Effects of Chlorphenamidine and Its Metabolites on HeLa Cells

by

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Chlorphenamidine, N'-(4-chloro-o-tolyl)-N,N-dimethylformamidine (I), has been widely used for plant protection purposes since its initial introduction as an acaricide in 1964. Knowles and his coworkers studied the metabolism of radioactive chlorphenamidine in plants and animals. They indicated that chlorphenamidine was partially demethylated to yield demethylchlorphenamidine, N'-(4-chloro-o-tolyl)-N-methylformamidine (II), and both compounds (I and II) were then hydrolyzed to form N-formyl-4-chloro-o-toluidine(III). This metabolite was subjected to deformylation and 4-chloro-2-methylaniline (IV) was produced. Furthermore they detected N-formyl-5-chloroanthranilic acid and 5-chloroanthranilic acid in mammals. The metabolic relationship among chlorphenamidine and its metabolites was summarized by KNOWLES (1970). In our laboratory Morikawa and Fukami studied chlorphenamidine metabolism in mammals and insects, and they identified compounds II, III, and IV as metabolites of chlorphenamidine (unpublished work).

The elucidation of the effects and the mechanisms of action of the pesticide on cultured human cells might be helpful in evaluation of safety of this effective chemical on humans. The present study deals with the effect of chlorphenamidine and its possible metabolites on cell growth and on the synthesis of DNA, RNA, and protein in HeLa cell cultures.

Materials and Methods

Compounds I, II, III, and IV were prepared and purified by M. Morikawa in our laboratory, and details of the procedures will be described elsewhere. A chlorphenamidine analog, 5-chloro-2-nitrotoluene (V) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin.

HeLa cells were provided by Dr. S. Suzuki, Research Laboratories, Chugai Pharmaceutical Co., Tokyo. Cells were grown as monolayer cultures in tightly stoppered culture bottles at 37°C. The culture medium (100 ml) consisted of 88 ml of Eagle's minimum essential medium (EAGLE 1959), 10 ml of calf serum, 1 ml of 7.5 percent solution of $NaHCO_3$

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and 1 ml of penicillin-streptomycin mixture (5000 units each). All components were obtained from Chiba Serum Laboratory, Chiba, Japan, except the antibiotics mixture which was purchased from Microbiological Associates, Bethesda, Maryland.

Effects of chemicals on synthesis of nucleic acids and protein were measured according to the method of LITTERST et al. (1969). Cells in 5 ml of growth medium were planted in culture bottles. After 24 hours of incubation, this starter medium was replaced with 5 ml of medium, and cells in the fresh medium were incubated for an additional 24 hours. Various concentrations of the test compounds in 0.05 ml of ethanol were added to the cultures. Controls were cultures that had been treated only with ethanol. Two hours later one of the following radioactive precursors were added: Uniformly labeled L-leucine-C14 (0.5 μCi; 282 mCi/mmole), uridine-5-T (5 μCi; 15 Ci/mmole), or thymidine-2-C14 (0.5 μCi; 57 mCi/mmole). Leucine and uridine were purchased from Daiichi Pure Chemicals Co. Ltd., Tokyo and thymidine was obtained from The Radiochemical Centre, Amersham, England. After one hour incubation, the cells were rinsed twice with 5 ml of cold 0.9 percent saline. The cell layer was then immersed in cold 4 percent perchloric acid for 40 minutes to remove acid-soluble materials, rinsed successively with 80 and 100 percent ethanol, and anhydrous diethyl ether. After drying, the fixed monolayer was dissolved in 2 ml of 85 percent formic acid for 20 minutes. Aliquots of 0.5 ml were added into liquid scintillation counting vials. Ethanol (2 ml) was added, followed by 10 ml of the scintillation medium. The scintillation medium consisted of 1000 ml toluene, 4 g 2,5-diphenyloxazole (PPO), and 40 mg 1,4-bis(2-(4-methyl-5-phenyloxazolyl)) benzene (Dimethyl POPOP). The samples were counted for 5 minutes on a Beckman LS-150 Liquid Scintillation System.

To determine the effect of chlorphenamidine or its related compounds on cell growth, about 1 million cells in 5 ml of culture medium were planted in each culture bottle. The test compounds dissolved in 0.05 ml of ethanol were incorporated in the growth medium at 4 different concentrations in triplicate. After 48 hours of incubation, this medium was replaced with 5 ml of fresh medium containing same concentrations of the test compounds. After 72 hours of incubation, the medium was removed from the cultures. The cell monolayers were washed twice with calcium and magnesium free phosphate buffered saline (MERCHANT et al. 1964). The amount of cell protein was determined according to the method of LOWRY et al. (1951) as modified by OYAMA and EAGLE (1956) for tissue cultures. The growth of treated cultures was expressed as the percentage of the control growth. The percent inhibition in the amount of cell protein was plotted on log-probit graph paper against the concentration of the compound. The test compound concentration for 50 percent inhibition

of cell growth (ID_{50}) was determined by graphical interpolation

Results and Discussion

The effect of varying concentrations of chlorphenamidine on nucleic acid and protein synthesis in HeLa cells is illustrated in Fig. 1. None of the macromolecules appeared particularly sensitive to the action of chlorphenamidine. The rates of the synthesis of macromolecules were inhibited by 40 to 50 percent at a chlorphenamidine concentration of 10^{-3} M. The rate of RNA synthesis was slightly inhibited at a concentration of 10^{-4} M, but DNA and protein synthesis was virtually unaffected at the same concentration. No significant inhibition of the synthesis of macromolecules was seen at a concentration of 10^{-5} M.

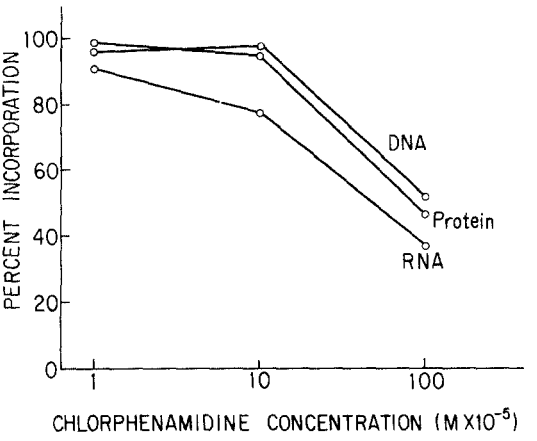


Fig. 1. Effect of varying concentrations of chlorphenamidine on the synthesis of DNA, RNA, and protein in HeLa cells.

Ineffectiveness at considerably high dose levels (10^{-5} M and 10^{-4} M) on the synthesis of macromolecules suggests that the toxicity of this chemical is low.

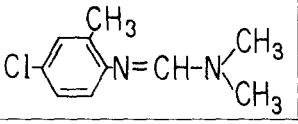
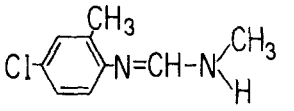
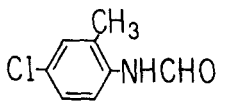
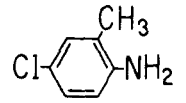
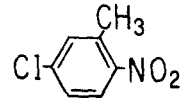
Table 1 compares the cytotoxicity and the inhibition of RNA synthesis at a concentration of 10^{-3} M by possible metabolites and an analog (compound V) of chlorphenamidine. It can be seen that chlorphenamidine and its two metabolites, demethylchlorphenamidine and N-formyl-4-chloro-o-toluidine, had almost equal toxicity and were more toxic than 4-chloro-2-methylaniline. Compound V, 5-chloro-2-nitrotoluene was the most toxic material. The relative cytotoxicity of the compounds tested corresponds closely to their comparative activity for inhibition of RNA synthesis. Again 4-chloro-2-methylaniline was the least effective compound against RNA synthesis, and 5-chloro-2-nitrotoluene was most effective.

Perhaps the most obvious conclusion to be drawn from the data presented in Table 1 is that rapid transformation of chlorphenamidine to the proposed final metabolite,

TABLE 1

Cytotoxicity and Inhibition of RNA Synthesis by Chlorphenamidine, Its Metabolites and 5-Chloro-2-nitrotoluene

RNA synthesis was measured at 10^{-3} M concentrations of test compounds.

Compound		ID_{50} ($MX10^{-5}$)	Inhibition of RNA Synthesis (%)
I		7.6	63
II		6.7	52
III		6.4	57
IV		10.1	38
V		6.0	77

4-chloro-2-methylaniline *in vivo* may be harmless for humans. We are now studying the metabolism of chlorphenamidine and other pesticides or environmental chemicals in cultured human cells.

Summary

Monolayer cultures of HeLa cells were exposed to an insecticidal chemical, chlorphenamidine, and its effect on nucleic acid and protein synthesis was measured. No significant differences among the effects of the chemical on DNA, RNA, and protein synthesis were observed. The rates of the synthesis of macromolecules were inhibited

by 40 to 50 percent at a concentration of 10^{-3} M. At lower dose levels (10^{-4} M and 10^{-5} M), the synthesis of macromolecules was virtually unaffected.

The cytotoxicity of chlorphenamidine, its potential metabolites, and an analog, 5-chloro-2-nitrotoluene, was studied and the growth inhibition (ID_{50}) based on inhibition of cell protein synthesis was determined. Chlorphenamidine and its two sequential metabolites, demethylchlorphenamidine and N-formyl-4-chloro-o-toluidine, had almost equal toxicity. The proposed final metabolite, 4-chloro-2-methylaniline was the least toxic compound and a chlorphenamidine analog, 5-chloro-2-nitrotoluene, was most toxic. These results closely correlated with the comparative inhibitory activity of these compounds for RNA synthesis.

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