. Conclusions from such studies are difficult to draw in view of the complex nature of DNA synthesis.

Agents showing capacity to inhibit DNA synthesis were also inhibitors of RNA and protein synthesis, but the more striking effect was on synthesis of DNA. Bacteria were relatively insensitive to the action of these compounds.

These compounds represent a new class of *bis*-thiosemicarbazones, not previously studied in detail. Some activity of the present class of drugs against Sarcoma 180 *in vivo* has been reported [17–19], but a methodical study of antitumor effects of these compounds against murine leukemias has not yet been carried out.

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REFERENCES

1. J. A. Stock, in *Experimental Chemotherapy* (Eds. R. J. Schnitzer and F. Hawking), Vol. 5, p. 387. Academic Press, New York (1967).
2. F. A. French and B. L. Freedlander, *Cancer Res.* **20,** part 2, 505 (1960).
3. H. G. Petering and H. H. Buskirk, *Cancer Res.* **24,** 367 (1964).
4. H. G. Petering, H. H. Buskirk and J. A. Crim, *Cancer Res.* **27,** 1115 (1967).
5. J. A. Crim and H. G. Petering, *Cancer Res.* **27,** 1287 (1967).
6. E. Mihich and C. A. Nichol, *Cancer Res.* **25,** 1410 (1965).
7. B. A. Booth and A. C. Sartorelli, *Nature, Lond.* **210,** 104 (1966).
8. B. A. Booth, D. G. Johns, J. R. Bertino and A. C. Sartorelli, *Nature, Lond.* **217,** 250 (1968).
9. B. K. Bhuyan and T. Betz, *Cancer Res.* **28,** 758 (1968).
10. R. W. Brockman, J. R. Thompson, M. J. Bell and H. E. Skipper, *Cancer Res.* **16,** 167 (1956).
11. E. J. Blanz, Jr. and F. A. French, *Cancer Res.* **28,** 2419 (1968).
12. B. A. Booth, E. C. Moore and A. C. Sartorelli, *Cancer Res.* **31,** 228 (1971).
13. E. C. Moore, M. S. Zedeck, K. C. Agrawal and A. C. Sartorelli, *Biochemistry, N.Y.* **9,** 4492 (1970).
14. E. C. Moore, B. A. Booth and A. C. Sartorelli, *Cancer Res.* **31,** 325 (1971).
15. A. C. Sartorelli, K. C. Agrawal and E. C. Moore, *Biochem. Pharmac.* **20,** 3119 (1971).
16. F. A. French, E. J. Blanz, Jr., J. R. DoAmral and D. A. French, *J. med. Chem.* **13,** 1117 (1970).
17. V. C. Barry, M. L. Conalty and J. F. O'Sullivan, *Cancer Res.* **26,** 2165 (1966).
18. V. C. Barry, M. L. Conalty, C. N. O'Callaghan and D. Twomey, *Proc. R. Ir. Acad.* **65,** 309 (1967).
19. V. C. Barry, M. L. Conalty, J. E. McCormick, R. S. McElhinney, M. R. McInerney and J. F. O'Sullivan, *J. med. Chem.* **13,** 421 (1970).
20. M. Gannon, J. E. McCormick and R. S. McElhinney, *Proc. R. Ir. Acad.*, in press.
21. D. Kessel, *Cancer Res.* **31,** 1883 (1971).
22. D. Kessel and H. B. Bosmann, *Cancer Res.* **30,** 3695 (1970).
23. I. C. Caldwell and J. F. Henderson, *Analyt. Biochem.* **34,** 303 (1970).
24. B. D. Davis and E. Mingioli, *J. Bact.* **60,** 17 (1950).
25. K. H. Mayer and D. Laverer, *Justus Liebigs Ann. Chem.* **731,** 142 (1970).
26. J. G. Cory, *Cancer Res.* **33,** 933 (1973).

Note Added in Proof—Further studies, to be published in detail elsewhere, have confirmed that the primary toxic phenomenon involved is the marked potentiation by certain *bis*-thiosemicarbazones of uptake of Cu^{2+} by L1210 cells. In the absence of added metal ion, the drugs probably scavenge traces of the metal from growth media, since their inhibitory effect is abolished by addition of EDTA.