Selective Inhibition by Sorbylhydroxamic Acid of Deoxyribonucleic Acid Synthesis in Ehrlich Ascites Tumor Cells

Glen R. Gale, Alayne B. Smith, and Ernest M. Walker, Jr.

SUMMARY

Sorbylhydroxamic acid (SOHA) inhibited the incorporation in vitro of thymidine into deoxyribonucleic acid in Ehrlich ascites tumor cells, with no appreciable effect on the incorporation of uridine into ribonucleic acid or of L-leucine into protein. Hexanoylhydroxamic acid, devoid of the conjugated double-bond system, had no such activity. The concentration of SOHA conferring 50% inhibition of DNA synthesis was approximately $6 \times 10^{-5}$ M. Inhibition was almost totally abolished when the cells were washed free of the compound but was only partially antagonized by supplementing the medium with a mixture of deoxyribonucleosides; this latter was interpreted as indicating that only part of the inhibitory action may be attributed to suppression of ribonucleotide bioreduction. No conversion by SOHA of intracellular DNA to an acid-soluble state was demonstrated. Electron microscopy of SOHA-sensitive bacteria grown in the presence of the inhibitor yielded cytological evidence of a reduced rate of DNA synthesis which was compatible with the biochemical data. A moderate reduction of the rate of development of Ehrlich ascites tumor cells in vivo was demonstrated at dosages which closely approximated toxic levels. Caution was suggested in utilization of this hydroxamate as a food preservative.

INTRODUCTION

Initial investigations of the pharmacology of sorbylhydroxamic acid dealt solely with its antagonism of cholinesterase inactivation induced by certain organophosphorus compounds (3, 11, 14). Structural relationships of this hydroxamate to the parent carboxylic acid as well as to the fully saturated congener are shown in Chart 1. More recently, Dudman (2) investigated its antifungal action with the apparent aim of utilizing it as a food preservative. The parent carboxylic acid, while widely used in the food industry as a preservative, suffers from the principal disadvantage of being relatively inactive at pH values appreciably greater than 5. The antifungal action of SOHA was shown to be unaffected by the hydrogen ion concentration of the medium over a broad range. This property was attributed to the reduced tendency of SOHA to dissociate at the higher pH values.

As part of a program of investigations of the action of a number of hydroxamic acids on nucleic acid synthesis, SOHA was assessed as a possible selective inhibitor in an in vitro system.

MATERIALS AND METHODS

SOHA and hexanoylhydroxamic acid were synthesized by Dr. John B. Hynes, School of Pharmacy, Medical College of South Carolina. Eagle’s minimal essential medium with Hanks’ balanced salt solution was from Microbiological Associates, Bethesda, Md. The strain of ascites tumor was maintained in BALB/c mice and was used 6 to 10 days after inoculation. Microorganisms were stock laboratory strains or recent isolates from the clinical microbiology laboratory of the Charleston VA Hospital. Thymidine-methyl-3H, uridine-5-3H, and L-leucine-14C (uniformly labeled) were from New England Nuclear Corporation, Boston, Mass.

The following parameters which were examined have been described in detail in earlier reports as indicated: rates of synthesis of DNA, RNA, and protein (5); dose-response relationships (5); reversal of inhibitory action by washing the cells (5); antagonism of inhibition by exogenous deoxyribonucleosides (6); conversion of intracellular DNA to an

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1Aided by NIH Grant GM-13958.

2The abbreviations used are: SOHA, sorbylhydroxamic acid; MEM, Eagle’s minimal essential medium with Hanks’ balanced salt solution; TCA, trichloroacetic acid; DMSO, dimethylsulfoxide.

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acid-soluble state (8); and effects on growth and morphology of selected microorganisms (4, 12). Vestopel-embedded sections for electron microscopy were cut with an LKB Ultratome and examined with an RCA EMU3-H electron microscope. The effect of SOHA on development of the Ehrlich ascites tumor in mice was assessed by a minor modification of the method of Wilson et al. (15); this modification consisted of administering 1 injection/day instead of 2 and the weighing of mice on an individual basis rather than computing the average of the group for calculating dosages.

RESULTS AND DISCUSSION

The effect of graded concentrations of SOHA on DNA synthesis by ascites tumor cells in vitro is shown in Chart 2. To achieve linearity of the dose-response relationship, the probit of the percentage of control activity was plotted against the logarithm of the inhibitor concentration. The 50% and 90% inhibitory concentrations, calculated by the method of least squares, were $5.8 \times 10^{-4}$ M and $1.3 \times 10^{-3}$ M respectively.

Chart 3 shows the selectivity of inhibition of DNA synthesis by SOHA. The rates of DNA, RNA, and protein synthesis, with and without the inhibitor, were assessed simultaneously following 0, 1, and 2 hr of preincubation of the cells with SOHA at $10^{-3}$ M. The pattern of response was virtually the same at the 1- and 2-hr intervals as at 0 time. Inhibition of DNA synthesis was consistently 80 to 85% throughout the 2-hr period, while the rates of synthesis of RNA and protein were only minimally suppressed. A comparison with the action of hexanoylhydroxamic acid, which is devoid of the conjugated system of double bonds, showed that the saturated compound was substantially inactive on all of the 3 parameters (Chart 3).

Virtually complete reversibility of the action of SOHA on DNA synthesis upon removal of the inhibitor by washing the...
cells is shown in Table 1. Cells which had been preincubated for 20 min with the inhibitor at $6.5 \times 10^{-4}$ and $10^{-3}$ M and then washed 3 times with MEM maintained the same rate of DNA synthesis as control cells which had not been exposed to SOHA. Those cells which had been preincubated similarly and then washed with MEM containing SOHA at the same concentrations displayed a markedly impaired rate of DNA synthesis.

Inhibition of DNA synthesis by hydroxyurea, a hydroxamic acid with an established role in chemotherapy of certain types of leukemia, can be antagonized in mouse fibroblast (L) cells by the presence of exogenous deoxyribonucleosides (1). This reversal of activity was used to support the contention that the major metabolic defect conferred by hydroxyurea is inhibition of bioreduction of ribonucleotides to deoxyribonucleotides. The inhibitory action of certain other hydroxamates on DNA synthesis in ascites tumor cells can also be overcome by the presence of exogenous deoxyribonucleosides (6). Table 2 shows that a mixture of thymidine, deoxyctydine, deoxyguanosine, and deoxyadenosine at concentrations which totally antagonized the action of hydroxyurea and oxamylhydroxamic acid in the same test system (6) only partially overcame the action of SOHA. Higher concentrations of these nucleosides were themselves inhibitory and yielded equivocal data. Thus, there appears to be evidence that even though part of the inhibitory action of SOHA is mediated through inhibition of ribonucleotide reduction, at least 1 other site of action must be involved; indeed, an additional site of action of hydroxyurea on de novo pyrimidine synthesis in vivo has been suggested (13).

Ascites cells which were labeled with thymidine-$^3$H, washed free of the unpolymerized nucleoside, and exposed to SOHA at $10^{-3}$ M for up to 3 hr at $37^\circ$ yielded the same specific activity in the acid-insoluble fraction as did cells treated identically with the exclusion of SOHA. No conversion of intracellular DNA to an acid-soluble state could be demonstrated.

The antifungal activity of SOHA (2) was confirmed with the testing of strains of Candida albicans, Saccharomyces cerevisiae, Aspergillus fumigatus, Penicillium sp., and Mucor corymbifera. Filter paper discs were impregnated with a 1% methanolic solution of SOHA, the solvent was removed by vacuum, and the discs were placed onto seeded agar plates. Following incubation for 24 to 48 hr, zones of complete inhibition of $5$ to $17$ mm from the periphery of the discs were noted. In addition to this antifungal action, it was observed that a number of recent clinical isolates of Escherichia coli and Pseudomonas aeruginosa were also inhibited. Older stock laboratory strains of these gram-negative organisms were quite insensitive, as were a number of gram-positive bacteria. Light microscopic examination of gentian violet-stained cells from a culture of E. coli grown in tryptic soy broth with SOHA at $10^{-2}$ M revealed the type of cell elongation previously noted to occur in the presence of hydroxyurea (9), oxamylhydroxamic acid (4), 1-methyl-1-hydroxyurea (7), and 3-hydroxybiuret (10), and which is typical of unbalanced growth (Fig. 1, A and B). Electron microscopic examination showed that control cells were typical of those described in numerous reports, with discrete ribosomes, nucleoids as represented by areas of low electron density, and ordered cytoplasmic membranes (Fig. 1, C to E). Cells grown in the presence of SOHA showed some modification of the cytoplasmic membrane, along with a virtually total absence of the nucleoid. The cytoplasm was devoid of discrete inclusions, and the general appearance was one of a compromised viability (Fig. 1, F to H). Morphological manifestations are thus concordant with the bio-

<table>
<thead>
<tr>
<th>Deoxyribonucleosides</th>
<th>$6 \times 10^{-4}$ M SOHA</th>
<th>$1.5 \times 10^{-3}$ M SOHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>75</td>
<td>96</td>
</tr>
<tr>
<td>+</td>
<td>59</td>
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**Table 2** Partial antagonism by a mixture of deoxyribonucleosides of the inhibitory action of SOHA on DNA synthesis in Ehrlich ascites tumor cells in vitro

Aliquots, 3 ml each, of a 1% cell suspension in MEM containing supplemental thymidine-$^3$H at $10^{-6}$ M were incubated for 10 min at $37^\circ$ with and without deoxyadenosine ($3.5 \times 10^{-6}$ M), deoxyguanosine ($10^{-6}$ M), and deoxythymidine ($5 \times 10^{-6}$ M). SOHA was added in 0.03 ml DMSO to yield the indicated final concentrations. Controls received DMSO alone. Isotopic thymidine was then added (1.5 µCi). After 20 min of further incubation, 2.0-ml aliquots were added to 2.0 ml cold 10% TCA, and samples were processed for liquid scintillation counting.

**Table 1** Restoration of DNA synthesis upon washing ascites tumor cells free of SOHA

Aliquots, 5 ml each, of a 1% cell suspension in MEM were preincubated for 20 min at $37^\circ$ with SOHA at $6.5 \times 10^{-4}$ M and $10^{-3}$ M; control vessels contained vehicle only. Control mixtures and 1 set which was preincubated with SOHA at both concentrations were then washed 3 times with fresh MEM. The other set was washed 3 times with MEM containing the inhibitor at the same concentration as was present during the preincubation. The volume of each was restored to 5 ml with the appropriate medium, and 3-ml aliquots were added to vessels containing 1.5 µCi thymidine-methyl-$^3$H. After 20 min further incubation at $37^\circ$, 2-ml samples were added to 2 ml of cold 10% TCA and processed for liquid scintillation counting.

<table>
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<tr>
<th>Incubated with SOHA at (M)</th>
<th>SOHA in washing medium</th>
<th>Radioactivity (cpm)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>0</td>
<td>-</td>
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<tr>
<td>$6.5 \times 10^{-4}$</td>
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<td>$10^{-3}$</td>
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chemical findings as regards a diminution of the rate of DNA synthesis. The effect of SOHA on the development in vivo of Ehrlich ascites tumor cells in mice is shown in Table 3. The dose range tested was from 200 to 400 mg/kg/day. No significant inhibition was shown at doses less than 300 mg/kg/day. A moderate degree of inhibition was noted at doses of 300 mg/kg/day and higher; however, toxicity was evident at 352 mg/kg/day and became more pronounced at 392 mg/kg/day.

The current report thus describes selective inhibition of DNA synthesis by another aliphatic hydroxamic acid and demonstrates the dependence of this pharmacological property upon the presence of the conjugated system of double bonds in the 6-carbon aliphatic chain. The fully saturated congener, hexanoylhydroxamic acid, was without effect in the in vitro test system. Such a relatively simple property upon the presence of the conjugated system of moderate degree of inhibition was noted at doses of 300 mg/kg/day and became more pronounced at 392 mg/kg/day.

The earlier antimicrobial investigations of SOHA by Dudman (2) were apparently directed at its potential application as an antifungal additive for food. However, in view of the demonstrated effects of this compound on mammalian macromolecular synthesis, such an application would seem to be one to be approached with considerable caution.

ACKNOWLEDGMENTS

The skillful technical assistance of Mrs. Geneva Williams is gratefully acknowledged.

REFERENCES

Fig. 1. (composite). A, photomicrograph of control cells of *Escherichia coli*; B, photomicrograph of cells of *E. coli* grown in the presence of $10^{-2}$ M SOHA; C to E, Electron micrographs of control cells of *E. coli*. In C, the cell is undergoing division and the low-density nucleoid is predominant and diffuse. Cell wall and/or membrane is apparently being formed at the divisional junction. In D, nuclear material is more discretely localized; F to H, electron micrographs of cells of *E. coli* grown in the presence of $10^{-2}$ M SOHA. In F and G, no nucleoid is clearly discernible and the outer limiting membrane has acquired an enhanced electron density. The cytoplasm is virtually homogeneous. In H, a modicum of nuclear material is detectable, but the ratio of nucleoid to cytoplasm is markedly diminished. A and B, X 1,100; C to H, X 60,000.
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