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Cytotoxic Chelators and Chelates
1. Inhibition of DNA Synthesis in Cultured Rodent and Human Cells by Aroylhydrazones and by a Copper(II) Complex of Salicylaldehyde Benzoyl Hydrazone

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Aroylhydrazones of pyridoxal and of salicylaldehyde, a series of tridentate chelating agents, are potent inhibitors of DNA synthesis and cell growth in a number of human and rodent cell lines grown in culture. A copper(II) complex of the most potent of the chelators, salicylaldehyde benzoyl hydrazone (SBH), exhibits significantly greater inhibitory activity than does SBH itself. Although the bioactive forms and mechanism of action of these agents are uncertain, their cytotoxic activity can equal or exceed that of many chelators and chelates previously known to possess such properties, including compounds used clinically. SBH and its copper complex are relatively non-toxic to mice and show some measure of selectivity in their effects on different cell types. It is possible that aroylhydrazones of this type and/or their metal complexes could prove to be useful therapeutic agents.

Introduction

The development of agents which selectively inhibit the growth of certain types of cells is an area of much current interest. Such compounds can potentially find use as anti-neoplastic, anti-viral and anti-inflammatory agents, as bacteriostats or as fungicides, algicides and herbicides. It has long been recognized that some chelating agents can possess cytotoxic properties [1–3] and that chelation of endogenous metal ions may underlie the mode of action of anti-neoplastic agents such as hydroxyurea [4] and of fungicides such as 8-hydroxyquinoline [5]. Complexes of both non-physiological metals (*e.g.* platinum [6], palladium [7] and rhodium [8]) and of essential metal ions (*e.g.* iron [9], copper [10] and zinc [11]) can also exhibit potent cytotoxicity, perhaps best exemplified by the use of *cis*-$[(NH_3)_2PtCl_2]$ in the treatment of certain human cancers [12]. The range of chelating agents and their metal complexes currently known to be inhibitors of cell growth is sufficiently diverse to suggest that such compounds, as a class, may warrant more extensive investigation.

This paper describes the effects of a series of tridentate aroylhydrazone chelators on DNA synthesis and cell growth in a variety of rodent and human cell lines grown in culture. The inhibitory activity of the most potent of the free chelating agents, salicylaldehyde benzoyl hydrazone (abbreviated SBH), is compared with that of the copper complex $[(SBH)Cu(Cl)(H_2O)]$.

Experimental

Materials

The following compounds were obtained from the commercial sources indicated and were used without further purification: pyridoxal hydrochloride, ethyl picolinate and nicotinoylhydrazide (Sigma Chemical Co., St. Louis, Missouri); benzoylhydrazide, isonicotinoylhydrazide and picolinic acid (Aldrich Chemical Co., Milwaukee, Wisconsin); salicylaldehyde (Eastman Organic Chemicals, Rochester, New York); copper(II) chloride dihydrate (J. T. Baker Chemical Co., Phillipsburg, New Jersey); desferrioxamine B, as the methanesulfonate (CIBA Pharmaceuticals, Summit, New Jersey); *cis*-dichloro-

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diammine platinum(II) (Parke, Davis & Co., Detroit, Michigan); bleomycin sulftate (Miles Laboratories, Elkhart, Indiana). Picolinoylhydrazide was prepared from ethyl picolinate plus hydrazine hydrate [13].

Preparation of Chelating Agents [14–17]

Benzoyl, nicotinoyl, isonicotinoyl and picolinoyl hydrazones of salicylaldehyde were prepared as follows: The appropriate acid hydrazide (20 mmol) was dissolved in an ethanol:water mixture (1:3, v/v, 40 ml). A solution of salicylaldehyde (2.44 g, 20 mmol) in ethanol (20 ml) was added to the hydrazide solution with stirring and the resulting mixture placed on a steam bath for 20 min. On cooling to room temperature, crystals of the hydrazone separated and these were filtered off and dried under vacuum. Recrystallization from ethanol, aqueous ethanol or *i*-propanol yielded the pure product. Yields: 50–80%.

The corresponding pyridoxal hydrazones were obtained as follows: The acid hydrazide (10 mmol) was dissolved in an ethanol:water mixture (1:1, v/v, 20 ml) and added to a solution of pyridoxal hydrochloride (2.04 g, 10 mmol) in water (20 ml). Sodium acetate (1.0 g) in water (10 ml) was added and the resulting solution was stirred briefly then placed on a steam bath for 15 min. The solution was then allowed to cool and stand at room temperature for 24 hours, during which time crystals of the product separated. These were collected by filtration and recrystallized from methanol/petroleum ether, ethanol/ether or ethanol/hexane to give the pure hydrazone. Yields: 50–80%.

Melting points, IR and ^{1}H NMR data for these eight hydrazones were in good agreement with values previously reported [14–17].

Preparation of (Salicylaldehyde benzoyl hydrazonato)copper(II) Monochloride Monohydrate

Method 1

A solution of salicylaldehyde benzoyl hydrazone (0.50 g, 2.1 mmol) in boiling 95% ethanol (20 ml) was added to a solution of copper(II) chloride dihydrate (0.34 g, 2.0 mmol) in boiling 95% ethanol (20 ml). A deep green-brown solution was immediately formed. The boiling solution was allowed to cool and stand undisturbed overnight at room temperature. Black, rod-like crystals separated and these were filtered off, washed with ethanol (2 X 10 ml) and ether (2 X 10 ml) and dried under vacuum. Yield: 0.55 g (77%). M.p. 156 °C (dec.). IR (nujol mull) ν 3450 (w), 3350 (w), 3160 (w), 1620 (s), 1610 (s), 1600 (sh), 1580 (sh), 1550 (s), 1540 (sh) cm^{-1}. *Anal.*: calculated for $C_{14}H_{12}O_3N_2CuCl$: 47.33 %C, 3.40 %H, 7.88 %N, 17.89 %Cu, 9.98 %Cl. Found: 47.74 %C, 3.47 %H, 7.85 %N, 18.15 %Cu, 9.44 %Cl.

Method 2

Finely-ground salicylaldehyde benzoyl hydrazone (9.6 g, 40 mmol) was suspended in a solution of copper(II) chloride dihydrate (6.8 g, 40 mmol) in water (400 ml). The suspension was vigorously stirred at room temperature for 4 days. After this time the reaction mixture consisted of a green amorphous solid suspended in a pale yellow-green solution. The floculent green solid was filtered off, washed with water (2 X 75 ml) and acetone (2 X 75 ml) then dried under vacuum. Yield: 12.47 g (87%). The melting point and IR spectrum of this product were identical to those of the product obtained by Method 1.

Cell Culture Procedures

The following cell lines were employed: normal human kidney cells, normal human fibroblasts, human bladder carcinoma (T-24), human lung epithelial carcinoma (SKMES-1), human melanoma (effron), rat hepatoma (HTC_4) and mouse lymphoid leukemia (L1210). The human fibroblasts, kidney cells, melanoma and lung carcinoma lines were cultured in Eagle's minimum essential medium containing 10% fetal calf serum. The rat hepatoma and human bladder lines were grown in Swims' S-77 medium plus 10% calf serum and the L1210 cells in L-15 medium plus 10% horse serum.

Suitable solutions of the free chelators in growth medium were obtained by raising the pH of an aqueous suspension (3.3 mg/ml) to 9.5, then adding 30 μl of the resulting solution to 0.97 ml of growth medium. This did not significantly alter the pH of the medium and the chelators remained in solution, perhaps as a result of binding to serum proteins. The resulting medium contained 100 μg of chelator/ml, additional dilutions being made with untreated growth medium to obtain the lower concentrations used in testing. [(SBH)Cu(Cl)(H_2O)] was dissolved in DMSO at a concentration of 2mg/ml then mixed with appropriate amounts of growth medium to obtain the desired concentration. DMSO was added to control cultures (and to cultures containing free SBH) in amounts equivalent to those present in the cultures containing the copper complex, at each of the concentrations tested. DMSO itself had no effect on DNA synthesis at the concentrations added and further control cultures containing only $CuCl_2 \cdot 2H_2O$ revealed no inhibitory effect in the concentration range from 0.01–10 μg/ml.

The testing procedure consisted of plating the cells (generally between 5 X 10^3 and 2 X 10^4 cells in 0.2 ml of medium) in microtiter wells (Falcon # 3042 or Corning # 25860) which were incubated under 5% CO_2–95% air at 100% humidity and 37 °C. After allowing 24 hours for attachment, the regular culture medium was replaced by an equivalent volume of medium containing the test compound.

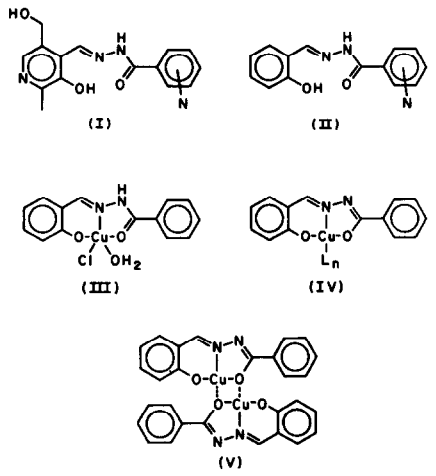

Fig. 1. Structures of aroylhydrazone chelating agents and of copper(II) complexes of salicylaldehyde benzoyl hydrazone.

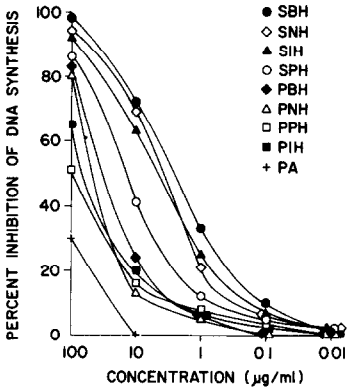

Fig. 2. Effect of aroylhydrazones on DNA synthesis in four cell lines: rat hepatoma (HTC_4), human bladder carcinoma (T-24), human lung epithelial carcinoma (SKMES–1) and human melanoma (effron). The data points shown represent average values for all four cell lines (6 culture wells/cell line) at a given concentration of chelator. For each individual cell line the standard deviation from the mean averaged 5% for all five data points and did not exceed 9% for any given point. The following abbreviations are used: SBH = salicylaldehyde benzoyl hydrazone, SNH = salicylaldehyde nicotinoyl hydrazone, SIH = salicylaldehyde isonicotinoyl hydrazone, SPH = salicylaldehyde picolinoyl hydrazone, PBH = pyridoxal benzoyl hydrazone, PNH = pyridoxal nicotinoyl hydrazone, PPH = pyridoxal picolinoyl hydrazone, PIH = pyridoxal isonicotinoyl hydrazone, PA = picolinic acid. All data were obtained by carrying out dilutions in plasticware.

After a total of 48 hours incubation in the presence of the chelator or chelate, the cells were pulsed with ^3H thymidine (20 Ci/mmol) for 4 hours at 0.05 μCi/well. DNA synthesis was then determined from the extent of incorporation of the radiolabel, using methods described previously [18].

In initial experiments, plastic containers and pipettes were used in carrying out successive dilutions of the test compounds in growth medium. However, it was found that results became erratic at low concentrations in that the value for % inhibition of DNA synthesis remained highly reproducible but step-wise dose response curves were not always obtained. When dilutions were made in glass tubes and using glass pipettes, smooth dose response curves were obtained. It thus seems that these compounds have a tendency to adsorb to plastic surfaces and that this effectively reduces the concentration of the compound that is available for inhibiting cell growth. This problem has not been completely resolved since the culture wells used in testing are also made of plastic (oxidized polystyrene). Suitable annotations appear throughout the text to indicate whether dilutions of the test compound were carried out in glassware or in plasticware.

Results and Discussion

Inhibition of DNA Synthesis by Uncomplexed Aroylhydrazones

Aroylhydrazones of 2-hydroxy- substituted aromatic aldehydes have recently been shown to be capable of chelating iron *in vivo* [19–22], probably through the formation of tridentate complexes of the type which we have characterized by X-ray crystallography [23]. This demonstrated ability to penetrate cell membranes and bind an essential metal ion in competition with endogenous ligands led us to examine the effects of such agents on DNA synthesis in cultured cells. We initially surveyed eight structurally-related hydrazones, namely the benzoyl, nicotinoyl, isonicotinoyl and picolinoyl hydrazones of pyridoxal (Fig. 1, I) and of salicylaldehyde (Fig. 1, II). Each chelator was tested against four neoplastic cell lines: a rat hepatoma and human melanoma, bladder carcinoma and lung epithelial carcinoma lines. These data appear in Fig. 2. The chelating agents markedly inhibited DNA synthesis in these cell lines at concentrations down to ~1 μg of chelator/ml of growth medium (*i.e.* $\sim 4 \times 10^{-6}$ *M*), salicylaldehyde hydrazones (II) being consistently more potent than the corresponding pyridoxal derivatives (I). This inhibition of DNA synthesis was followed by a decrease in cell number over 48 hours.

It is noteworthy that the most active of the eight compounds tested, SBH, is also the derivative which contains the smallest number of hydrophilic sites. The pyridoxal hydrazones and all of the salicylaldehyde hydrazones except SBH contain one or more heterocyclic nitrogen atoms which are not

TABLE I Concentrations of SBH and [(SBH)Cu(Cl)(H_2O)] which reduce DNA Synthesis by 50% in Various Cell lines, in μg/ml.*

Cell line	SBH	[(SBH)Cu(Cl)(H_2O)]
Rodent tumor lines:		
Mouse leukemia (L1210)	P 4	P 5
Rat hepatoma (HTC_4)	P 30	P 0.5
Human tumor lines:		
Bladded cancer (T-24)	P 0.5	P 0.05
Lung carcinoma (SKMES-1)	P 8	P 0.4
Melanoma (effron)	P 1	P 0.2
	G 0.3	G 0.04
Normal human cell lines:		
Kidney cells	G 0.1	G 0.04
Fibroblasts	P 0.07	P 0.005
	G 0.0026	G 0.00015

*Values prefixed by P denote dilutions were made in plasticware, those by G in glassware.

involved in coordination to a metal. These sites would be expected to impart hydrophilic character to the molecule, particularly if protonated at physiological pH. Similarly, the greater inhibitory activity of the salicylaldehyde hydrazones compared to their pyridoxal analogs may reflect the presence in the latter of an additional hydrophilic moiety *i.e.* the $-CH_2OH$ group.

Inhibition of DNA Synthesis by a Copper(II) Complex of SBH

There is ample evidence from studies of cytotoxic thiosemicarbazones [3] that the inhibitory activity of such chelators can be increased by complexation with copper. Accordingly, we synthesized and tested a copper complex of the most active of the eight aroylhydrazones (SBH), in order to investigate whether this might also exhibit enhanced cytotoxic properties. Copper complexes of SBH have previously been described [17, 24–26] and it has been suggested that SBH might be a useful chromogenic reagent for copper(II) [26]. The complex [(SBH)-Cu(Cl)(H_2O)] was obtained by allowing equimolar quantities of SBH and $CuCl_2 \cdot 2H_2O$ to react either in ethanol, as described by Iskander *et al.* [17], or in water at a concentration of ~100 m*M*. This complex is believed to have the structure shown in Fig. 1 (structure III), in which the aroylhydrazone functions as a uninegative tridentate ligand, the coordination polyhedron being completed by a coordinated chloride ion and a water molecule. When the solid complex is heated briefly its color changes from green to red-brown, this color change being reversed on allowing the solid to cool in air. This is consistent with the observations of Iskander *et al.* [17] and probably reflects reversible loss of the coordinated water molecule.

When tested against a range of normal and neoplastic cell lines, complex III was consistently more effective in inhibiting DNA synthesis than was SBH itself (Table I). This enhancement of inhibitory activity ranged from 2.5-fold to 60-fold depending upon the particular cell line, the mouse leukemia line being the only one where no effect was seen. SBH and its copper complex are not merely inhibitory but exhibit pronounced cytotoxicity. For example, in the human bladder cancer line the number of viable cells in control cultures increased 215% after 7 days in growth medium. In contrast, addition of 10 μg/ml (~3×10^{-5} *M*) of complex III to the growth medium resulted in a 94% decrease in the number of viable cells over the same period. If the chelate-containing medium was replaced after 48 hours by regular growth medium, cell number did not increase.

Possible Bioactive Forms and Mechanisms of Action

Before being introduced into the culture medium, the copper complex of SBH probably has the structure shown in Fig. 1, III. However, it seems likely that this structure is modified once exposed to the conditions prevailing in culture *i.e.* at physiological pH and in the presence of calf or horse serum. As a result, the structure of the species which actually interacts with the cells is uncertain. Based on what is known of the coordination chemistry of copper complexes of SBH [17, 24, 25], deprotonation of the hydrazidic nitrogen atom and concomitant loss of the coordinated chloride ion might be expected to occur at physiological pH. Such complexes, in which SBH acts as a dinegative tridentate ligand, are well

TABLE II. Comparison of the Inhibitory Activities of Various Chelating Agents and Metal Complexes in Two Neoplastic Cell Lines.

Compound	Concentration which reduces DNA synthesis by 50%, in μg/ml	
	Human Bladder carcinoma (T-24)	Rat hepatoma (HTC_4)
SBH	0.5	30
[(SBH)Cu(Cl)(H_2O)]	0.05	0.5
Desferrioxamine B	–	>10
Picolinic acid	>100	>100
cis-[$(NH_3)_2PtCl_2$]	0.1	0 5
Bleomycin sulfate	10**	–

*All values refer to dilutions made in plasticware. **In nano Units/ml. 1 Unit of bleomycin sulfate = ~1 mg

known [17, 24, 25] and take the form of dimers (Fig. 1, structure V). There is disagreement as to whether the dimeric bridges are formed via the carboxylic oxygen atoms [17] (as shown) or through the phenolic groups [24]. In either case, it is unlikely that such oxygen bridges would remain intact in the presence of the wide variety of endogenous ligands (polypeptides, amino acids, sugars, *etc.*) contained in the culture medium. It is therefore our working hypothesis that the active form of the copper complex in cell culture may be that shown in structure IV, Fig. 1, in which SBH acts as a divalent tridentate ligand and the remaining coordination sites are occupied by an unknown number of endogenous ligands, L, of unknown identity.

Even less clear is the mechanism, or mechanisms, by which the aroylhydrazones and the copper complex of SBH exert their growth-inhibitory effect on cells. There is good evidence that SBH is able to cross the cell membranes of reticulocytes *in vitro* [19] and of hepatocytes *in vivo* [22]. This suggests that such agents are probably not restricted to acting only at the external surface of the cell membrane (*e.g.* by extracellular sequestering of essential metal ions required for cellular replication) but rather that they may act intracellularly in inhibiting cell growth. However, whether such inhibition is a result of binding to intracellular proteins, direct binding to nucleic acids or disruption of metal transport (three mechanisms which have been advanced to explain the cytotoxicity of thiosemicarbazones [3]) or of still other mechanisms, is unknown.

Potential Utility

In order to be therapeutically useful, any cytotoxic agent must meet three criteria. Firstly, it must be effective *i.e.* it should be a potent inhibitor of the growth of target cells. Secondly, it must be selective, so that other cells in the body are relatively unaffected by it. Lastly, it must be safe *i.e.* free not only from non-selective cytotoxicity but also from all other toxic effects when administered at the doses needed to achieve the desired therapeutic effect. In practice, many agents which are used clinically do not completely fulfill all of these requirements. However, total failure to satisfy any one of these criteria is usually sufficient to preclude any potential utility in man. On these grounds, the possibility that aroylhydrazone chelators and chelates could be developed into useful drugs cannot be totally ruled out, given the data obtained thus far.

The aroylhydrazones, and particularly the copper complex of SBH, appear to remain potent inhibitors of DNA synthesis at concentrations well below those at which many other chelating agents and metal complexes cease to show such activity. For example, in a recent study [27] of the effects of 37 different chelating agents (mainly hydroxamates and catecholates) on PHA-stimulated normal human lymphocytes, only three showed significant inhibition of DNA synthesis at 10^{-3} *M*, most being inactive or only marginally active even at this concentration. When tested in our rat hepatoma line, the hexadentate hydroxamate ligand desferrioxamine B reduced DNA synthesis by 50% at concentrations above 10 μg/ml (~2×10^{-5} *M*, Table II). Similarly, the bidentate chelator picolinic acid, which inhibits the growth of SV-40 transformed normal rat kidney cells by a mechanism thought to involve the chelation of iron [28, 29], requires concentrations of 10^{-3} *M* to exert this effect in the transformed cells and was inactive in our neoplastic cell lines at concentrations below ~100 μg/ml (~7×10^{-4} *M*, Fig. 2 and Table II).

In the two cell lines where direct comparisons were made, [(SBH)Cu(Cl)(H_2O)] and *cis*-[$(NH_3)_2PtCl_2$] exhibited approximately equivalent activities (Table II) while bleomycin, another agent used clinically, was much more active than either complex. In general, the activities of SBH and its copper complex appear comparable to those of the most potent

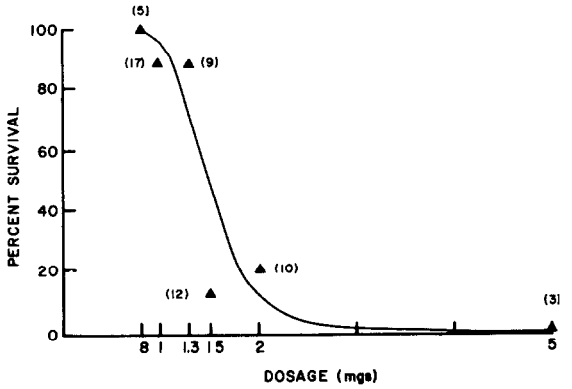

Fig. 3. Effect of [(SBH)Cu(Cl)(H_2O)] on the survival of male Swiss–Webster mice of average body weight 25 g. The complex was administered in the form of an aqueous suspension by intraperitoneal injection. The values shown in parentheses are the number of animals in each group.

members of the thiosemicarbazone group of chelating agents and their copper chelates. These latter compounds have been extensively studied as potential anti-viral and anti-neoplastic agents [3] and one such compound has undergone clinical trials [30]. Despite the fact that the particular agent chosen for this trial (5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone) was one of the least toxic of such derivatives when tested in animals, severe toxic side effects were noted in the clinical study and these have apparently discouraged further therapeutic use of thiosemicarbazones in man.

Preliminary studies of the toxicity of SBH and its copper complex in mice suggest that these compounds are relatively well tolerated. Figure 3 shows the effects on the survival of male Swiss–Webster mice of a single intraperitoneal injection of an aqueous suspension of [(SBH)Cu(Cl)(H_2O)]. The LD_{50} dosage for the copper complex when given in this manner is approximately 60 mg/kg. The corresponding value for SBH itself, administered in the same form and by the same route, is 1.9 g/kg, showing that complexation with copper increases not only the cytotoxicity of this chelator but also its acute toxicity to the intact animal. When mice were injected daily with an intraperitoneal dose of the copper complex equivalent to one half of the LD_{50} dosage, the animals progressively lost weight. However, this effect was reversed once administration of the chelate was discontinued and the mice showed no long-term ill effects.

Inspection of Table I shows that both SBH and its copper complex exhibit inhibitory activities which vary significantly from one cell line to another. If comparisons are limited to the human cell lines and to values obtained under the same conditions (*i.e.* in plasticware), the concentration of either agent required to reduce DNA synthesis by 50% in the most sensitive cell line (the normal fibroblasts) differs by some two orders of magnitude from the analogous value for the most resistant cells (the lung carcinoma). Although this suggests a potential for selective cytotoxicity, much more extensive testing using a wider range of derivatives and a broader spectrum of cell types will be required before the extent of such selectivity can be established. Only then will it be possible to determine the types of application toward which future development of these agents should be directed.

Acknowledgements

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Note added in proof: We have recently completed a single crystal X-ray diffraction study of the copper complex of SBH (A. A. Aruffo, T. B. Murphy, D. K. Johnson, N. J. Rose and V. Schomaker, *Inorg. Chim. Acta, 67,* L25 (1982)) which showed that this species is in fact 4-coordinate, the correct formulation being [(SBH)Cu(Cl)] $\cdot H_2O$.