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2,9-DIMETHYL-1,10-PHENANTHROLINE (NEOCUPROINE): A POTENT, COPPER-DEPENDENT CYTOTOXIN WITH ANTI-TUMOR ACTIVITY

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Abstract—2,9-Dimethyl-1,10-phenanthroline (2,9-DMP), a copper-specific chelator, was a potent cytotoxin against L1210 cells *in vitro*; its activity was dependent upon available Cu^{2+} in the medium. Other divalent ions, Fe^{2+} and Zn^{2+}, were ineffective as promotors of growth inhibition. As the copper chelate, a 4 μM solution produced a 4 log kill after a 1-hr incubation. This was in marked contrast to 1,10-phenanthroline, whose inhibition of cell growth was overcome by added Cu^{2+}, Fe^{2+} and Zn^{2+}. Cellular uptake of labeled 2,9-dimethyl-1,10-phenanthroline also required added Cu^{2+} in the medium. This transport was energy dependent, and the drug was concentrated over 200-fold by the cells. In preliminary evaluations, copper-2,9-DMP showed significant chemotherapeutic activity against the P388 murine lymphoma *in vivo*.

Metal binding agents, chelators, interfere with metabolic processes of whole animals and with those of mammalian or microbial cells in culture [1]. The 1,10-phenanthrolines, a class of chelators, have antibacterial [2, 3], antifungal [4], antiviral [5], and antineoplastic properties [6], and have been applied as topical antimicrobials [7, 8]. The action of 1,10-phenanthroline may be of a chelator or chelate type. As a chelator, its toxicity has been attributed to its ability to combine with zinc and thus inhibit zinc-containing nucleotidyl transferases [9, 10] and DNA synthesis [11, 12]. In these systems, added divalent metal ions reverse this inhibition [11, 12]. Alternatively, chelate complexes of 1,10-phenanthroline with the divalent metal ions, Cu^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+} and Ru^{2+}, are reported to be cytotoxic [6], and the cuprous chelate promotes the degradation of DNA in a model cell-free system [13, 14].

Substitution of 1,10-phenanthroline at the 2 and 9 positions confers upon this derivative specificity for the cuprous ion, which has been attributed to steric hindrance to planar configuration of bivalent transition metal cations at the coordination site [15, 16]. We here report that 2,9-dimethyl-1,10-phenanthroline (2,9-DMP) is a potent copper-dependent cytotoxin which is biologically active only as the coordinated metal complex. Copper-2,9-DMP is only marginally effective against the L1210 leukemia *in vivo* but significantly increased the life span of P388 leukemia-bearing mice.

MATERIALS AND METHODS

Growth in cell culture. The L1210 established murine lymphoma line was maintained in RPMI 1630 growth medium (K.C. Biologicals, Lenexa, KS) containing 16.5% fetal bovine serum (Sterile Systems, Logan, UT) and routinely tested for mycoplasma contamination. Cytotoxicity of 2,9-dimethyl-1,10-phenanthroline (G. Frederick Smith Chemical Co., Columbus, OH) was assessed as follows: cells were harvested at mid-log phase ($8–10 \times 10^5$ cells/ml), washed three times with fresh growth medium containing 40 μg gentamicin (Schering Corp., Kenilworth, NJ) per ml, and resuspended at 1×10^5 cells per ml as determined by a model ZBI Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). Suspensions (7 ml) were added to 25 cm^3 Corning flasks, and 2,9-DMP, dissolved in ethanol (final concentration 1 μl/ml), was introduced at appropriate concentrations alone or combined with 25 μM $CuSO_4$ or 25 μM bathocuproine sulfonate, whose systematic name is 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonic acid, disodium salt (G. Frederick Smith Chemical Co.) in H_2O (10 μl/ml). Cells were grown at 37° for 45 hr in tightly stoppered flasks, and cell density was determined as indicated above. The results are expressed as percent growth fraction (N − No/No) compared to appropriate controls to which only the solvent was added.

Cell viability determination. Cell suspensions (1×10^5 cells/ml) were incubated with several concentrations of 2,9-DMP in the presence or absence of 25 μM copper sulfate for 1 hr at 37°. The treated cells were then washed with fresh medium containing 20% fetal bovine serum and suspended in growth medium supplemented with 0.4% soft nutrient-agar (Noble Agar, Difco Laboratories, Detroit, MI) at serial dilutions of 1×10^5 cells/tube. After 2 weeks at 37°, colony formation of treated cells was compared to that obtained with untreated controls or with cells exposed to appropriate amounts of the solvents, ethanol and water, used to dissolve 2,9-

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DMP and $CuSO_4$ respectively. The solvents at concentrations used for introduction of additives had no effect on cloning efficiency, which under these conditions ranged from 85–90%.

Transport and release of 2,9-DMP. Cells were incubated in complete RPMI 1630 medium supplemented with 16.5% fetal bovine serum and gentamicin (40 μg/ml) containing [^{3}H]-2,9-DMP (sp. act. 30 mCi/mmole; Moravek Biochemicals, Brea, CA). Incubations with [^{3}H]-2,9-DMP and [^{3}H]-tetraphenylphosphonium bromide (New England Nuclear Corp., Boston, MA) were also carried out in defined Krebs–Ringer medium containing Bis-Tris/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] buffer, 10 mM, at pH 6.0 and 7.8. These were performed at 37° with cell suspensions (1×10^5 cells/ml) under conditions identical to that used for growth inhibition and lethality experiments. Incubations were terminated by layering triplicate 1.0-ml aliquots of the incubation mixture over 0.6 ml of F50 Versilube silicone fluid (General Electric, Waterford, NY) in 1.5-ml polypropylene tubes (Eppendorf micro tubes, Brinkmann Instruments, Westbury, NY) and immediately centrifuging the cells through the oil at 12,000 *g* for 1 min in a Fisher Microcentrifuge (model 234; Fisher Scientific Co., Rockville, MD). The centrifuge tube tips containing the cells were cut off, the pellets were solubilized in 0.2 N NaOH and neutralized with acetic acid, and the associated radioactivity was estimated in a Beckman L-5000 scintillation counter (Beckman Instruments, Irvine, CA). Estimations of intracellular accumulation of the labeled compounds were made by comparing intracellular and extracellular tritiated water observed in concurrent incubations, and interstitial contamination was determined with the use of [^{3}H]-inulin (New England Nuclear).

Determination of chemotherapeutic activity. The P388 murine lymphocytic leukemia was maintained in ascites form in 8 to 12-week-old male CDF_1 (Balb C × DBA/2) mice and transplanted weekly with 1×10^6 cells/mouse. For determination of chemotherapeutic activity, mice (24–30 gm) were inoculated i.p. with 1×10^6 cells in 0.2 ml of Dulbecco's phosphate buffered saline (Gibco Laboratories, Grand Island, NY) containing 0.7% crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, MO) to stabilize the cell suspension. Treatment began 24 hr later and consisted of 0.2-ml injections, i.p., of 20 μmoles/kg 2,9-DMP, dissolved in 25% propylene glycol, and 10 μmoles/kg $CuSO_4$ with 20 μmoles/kg nitrilotriacetic acid to reduce copper toxicity [17]. Appropriate controls were maintained with injections of 25% propylene glycol.

RESULTS

Copper-dependent cytotoxicity of 2,9-dimethyl-1,10-phenanthroline. 2,9-Dimethyl-1,10-phenanthroline inhibited growth of L1210 cells at nanomolar concentrations and this inhibition reached a limiting value of 90% (Fig. 1). The copper requirement for cytotoxicity was demonstrated by (i) the addition of $CuSO_4$ which potentiated growth inhibition and (ii) the addition of bathocuproine sulfonate which abolished growth inhibition. We have reported that bathocuproine sulfonate is a reliable and specific indicator of copper-mediated cytotoxicity because of its ability to deplete the medium of available copper [18]. Bathophenanthroline sulfonate, which lacks the 2 and 9 methyl groups and, therefore, is not specific for copper, was ineffective in protecting the cells from 2,9-DMP cytotoxicity (Fig. 2). Neither $CuSO_4$ nor bathocuproine sulfonate was an effective inhibitor when added alone at the concentrations indicated in Fig. 2 (Fig. 3). Metal ion specificity for the potentiation of 2,9-DMP toxicity was further demonstrated by adding Fe^{2+} or Zn^{2+} instead of Cu^{2+} in combination with 2,9-DMP. As shown in Fig. 4, only

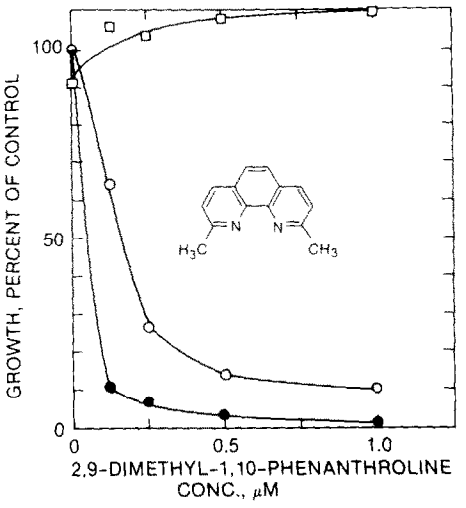

Fig. 1. Requirement for copper in the inhibition of cell growth by 2,9-dimethyl-1,10-phenanthroline (2,9-DMP). Increasing concentrations of 2,9-DMP were added to 7-ml suspensions of L1210 cells (1×10^5/ml) in growth medium and incubated at 37° for 45 hr as described in Materials and Methods. Toxicity was assessed as percent of control growth when the drug was added alone (○—○), in the presence of 25 μM $CuSO_4$ (●—●), or in the presence of 25 μM bathocuproine sulfonate (□—□).

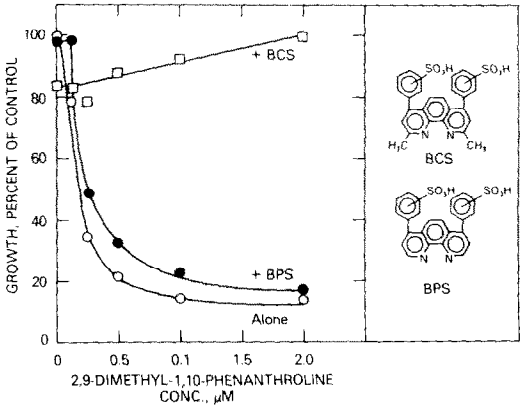

Fig. 2. Specificity of bathocuproine sulfonate protection against copper-dependent toxicity of 2,9-DMP. Cells were incubated with increasing concentrations of 2,9-DMP and growth was measured in the presence of 25 μM bathocuproine sulfonate (BCS) (□—□), and bathophenanthroline sulfonate (BPS) (●—●) and compared to sets containing 2,9-DMP only (○—○).

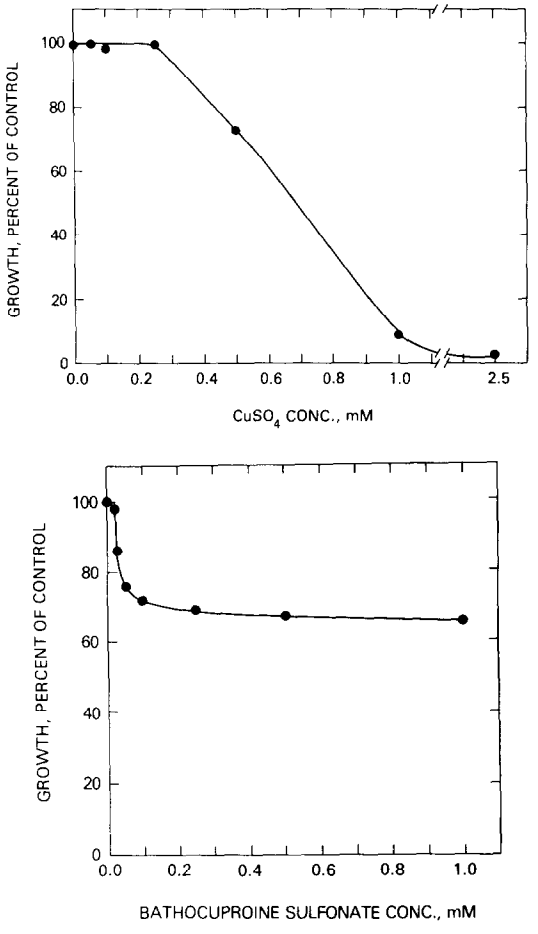

Fig. 3. Effect of $CuSO_4$ and bathocuproine sulfonate on the growth of L1210 cells. Increasing concentrations of $CuSO_4$ (top panel) and bathocuproine sulfonate (bottom panel) were added to 7-ml suspensions of log-phase L1210 cells (1×10^5/ml). Growth was measured, as described in Materials and Methods, after incubation for 45 hr at 37°.

Cu^{2+} potentiated the 2,9-DMP growth inhibition, while Fe^{2+} had no effect and Zn^{2+} caused a slight protection. The inhibition of growth by copper-2,9-DMP was due to a rapid cytocidal activity as indicated in Fig. 5. Incubation of the cells for 1 hr with 2,9-DMP in the absence of $CuSO_4$ resulted in minimal cytocidal activity, indicating the critical role of endogenous copper normally present in the medium for inhibition of growth during longer incubation periods. Copper sulfate, added alone, did not inhibit colony formation at the concentrations used.

Cellular transport of 2,9-dimethyl-1,10-phenanthroline. Cellular uptake of tritium-labeled 2,9-DMP in complete growth medium was dependent upon the concentration of added copper (Fig. 6). Also, as shown in Fig. 7, the uptake of 2,9-DMP under growth conditions was potentiated in the presence of copper and abolished in the presence of bathocuproine sulfonate, indicating that a copper chelate-dependent uptake system was the principal factor in the copper requirement for cytotoxicity.

The metal-ion-dependent uptake of [^{3}H]-2,9-DMP was specific for copper. As indicated in Fig. 8, Fe^{2+}, Zn^{2+}, or Co^{2+} did not promote the accumulation of

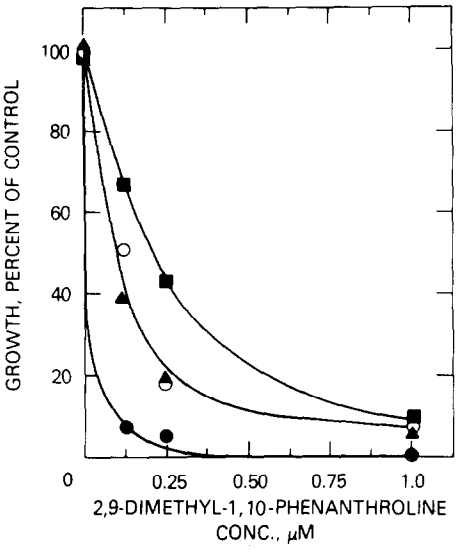

Fig. 4. Metal ion specificity in the cytotoxicity of 2,9-DMP. Cells (1×10^5/ml) were incubated with increasing concentrations of 2,9-DMP alone (○—○), or 2,9-DMP with 100 μM $CuSO_4$ (●—●), 100 μM $FeSO_4$ (▲—▲) or 100 μM $ZnCl_2$ (■—■). Growth was measured after 45 hr at 37° and compared to control sets containing solvents only.

label into L1210 cells, results which agree with the inability of these ions to potentiate 2,9-DMP cytotoxicity. The uptake of copper-2,9-DMP reached a

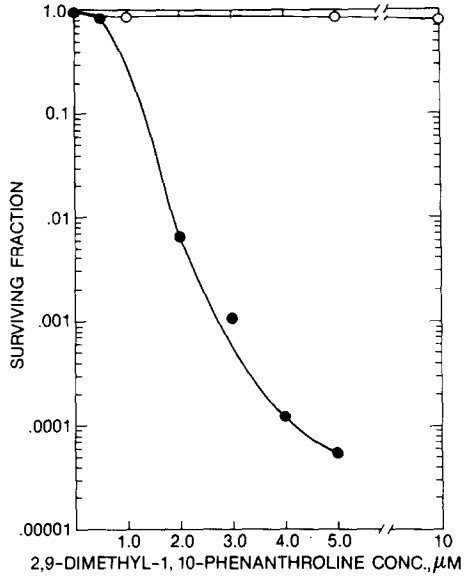

Fig. 5. Viability of L1210 cells incubated with 2,9-DMP and copper. Suspensions (10 ml) of L1210 cells (1×10^5/ml) were incubated with micromolar concentrations of 2,9-DMP alone (○—○) or in the presence of 25 μM $CuSO_4$ (●—●) for 1 hr at 37° in complete growth medium. The cells were then washed with fresh medium lacking 2,9-DMP and copper and suspended in 0.4% soft nutrient-agar containing growth medium with 20% fetal bovine serum, as described under Materials and Methods. Colony formation was assessed after 2 weeks at 37°, and results are expressed as the fraction of viable cells compared to appropriate control sets. $CuSO_4$ (25 μM) alone had no measurable effect on L1210 cloning efficiency.

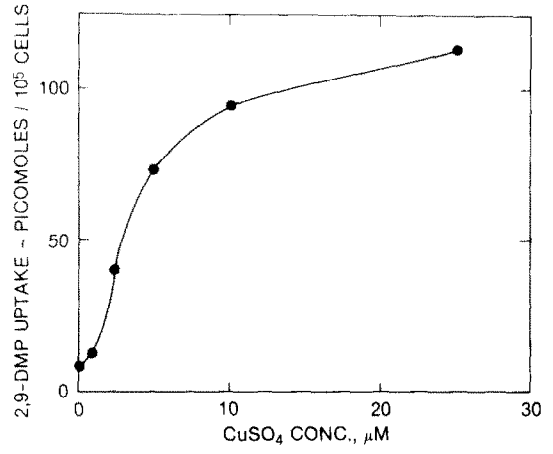

Fig. 6. Requirement for copper in the uptake of [^{3}H]-2,9-DMP by L1210 cells. Cells (1×10^5/ml) were incubated with 5 μM [^{3}H]-2,9-DMP for 1 hr at 37° in complete growth medium, in the presence of increasing concentrations of $CuSO_4$. The uptake of 2,9-DMP was estimated as described in Materials and Methods.

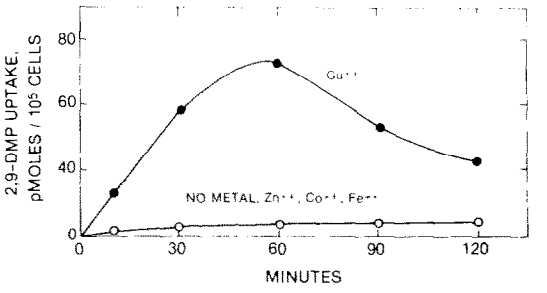

Fig. 8. Metal ion specificity in support of uptake of 2,9-DMP. Cells were incubated with 5 μM [^{3}H]-2,9-DMP in complete growth medium alone (○—○) and in the presence of 25 μM Zn^{2+}, Co^{2+} or Fe^{2+} (○—○), or 25 μM Cu^{2+} (●—●). At specified intervals, 1.0-ml aliquots were removed in triplicate, and the amount of radioactivity was determined in the separated cell pellet.

maximum in 60 min, and then the radioactivity gradually escaped from the cells (Fig. 9). It is not apparent at this time whether 2,9-DMP was released as the free ligand or as metal-bound complex. However, a concentrative uptake of copper-2,9-DMP occurred during the first 60 min, and a ratio of intracellular to extracellular concentrations greater than 200-fold was observed with 5 μM ligand at that time.

Since the results indicated a concentrative uptake for copper-2,9-DMP, experiments were conducted to determine if this accumulation was dependent upon a physiological transport system. Both the bidentate, distorted, planar cupric chelate and the bidentate, tetrahedral cuprous chelate of 2,9-DMP [15, 16] resemble the structure of a well characterized membrane potential agent, the tetraphenyl phosphonium ion (TPP^+) [19] by having a positive charge shielded by lipophilic groups. This property allows TPP^+ to rapidly equilibrate across the cell membrane because of the negatively-charged intracellular potential [19]. Studies have shown that altering the extracellular pH or inhibiting oxidative phosphorylation will influence the uptake of TPP^+, or its congener, the triphenylmethyl phosphonium ion, into Ehrlich ascites cells [20]. We examined the effects of medium pH, added glucose and dinitrophenol on the uptake of copper-[^{3}H]-2,9-DMP by L1210 cells, and observed that it was inhibited by dinitrophenol but was not entirely dependent upon the intracellular ionic gradient (Fig. 10). The uptake of TPP^+ was observed only at pH 7.8, was inhibited by dinitrophenol, and was partially restored by added glucose, as described for Ehrlich ascites cells by Johnstone [20]. In contrast, the L1210 cells incorporated copper-2,9-DMP under both acid and alkaline conditions, and glucose did not relieve the inhibition by dinitrophenol. Such differences may be due to the

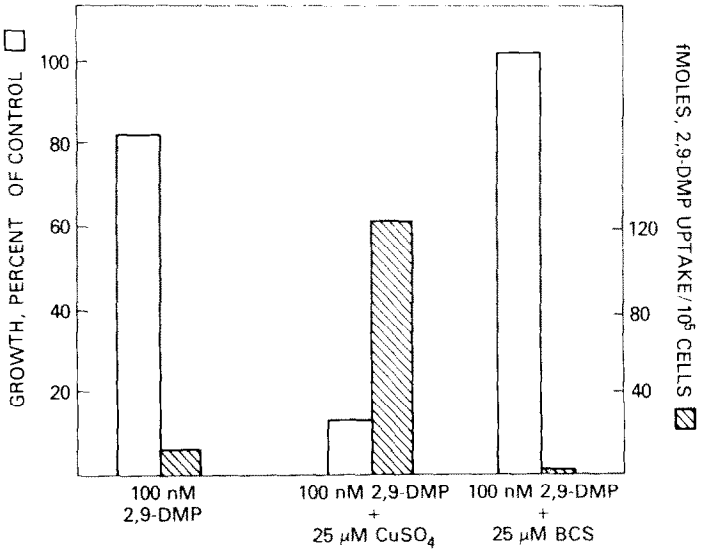

Fig. 7. Evidence that copper-dependent uptake of [^{3}H]-2,9-DMP determines its copper-requiring cytotoxicity under conditions of cell growth. Cytotoxicity of 100 nM [^{3}H]-2,9-DMP alone, in the presence of 25 μM $CuSO_4$ or 25 μM bathocuproine sulfonate, was assessed as described in Materials and Methods. Cell growth and the amount of associated [^{3}H]-2,9-DMP were determined after 45 hr at 37°.

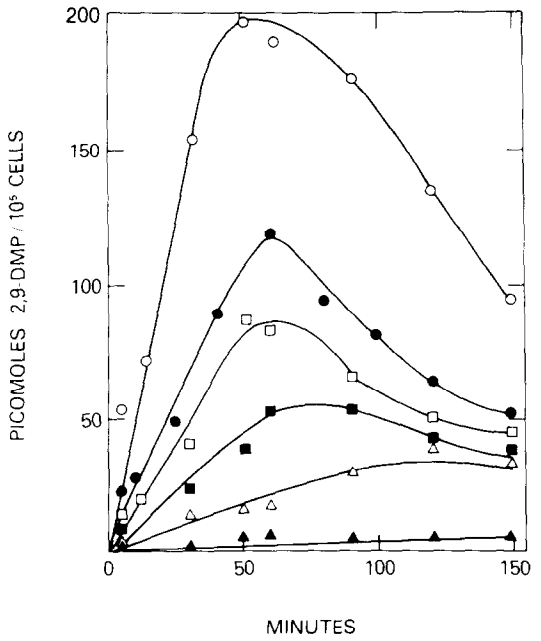

Fig. 9. Uptake and release of copper-[^{3}H]-2,9-DMP incubated with L1210 cells. Log-phase cells (1×10^5/ml) were incubated with 1.0 (▲—▲), 2.0 (△—△), 3.0 (■—■), 4.0 (□—□), 5.0 (●—●), and 8.0 μM (○—○) [^{3}H]-2,9-DMP in the presence of 25 μM $CuSO_4$. Radioactivity associated with the cells was determined as described in Materials and Methods at various time periods.

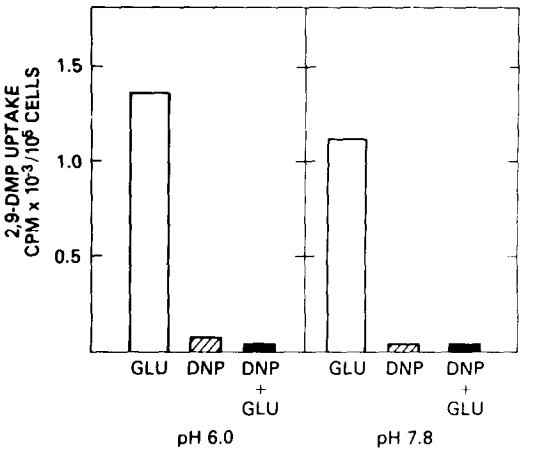

Fig. 10. Effects of medium pH, added glucose and dinitrophenol on the uptake of copper-[^{3}H]-2,9-DMP by L1210 cells. Log-phase cells (1×10^5/ml) were washed free of growth medium with Bis-Tris/Hepes buffer, 10 mM, at pH 6.0 and 7.8 respectively, and resuspended at 1×10^5 cells/ml in the same buffer. Uptake from a concentration of 5 μM [^{3}H]-2,9-DMP was in the presence of 10 mM glucose, 100 μM dinitrophenol (DNP) or glucose and DNP added together. Incubations were carried out for 30 min at pH 6.0 and pH 7.8.

dissociation of the copper 2,9-DMP chelate within the cells, while the ionic and lipoidal moieties of TPP^+ are covalently bonded and undissociable.

Chemotherapy with copper-2,9-dimethyl-1,10-phenanthroline. When the chemotherapeutic activity of copper-2,9-DMP was evaluated against L1210 tumor-bearing mice, borderline responses were observed, with increased survival of only 2 days beyond controls. However, the transplanted P388 leukemia responded well to treatment, and significant increases in life span, as judged by NCI guidelines for antitumor activity, were obtained with a 45% increase in life span in the treated group (Table 1). Increased therapeutic index was limited by host toxicity due to possible neuromuscular blockade [21].

DISCUSSION

Several early reports have suggested that of the many derivatives of 1,10-phenanthroline, the 2,9-dimethyl form possesses a marked specificity for copper and stereochemistry that confers upon the bidentate cupric chelate of 2,9-DMP potent oxidative properties [22, 23]. This is attributed to the steric hindrance to normal planar configuration caused

Table 1. Chemotherapy of P388 murine leukemia with copper-2,9-DMP*

Days of treatment	Median survival time (days)	Increase in life span (%)
None	11.6	0
1,2	15.0	29
1,2,4,5	16.8	45
1,2,4,5,7,8	15.8	36

* Mice (CDF_1, 24–30 g) were inoculated, i.p., with 10^6 freshly harvested P388 lymphoma cells in 0.2 ml of Dulbecco's PBS containing 0.7% bovine serum albumin. Treatment began 24 hr later and continued once a day according to the indicated schedules. Two injections were made i.p., one containing 20 μmoles/kg 2,9-DMP in 25% propylene glycol, and the second containing 10 μmoles/kg $CuSO_4$ with 20 μmoles/kg nitrilotriacetic acid in normal saline. Mice treated with 2,9-DMP alone or with copper-nitrilotriacetic acid alone died on the same day as the vehicle controls. Increase in life span is expressed as the percent of respective median survival times compared to the median survival times of controls. The untreated group contained fourteen mice and each treated group ten mice. The survival times of all treated groups were increased beyond that of the control group to a highly significant extent ($P < 0.001$, two-sided) as determined by the Wilcoxon Rank Sum Test [E. L. Lehmann, *Nonparametrics*, pp. 5–23. Holden-Day, San Francisco (1975)].

by the presence of the methyl groups proximate to the metal coordination site [15, 16], a feature not shared by 1,10-phenanthroline or other methylated derivatives. Thus, large differences between the standard redox potential of cupric-2,9-DMP (+594 mV) and cupric-1,10-phenanthroline (+174 mV) have been reported [22].

It is of interest that, in a model biochemical system, rapid DNA degradation was promoted by the cuprous complex of 1,10-phenanthroline [13, 14]. This activity was due to the generation of reactive oxygen species as indicated by the requirement for hydrogen peroxide and thiols. The ligand appeared to intercalate between the bases of DNA as indicated by competitive inhibition with ethidium bromide and a preference for double-stranded substrate [14]. The copper complex of 2,9-DMP was, however, ineffective in degrading DNA under these same conditions [14]. In contrast, others have presented evidence that 2,9-dimethyl-*N*-methylphenanthrolinium is the superior intercalator of DNA [24]. Since in *intact-cell* systems, such as the L1210 lymphoma, Cu^{2+} promotes the cytotoxicity of 2,9-DMP only and actually overcomes growth inhibition by 1,10-phenanthroline, the bases for differences between the acellular model system and our observations with intact cells are yet to be reconciled.

The present study also demonstrates that differences between the copper chelates of 2,9-dimethyl-1,10-phenanthroline and 1,10-phenanthroline are not limited to their metal-binding specificity but that striking *qualitative* differences exist in their biological activities and mechanisms of action. Whereas 2,9-DMP is cytotoxic in tissue culture only in the presence of available copper, as demonstrated in this study, the lethal action of 1,10-phenanthroline is overcome by the addition of copper or other divalent metal ions, as reported by others [11, 12]. Using the *in vitro* L1210 growth assay, we have also confirmed that added copper completely protects the cells from 1,10-phenanthroline cytotoxicity. *Therefore, the addition of the 2- and 9-methyl groups converts a ligand which inhibits as a chelator to one which inhibits as a copper chelate.*

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