

Inhibition of the growth of cultured cells and an implanted fibrosarcoma by aroylhydrazone analogs of the Gly-His-Lys-Cu(II) complex

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The growth-modulating plasma tripeptide, GHL^* , alters the growth rate or state of differentiation of a wide variety of cultured cells and organisms (e.g. hepatocytes, neurons, mycoplasma, fungi, and *Ascaris* larvae) and may mediate a functional or nutritional need common to diverse organisms [1, 2]. GHL chelates copper with an affinity equivalent to that of the copper transport site on plasma albumin [3] to form GHL-Cu and may function to enhance the cellular uptake of copper under the particular conditions of the culture system. *In vivo*, in rabbits, GHL-Cu possesses angiogenic activity and induces new capillary growth. On the basis of X-ray, electron paramagnetic resonance, electron spin echo, and nuclear magnetic resonance studies, we have determined that copper is bound to GHL to form a near-planar, triaza, tridentate complex (GHL-Cu) in solution (Fig. 1a) [4-7].

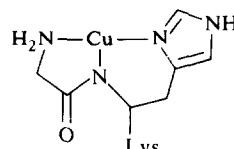
Aroylhydrazones of pyridoxal and of salicylaldehyde chelate transition metal ions in an analogous manner forming similar planar tridentate complexes around the metal ion (Fig. 1, b, and c) [7-9]. Therefore, we were prompted to investigate whether such synthetic tridentate systems might also affect cell growth. In recent studies of a variety of aroylhydrazone-copper complexes, we found two compounds, PCPH-Cu and SBH-Cu , that possess potent growth-inhibitory properties when tested *in vitro* [10]. Complexation with copper, but not iron, increases the cytotoxicity of these aroylhydrazones [11]. Furthermore, X-ray analysis of SBH-Cu crystals has demonstrated that SBH chelates the copper to form a tridentate, near-planar complex similar to that observed in GHL-Cu [12].

In this paper we report that these two compounds, when tested *in vitro* on neoplastic cells, inhibited DNA synthesis at concentrations similar to those found for three commonly used anti-tumor drugs (bleomycin sulfate, cisplatin, and

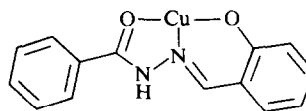
thiotepa). Furthermore, PCPH-Cu and SBH-Cu markedly inhibited the growth of an implanted fibrosarcoma in mice.

Materials and Methods

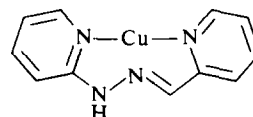
Materials. SBH and SBH-Cu were prepared as previously described [11, 12] and their melting points, infrared spectra, and nuclear magnetic resonance spectra were in agreement with literature values. PCPH was purchased from the Aldrich Chemical Co. (Milwaukee, WI). PCPH-Cu was prepared according to published methods [15, 16] by adding a solution of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.34 g, 2.0 mmoles) in 40 ml ethanol to a solution of PCPH (0.40 g, 2.0 mmoles) in 40 ml ethanol. On standing at room temperature, the resulting solution deposited green, needle-like crystals which were filtered off, washed with ethanol, and dried under vacuum



(a)



(b)



(c)

Fig. 1. Probable mode of binding to copper (II) by (a) GHL , (b) SBH and (c) PCPH .+ (a) GHL binds to copper via the N-terminal nitrogen atom of glycine, the nitrogen atom of the adjacent amide bond, and the unprotonated nitrogen of the imidazole ring of histidine, forming a tridentate system which is approximately planar. (b) In $(\text{SBH})\text{Cu}(\text{Cl})(\text{H}_2\text{O})$, X-ray crystallography has demonstrated that SBH binds copper via the phenolic and carboxylic oxygen atoms and by the imine nitrogen atom of the hydrazone linkage [12]. X-ray data plus spectroscopic and nuclear magnetic resonance measurements suggest a similar binding in asolution and that the disubstituted phenyl ring and the two chelate rings would be approximately co-planar, while the phenyl ring of the benzoyl moiety would rotate freely. (c) In $(\text{PCPH})\text{Cu}(\text{Cl})_2$, PCPH is believed to bind copper through the two heterocyclic nitrogen atoms and the imine nitrogen atom of the hydrazone linkage [13, 14]. All four rings would be expected to lie in the same plane.

* Abbreviations: GHL , glycyl-1-histidyl-1-lysine; GHL-Cu glycyl-1-histidyl-1-lysine-copper(II); PCPH , pyridine-2-carboxaldehyde-2'-pyridylhydrazone; PCPH-Cu , (pyridine-2-carboxaldehyde-2'-pyridylhydrazone)-copper(II) dichloride; SBH , salicylaldehydebenzoylhydrazone; SBH-Cu , (salicylaldehydebenzoylhydrazone)-copper(II) monochloride monohydrate; thiotepa, N,N',N'' -triethylenethiophosphoramide; cisplatin, *cis*-diaminedichloroplatinum(II); EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum, and DMSO, dimethyl sulfoxide.

+ Structures are drawn so as to depict the probable manner in which the multidentate ligand is bound. The identity of species occupying the remaining sites in the coordination polyhedron, and indeed the coordination number of the copper atom itself, are unknown under the conditions prevailing in culture or *in vivo*. Deprotonation of the hydrazidic nitrogen atom, with a concomitant alteration in the charge on the multidentate ligand, would be expected to occur at physiological pH for both $(\text{SBH})\text{Cu}(\text{Cl})(\text{H}_2\text{O})$ and $(\text{PCPH})\text{Cu}(\text{Cl})_2$, based on their behavior *in vitro*. Similarly, displacement of the monodentate ligands present in these complexes (i.e. chloride ions and/or water molecules) by other ligands present in a biological milieu (e.g. amino acids) is highly likely.

to give the pure complex. Yield: 0.54 g (81%). Anal. Calcd. for $C_{11}H_{10}N_4Cl_2Cu$: C, 39.71%; H, 3.03%; N, 16.84%; Cu, 19.10%; Cl, 21.31%. Found: C, 39.93%; H, 2.67%; N, 16.64%; Cu, 18.31%; Cl, 21.23%. Bleomycin sulfate and cisplatin were purchased from Bristol Laboratories (Bedford, OH) and thiotepa from Lederle Laboratories (Pearl River, N.Y.).

Cell culture procedures. The human melanoma line (Effron), the normal fibroblast line (Goodwin), the human lung epithelial cancer line (SKMES-1), and the normal kidney line (Nelson) were grown in EMEM supplemented with 10 ml of non essential amino acids (100X), 292 mg L-glutamine, 2.26 g $NaHCO_3$, and 100 ml FBS per liter; the human bladder cancer line (T-24) in Swim's S-77 medium supplemented with 0.5 g $NaHCO_3$, 4 g D-glucose, 292 mg L-glutamine, 14 mg L-cystine, 17.9 g tricine and 100 ml FBS per liter; and the mouse fibrosarcoma line (MCA-1511) in RMPI-1640 medium containing 292 mg L-glutamine, 2.26 g $NaHCO_3$, and 200 ml FBS per liter. All media contained 10 mg streptomycin and 10,000 units penicillin per liter. Cells were cultured at 37° under an atmosphere of 5% CO_2 and 95% air at 100% relative humidity.

The testing procedure consisted of plating the cells (5,000 fibroblast, 10,000 normal kidney, or 20,000 of the other cell lines) in 0.2 ml medium into microtiter wells (Corning No. 25860). After 24 hr to allow attachment, the culture medium was replaced with an equivalent volume of chelator-containing medium. After 48 hr of incubation, the cells were pulsed with tritiated thymidine (20 Ci/mmol) for 4 hr at 0.5 μ Ci/well. DNA synthesis and numbers of viable cells (trypan blue exclusion) per well were determined by methods described previously [17, 18].

Dilutions of SBH-Cu and PCPH-Cu were performed with glass tubes and pipets since these compounds adsorb to plastic at low concentrations. SBH-Cu was dissolved in DMSO and PCPH-Cu in water to concentrations of 2 mg/ml and then mixed with appropriate amounts of growth medium to obtain concentrations (1 μ g/ml to 1 μ g/ml, by order of magnitude) for testing. DMSO was added to control cultures in amounts equivalent to those present in the SBH-Cu containing cultures at all concentrations tested. DMSO itself had no effect on DNA synthesis at the concentrations added, and further control cultures containing $CuCl_2 \cdot 2H_2O$ revealed no inhibitory effect at concentrations from 1 μ g/ml to 1 μ g/ml. Each concentration was tested on six culture wells. The standard deviation from the mean averaged 5% for all data points and did not exceed 9% for any given data point. Results were graphically plotted to estimate the concentration of compound that inhibited DNA synthesis by 50%.

Mouse tumor experiments. The acute toxicity of PCPH and SBH, and of their copper chelates, was determined in Balb/C mice (male, 20 g). The dosage of each compound that by intraperitoneal injection killed 50% of the mice within 24 hr was 1.9 g/kg for SBH, 60 mg/kg for SBH-Cu, 1.0 g/kg for PCPH, and 18 mg/kg for PCPH-Cu. Preliminary experiments indicated that the administration of

100 μ g PCPH-Cu or 350 μ g SBH-Cu, twice weekly, was the maximal dosage that did not reduce the normal body weight gain.

For anti-tumor testing, a methylcholanthrene-induced fibrosarcoma (MCA 1511) was implanted in Balb/C mice (male, 20 g) by the subcutaneous injection of 6×10^4 cells into the right flank. After allowing 24 hr for implantation of the tumor, treatment was initiated by injecting an aqueous suspension of the test compound either into the tumor area, intraperitoneally, or intravenously. Each experimental group of tumor-bearing mice consisted of five animals. Each experimental group received PCPH or PCPH-Cu (100 μ g dissolved in 0.1 ml water/ml) or SBH or SBH-Cu (350 μ g suspended in 0.1 ml water/mouse) twice weekly (Monday and Thursday) for 4 weeks. The control group received equivalent doses of phosphate-buffered saline at pH 7.4. A final group of tumor-free mice was injected with these compounds to determine their effects on normal animals. In addition, to minimize complications due to infections, all mice were injected intraperitoneally with 1000 units penicillin and 1 mg streptomycin in 0.1 ml saline concomitantly with the administration of chelators or saline.

Results

In vitro. Both PCPH-Cu and SBH-Cu inhibited DNA synthesis in cultured normal human fibroblasts when added at very low concentrations. PCPH-Cu gave 50% inhibition of DNA synthesis at 30 μ g/ml (9×10^{-14} M) while SBH-Cu gave this degree of inhibition at 150 μ g/ml (4.2×10^{-10} M) (Table 1). The human melanoma and mouse fibrosarcoma lines also gave 50% inhibition of DNA synthesis at low concentrations of PCPH-Cu (0.052 and 0.50 ng/ml respectively) and SBH-Cu (70 and 11 ng/ml respectively), while the remaining cell lines were somewhat less sensitive. This inhibition of DNA synthesis was followed by a decrease in cell number. At 48 hr, at the concentration of PCPH-Cu (0.50 ng/ml) calculated to reduce DNA synthesis by 50% below control values, 66% of cultured Fibrosarcoma remained viable. However, by 96 hr the number of viable cells was reduced to 47% of control values.

The degree of inhibition of DNA synthesis in the four cultured neoplastic cell lines was very comparable to that observed for the anti-tumor drugs bleomycin and cisplatin (Table 1). Interestingly, the human melanoma line was more sensitive to PCPH-Cu and SBH-Cu than to any of the tested anti-tumor drugs.

In vivo. In the mice implanted with tumor cells, after 4 weeks during which the test compounds were administered twice weekly into the tumor area, control animals grew tumors averaging 0.65 cm^3 . SBH-Cu-treated animals had marginally detectable tumors, while the PCPH-Cu-treated group was free of detectable tumors (Fig. 2). Treatment was ceased at this point. Tumors in PCPH-Cu- and SBH-Cu-treated mice remained quiescent for an additional 3 weeks after which growth resumed in all SBH-Cu-treated and most of the PCPH-Cu-treated mice. Five of fifteen

Table 1. Concentration of anti-tumor drug that inhibits DNA synthesis by 50% in six types of cultured cells*

Cell type	Drug concentration (ng/ml) for 50% inhibition				
	PCPH-Cu	SBH-Cu	Cisplatin	Bleomycin	Thiotepa
Normal human fibroblasts	0.000030	0.15			
Normal human kidney	4	5			
Mouse fibrosarcoma	0.50	11	2.4	32	3,600
Human bladder cancer	12	34	95	7	2,000
Human lung epithelial cancer	580	410	870	44	4,300
Human melanoma	0.052	70	6,000	80,000	50,000

* Experimental details are described in Materials and Methods.

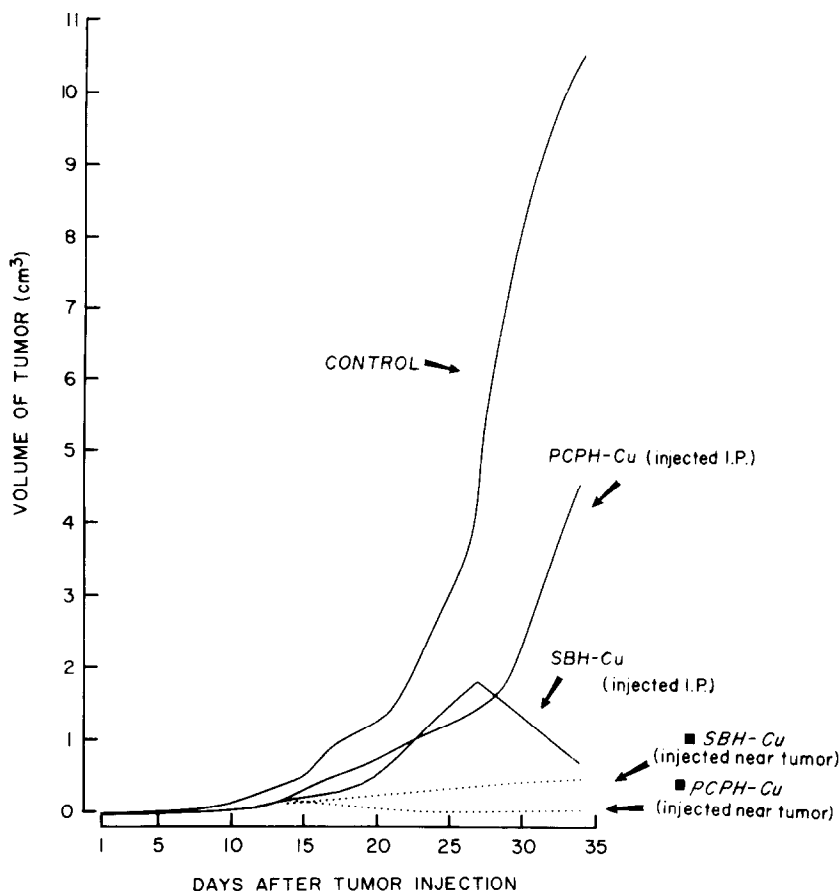


Fig. 2. Effect of PCPH-Cu and SBH-Cu on the growth of an implanted fibrosarcoma in mice. The tumor, which grew as a rounded ball immediately below the skin, was measured twice weekly. The P values for all days of measurement were averaged. For intraperitoneal injection: PCPH-Cu vs control, $P < 0.02$; SBH-Cu vs control, $P < 0.0005$. For injection into the tumor area, PCPH-Cu vs control, $P < 0.0001$; SBH-Cu vs control, $P < 0.0002$.

tumor-bearing mice (from three separate experiments) treated with PCPH-Cu remained free of the tumor for 5 months post-treatment at which time the experiment was terminated.

In experiments in which SBH-Cu and PCPH-Cu were injected intraperitoneally rather than near the site of the tumor, both SBH-Cu and PCPH-Cu reduced tumor growth about 75%, resulting in an average tumor size of 0.16 cm^3 after 4 weeks. Following cessation of treatment, tumors in the PCPH-Cu-treated mice resumed growth. In contrast, tumors in the SBH-Cu-treated group regressed to an average volume of 0.08 cm^3 by week 5, and then resumed growth after an additional 2 weeks. This post-treatment regression may be attributable to the delayed solubilization of slurry-injected SBH-Cu. Thus, the administration of the chelates in areas immediately adjacent to the tumor is more effective in reducing tumor size than intraperitoneal injection.

Repeated intravenous (tail vein) administration of SBH-Cu and PCPH-Cu proved very difficult. Localized venous necrosis developed around the injection site after one to three injections, with subsequent sloughing of the tail.

SBH and PCPH did not significantly reduce tumor size by either treatment regimen. Attempts to administer SBH and PCPH at higher dosages appeared to cause itching in the mice. Treatment of non-tumor bearing mice with similar dosages of SBH-Cu and PCPH-Cu produced a thickening of the skin at the area of injection. No other effects have been observed in these mice in the subsequent 5 months.

Discussion

These results demonstrate that two tridentate, hydrazone-copper complexes, PCPH-Cu and SBH-Cu, are potent mitotic inhibitors with antineoplastic activity against an implanted mouse fibrosarcoma. Structurally similar compounds, such as pyridine-2-carboxaldehyde thiosemicarbazone and related α - N -heterocyclic carboxaldehyde thiosemicarbazones, have long been recognized as potent cytotoxic agents which exhibit tumor-inhibitory actions [13, 14] that have been attributed to their abilities to function as tridentate chelators of transition metal ions [13, 19]. Since early structure-activity studies established that carbazones and salicylaldehyde analogs of pyridine-2-carboxaldehyde thiosemicarbazone were inactive, possession of an NNS set of ligating atoms was assumed to be an important determinant of the bioactivity of such tridentate chelators [20, 21]. Our results indicate that ligands that are similarly tridentate but which bind metals through an ONO or NNN array of donor atoms are also potent inhibitors of cell growth and that, as in the thiosemicarbazones [13, 22], complexation to copper increases inhibitory activity.

It is possible that this inhibitory activity may be related to structural similarities between those molecules and the GHL-Cu complex. Recently, GHL-Cu has been found to be a potent inducer of *in vivo* angiogenesis in rabbits [23]. Thus, the antineoplastic activity of these two hydrazone-copper analogs of GHL-Cu may reside either in their general cytotoxicity or might possibly be due to an interference with the mechanisms which concentrate the copper ions required for tumor angiogenesis.

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Effect of acute cocaine administration on the metabolism of antipyrine *in vivo*

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Cocaine, a potent central nervous system stimulant, has been studied extensively with respect to its psychopharmacology. Only recently, however, has cocaine been found to be acutely hepatotoxic in mice, causing necrosis, elevation in serum transaminase levels, and depression of several hepatic enzyme activities [1-3]. It has been proposed that free radical production and the resulting lipid peroxidation play an important role in the production of cocaine-induced hepatotoxicity [4-6].

To date, all studies involving acute cocaine-induced hepatotoxicity have characterized the hepatic damage in terms of histopathology and biochemical damage. Shuster *et al.* [1] have demonstrated previously that two sequential doses of cocaine to induced mice result in enhanced pentobarbital sleeping times and decreased pentobarbital metabolism *in vivo*. Unfortunately, this study chose to measure *in vivo* pentobarbital metabolism merely by monitoring the disappearance of pentobarbital [²⁻¹⁴C] from the blood; this method does not definitively reflect the cytochrome P-450-mediated metabolism but, rather, includes all types of metabolism centered on the 2-carbon of pentobarbital. Similarly, these investigators correlated an increase in plasma pentobarbital half-life after cocaine administration with enhanced sleeping times: no attempt was made to determine any changes in volume of distribution, a factor that plays an important role in determining

barbiturate-induced sleeping times. Freeman and Harbison [7] have also reported that low-dose, chronically administered (several weeks) cocaine to non-induced mice results in enhanced hexobarbital narcosis.

The intent of this study was to determine a physiological, *in vivo* correlate to the existing biochemical data on acute cocaine-induced hepatotoxicity in a non-induced mouse strain. We report that a single injection of cocaine produced a dramatic decrease in hepatic cytochrome P-450 content and a correspondingly marked depression of *in vivo* hepatic antipyrine metabolism in non-induced mice. It is suggested that cocaine administration may likewise alter the hepatic metabolism of a wide variety of xenobiotics and, in doing so, may alter the therapeutics and toxicity of these drugs.

Materials and Methods. DBA/2Ha male mice, 10 weeks of age, were obtained from Health Research Laboratories (West Seneca, NY) and were housed on corn cob bedding. Mice were given food (Purina Chow No. 5001) and tap water *ad lib*. Cocaine hydrochloride (Merck) was dissolved in a saline solution immediately prior to 60 mg/kg i.p. injection; control mice received only the vehicle solution. Cocaine was injected 24 hr prior to antipyrine administration, since we have noted maximal hepatic damage during this time period [3]. Serum glutamic-pyruvic transaminase was measured as described previously [8]. Antipyrine (25 mg/kg) was administered by intracardiac injection.