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MUTAGENICITY OF THE NUCLEASE ACTIVITY OF 1,10-PHENANTHROLINE - COPPER ION

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The nuclease activity of 1,10-phenanthroline-copper functions intracellularly. This was shown by its mutagenicity in the Ames Test using the tester strain TA102 and the in vivo nicking of plasmids derived from this strain. In vivo DNA strand scission requires all the components essential for the in vitro activity: 1,10-phenanthroline, cupric ion, thiol and hydrogen peroxide. Although ^{60}Co gamma radiation potentiates the nuclease activity of 1,10-phenanthroline-copper ion in vitro via a superoxide dependent pathway, it does not promote significant mutagenesis in vivo at exposure levels below cytotoxicity.

The nuclease activity of 1,10-phenanthroline-copper functions at physiological pH's and temperatures.[1] In vitro, the generation of the essential coreactants for the nucleolytic activity, the 2:1 1,10-phenanthroline-cuprous complex and hydrogen peroxide, can be accomplished using either thiol or superoxide as a one electron reductant. Once formed, the 2:1 1,10-phenanthroline-cuprous complex binds reversibly to DNA where it is oxidized by hydrogen peroxide. This oxidation forms a copper-oxo intermediate which causes strand scission by oxidative attack at the C-1 hydrogen of the deoxyribose.[1] The 3'phosphomonoester termini produced by this reaction are responsible for the potent in vitro inhibition of RNA and DNA polymerases by the 1,10-phenanthroline-cuprous complex.[1-5]

In this communciation, we demonstrate that the nucleolytic activity, previously shown in vitro,[1] is mutagenic in the Ames Test[6] and therefore can function intracellularly. Our results suggest that the nuclease activity of 1,10-phenanthroline-copper may be useful as an intracellular footprinting reagent and, because of its demonstrated efficiency as a viricide in vitro,[7] may also be adaptable for pharmacological applications.

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Methods

1,10-Phenanthroline (OP), and its derivatives were all purchased from G.F. Smith. Catalase was purchased from Sigma and 30% Hydrogen Peroxide was purchased from Fisher.

Mutagenicity was assayed using the Ames Test and the Salmonella typhimurium strain TA102,[8] a strain which is particularly sensitive to oxidative mutagens. The data is reported as the percent of control (100 x revertants/spontaneous reversion rate) which normalizes the data between different experiments. Within each experiment, all conditions were run in triplicate and the results were averaged.

Standard reaction conditions were 50 μM OP (or one of its derivatives), 25 μM $CuSO_4$, 5 mM MPA in 0.05 M phosphate buffer (pH 7.1) in a total volume of 0.2 ml of which 0.1 ml was overnight bacterial culture. Unless otherwise noted, cells were incubated with the mutagens for 1 minute at 37° C prior to plating and then for 40-48 hours at 37° C prior to counting the revertant colonies.

The mutagenicity experiments using ^{60}Co potentiation were conducted in 20 mM tris-HCl buffer (pH 8.0) to minimize the background reversion rate. The phosphate and the tris buffer systems were shown to be comparable in the non-gamma potentiated mutagenesis assays (data not shown). The cells were mixed with the appropriate mutagens and exposed to the gamma radiation inside a closed ^{60}Co gamma cell (13.5 rads/second).

In vivo nicking was done with 1.5 ml of an overnight growth culture of TA102 cells. The cells were incubated at 37° C with 50 μM OP, 25 μM $CuSO_4$, and 5 mM MPA in 50 mM phosphate buffer (pH 7.1) for various lengths of time. The plasmids were then isolated in mini-preparations using boiling for lysis as previously described.[9] After treatment with RNase A (50 μg/ml for 30 minutes at 37° C) the plasmids were purified by phenol:chloroform extraction and ethanol precipitation. The nicking was visualized by running the plasmids on 0.8% agarose gels and staining with ethidium bromide.

Results

The mutagenicity of 1,10-phenanthroline observed upon preincubation of the compound with Salmonella strain TA102 is absolutely dependent upon the presence of thiol (3-mercaptopropionic acid) and copper. Furthermore, the mutagenicity is linearly dependent upon both the length of the OP-Cu/thiol/cell incubation prior to plating and the concentration of OP-Cu (Figures 1A and 1B). Prolonged incubation is bacteriocidal.

In contrast to OP, 2,9-dimethyl-1,10-phenanthroline-cuprous ion, a compound which does not cleave DNA in vitro due to the steric constraints imposed by the methyl groups, is not mutagenic (see Table 1). Consistent with previous reports of the cytotoxicity of this complex in mammalian cells,[10] this coordination complex is bacteriocidal at high concentrations. The mechanism for this cytotoxicity has not been fully determined. The copper complexes of 5-substituted phenanthroline derivatives, which cleave DNA in vitro,[11] show mutagenic activity equivalent to that of OP-Cu (Table 1).

Hydrogen peroxide is an essential coreactant for the nuclease activity in vitro. It can be generated in situ by the oxidation of 1,10-phenanthroline cuprous or added exogenously.[2,12] If cells are preincubated with the coordination complex in the presence of catalase, mutagenesis is blocked. The addition of hydrogen peroxide enhances the mutagenesis unless it is added at

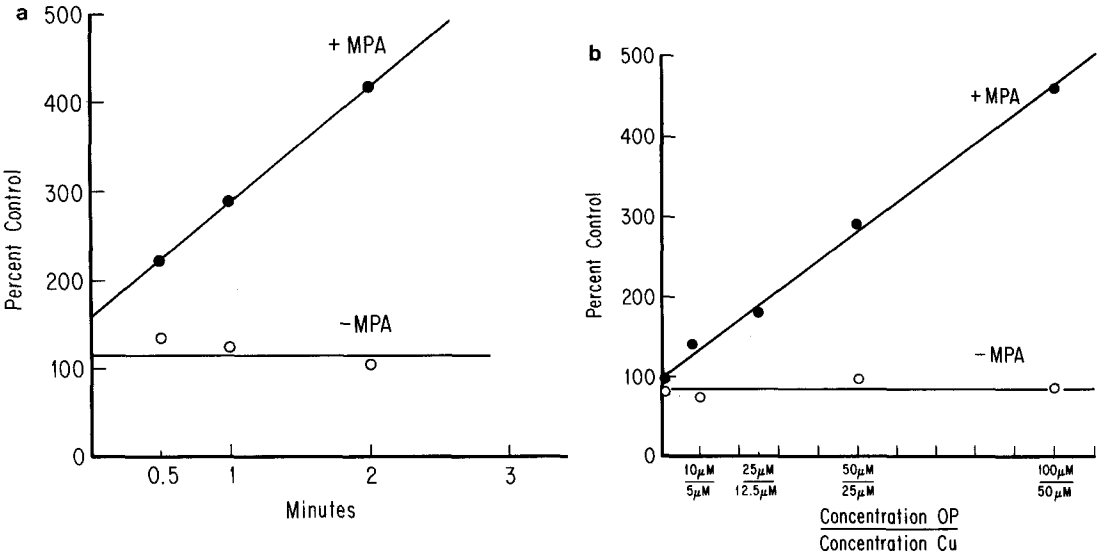

Figure 1. The Effects of Incubation Time and Concentration on OP Mutagenicity.

a) 0.1 ml of an overnight TA102 culture (at 37° C) was exposed to 50 μM OP, 25 μM $CuSO_4$, in the presence and absence of 5 mM MPA, for the indicated time prior to plating. The number of revertant colonies was scored 40-48 hours later. b) The experimental conditions were identical to those in 1a) except that the concentration of OP-Cu was varied with a constant incubation time of 1 minute at 37° C.

concentrations which result in the oxidation of all the thiol. As in the case of the DNA cleavage reaction, hydrogen peroxide is non-mutagenic at these concentrations in the absence of the 1,10-phenanthroline-cuprous complex (Fig 2).

The hypothesis that the mutagenesis by the 1,10-phenanthroline is due to DNA damage can be directly tested by examining the plasmids from TA102 cells which have been incubated with 1,10-phenanthroline-copper. As demonstrated in Figure 3, they are extensively nicked.

Table 1. The Mutagenicity of OP and its Derivatives

Mutagen	Mutagenicity as % Control			
	alone	+Cu	+MPA	+Cu/MPA
OP	97	107	125	265
5-Br OP	112	129	126	267
5-NO_2 OP	102	138	124	224
2,9-dimethyl OP	66	*	34	97

0.1 ml of an overnight TA102 culture was incubated for 1 minute at 37° C with 0.1 ml of mutagen (50 μM final concentration) and then plated. The number of revertant colonies was scored 40-48 hours later. * indicates that no background lawn was seen on these plates, an indication of cell death.

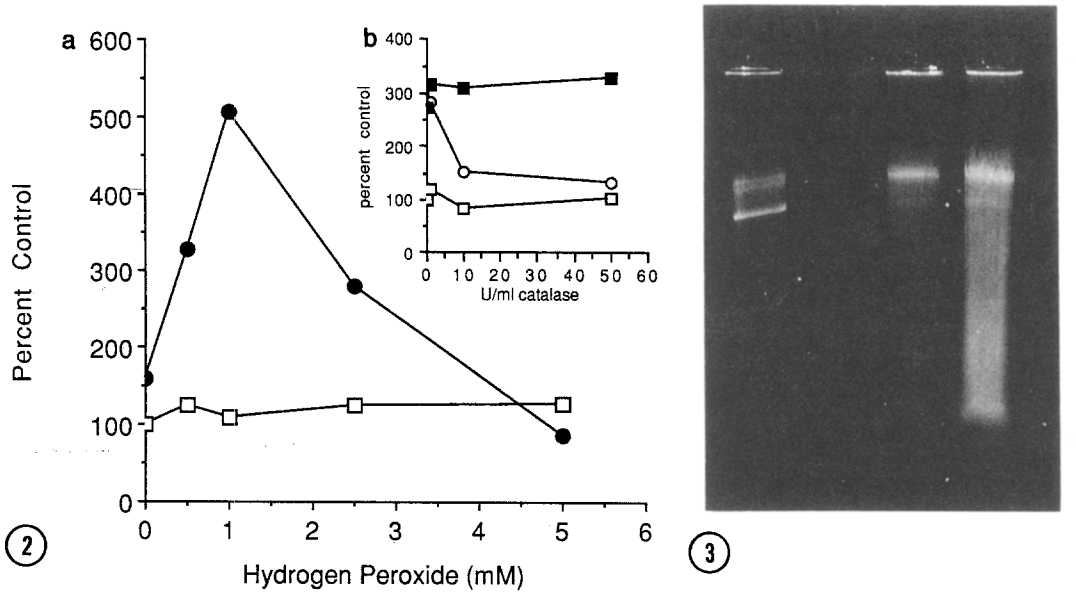

Figure 2. The Effect of Extracellular Hydrogen Peroxide on OP Mutagenicity.

In 2a, 50 μM OP, 25 μM $CuSO_4$ and 5 mM MPA were incubated for 1 minute at 37° C with 0.1 ml of overnight TA102 culture in the presence of active catalase (—o—) and catalase inactivated by placing in a 100° C water bath for 10 minutes (—■—). The control (—□—) was phosphate buffer in the presence of active catalase. 2b shows the effect of exogenously added hydrogen peroxide under the same reaction conditions. (—●—) denotes OP/Cu/MPA and (—□—) indicates the phosphate buffer control.

Figure 3. In vivo Nicking of Plasmid DNA.

1.5 ml of an overnight culture of TA102 cells was incubated in 50 μM OP, 25 μM $CuSO_4$, 5 mM MPA at 37° C for 5 minutes (lane 3) and 30 minutes (lane 4). The cells were washed twice with 50 mM phosphate buffer (pH 7.1) and the plasmids were purified as described in the Methods section. The plasmids were then treated with 50 μg/ml RNase A for 30 minutes at 37° C, purified by phenol:chloroform extraction and ethanol precipitation, and run on an 0.8% agarose gel. Lane 1 contains plasmid prepared from untreated cells.

^{60}Co gamma irradiation potentiates a superoxide-dependent cleavage reaction for OP-Cu and its derivatives, in vitro, under aerobic conditions.[11] To achieve cleavage levels equivalent to that of the thiol driven reaction, exposure to 10,000 rads was required. These conditions induced prohibitively high background mutagenesis and cytotoxicity when used in the Ames Test with the TA102 strain. At the maximum acceptable radiation dosage of 40 rads, no mutagenesis was observed.

Discussion

All the components required for the induction of the in vitro nuclease activity of 1,10-phenanthroline-copper are also necessary for mutagenesis, and the associated nicking of the

plasmids, in vivo. Our data, as well as those of others,[8,14,15] indicate that bacterial cells present no permeability barrier to 1,10-phenanthroline, and its complexes with cupric or cuprous ion. The copper requirement for OP-Cu mediated mutagenesis must therefore be due to insufficient levels of free intracellular cupric ion.

The requirement for thiol indicates that the intracellular reducing enviroment is not sufficient to generate the 2:1 1,10-phenanthroline-cuprous complex essential for strand scission. Since the coordination complexes appear to freely diffuse across the cell membrane, it is not possble to determine if the exogenously added thiol reduces the cupric complex outside or inside the cell.

Superoxide dependent pathways for activation of the nuclease activity of 1,10-phenanthroline-copper are ineffective intracellularly. Since the high background in the Ames Test indicates the production of superoxide even at low levels of irradiation, it is clear that the superoxide dependent, ^{60}Co potentiation of scission is not active intracellularly. Earlier work had demonstrated that in vitro DNA strand scission by 1,10-phenanthroline-cuprous complex could be potentiated by NADH and hydrogen peroxide in a reaction dependent on the intermediate production of superoxide.[16] Since the 1,10-phenanthroline cupric complex and hydrogen peroxide, in the absence of thiol, is not mutagenic, this mechanism is not significant under our experimental conditions. These results, contrast with recent studies on the in vivo significance of the Fenton reaction (ferrous ion and hydrogen peroxide),[14,15] which suggest that NADH may be a source of reducing equivalents to recycle the iron catalyst, possibly via superoxide, even in the presence of low levels of hydrogen peroxide.

An important determinant governing OP-Cu dependent mutagenesis is the level of hydrogen peroxide. Cellular catalase is not able to keep hydrogen peroxide levels low enough to prevent the efficient mutagenesis caused by the DNA bound 1,10-phenanthroline-cuprous complex. Extracellular catalase must be added to destroy the apparently freely diffusible hydrogen peroxide and block DNA damage. The inability of organic scavengers of metal ions (i.e. EDTA, 1,10-phenanthroline) to block completely the mutagenicity and cytotoxicity of the Fenton reaction has led to the suggestion that DNA bound iron must be responsible for the cell damage caused by low levels of hydrogen peroxide.[14]

The results reported in this communication demonstrate the feasibility of using the nuclease activity of 1,10-phenanthroline-copper for intracellular footprinting. Primer extension

assays should be useful in visualizing the results of these experiments.[17] Our demonstration of the intracellular action of the nuclease activity suggests that this reaction may serve as the basis for designing new cytotoxic and anti-viral agents. These agents, in addition to interfering with nucleic acid replication as a result of accumulated strand breaks, also will inactivate cellular polymerases by the production of 3'-phosphmonoester termini.

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