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SYNERGISTIC EFFECTS OF Cu(II) AND DIMETHYLAMMONIUM 2,4-DICHLOROPHENOXYACETATE (U46 D FLUID) ON PM2 DNA AND MECHANISM OF DNA DAMAGE

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Dimethylammonium 2,4-dichlorophenoxyacetate (2,4-D · DMA) induced strand breaks in PM2 DNA when incubated with $CuCl_2$, whereas 2,4-D · DMA alone or $CuCl_2$ alone did not show any or only a negligible effect. The formation of single strand breaks increased linearly with time and concentration of 2,4-D · DMA. Neocuproine, a specific Cu(I) chelator totally prevented strand break formation. So did catalase (up to 100 mM 2,4-D · DMA), but DMSO had only a small protective effect. 2,4-Dichlorophenol, CO_2 and formaldehyde were detected as reaction products of 2,4-D and $CuCl_2$. From these results a redox reaction of Cu(II) and 2,4-D is proposed, which could explain the DNA damaging properties of $CuCl_2$/2,4-D · DMA.

KEY WORDS: $CuCl_2$, 2,4-D, U46 D Fluid, synergistic effect, DNA strand breaks, site specific reaction, reactive oxygen species.

ABBREVIATIONS: 2,4-D, 2,4-dichlorophenoxyacetic acid; DMA, dimethylammonium; 2,4-D · DMA, dimethylammonium 2,4-dichlorophenoxyacetate; ROS, reactive oxygen species; SOD, superoxide dismutase; SSB, DNA single strand break; DSB, DNA double strand break.

INTRODUCTION

Recently we have shown that U46 D Fluid (a commercial formulation of dimethylammonium 2,4-dichlorophenoxyacetate) induced DNA repair in human fibroblasts after pretreatment of the cells with $CuCl_2$, whereas neither $CuCl_2$ nor U46 D Fluid alone showed any effect.[1] Furthermore, $CuCl_2$ enhanced drastically the effects of U46 D Fluid on cell growth and DNA synthesis.[1] These results suggested a DNA damage caused by the combined action of $CuCl_2$ and 2,4-D · DMA (in U46 D Fluid) by an unknown mechanism.

Cu(II) is able to react with DNA in various ways. It preferentially binds strongly to guanine-cytosine base pairs,[2–4] whereby the double helix is destabilized by breakage of the hydrogen bonds resulting in significant conformational changes.[2,4,5] These effects may facilitate an attack of 2,4-D · DMA on the DNA molecule.

Synergistic effects on DNA strand breakage caused by Cu(II) and chemicals able

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to reduce Cu(II) to Cu(I) have been described for *in vitro* experiments.[6-8] By reoxidation of Cu(I) reactive oxygen species (ROS) like O_2^-, H_2O_2 and $^{\cdot}OH$-radicals are formed, whereby the latter are well known to damage DNA.[9-11] For example, the combination of the reductant ascorbic acid and Cu(II) produces DNA strand breaks via the formation of $^{\cdot}OH$ radicals[6] as well as quercetin combined with Cu(II).[7] The enhancement of bleomycin induced strand breaks in SV40 DNA by Cu(II) in the presence of dithiothreitol also was explained by the reduction of the Cu(II)-bleomycin complex and the formation of ROS.[8]

In searching for an explanation concerning the synergistic effects of $CuCl_2$ and 2,4-D · DMA on DNA repair and on inhibition of DNA synthesis in human fibroblasts, we determined the DNA damaging properties of these two agents *in vitro*. Furthermore, we examined the possible involvement of ROS in the DNA damage. Based on the results a mechanism for the synergistic effect of Cu(II) and 2,4-D · DMA on DNA is proposed.

MATERIALS AND METHODS

Chemicals

U46 D Fluid (containing 620 g/l dimethylammonium 2,4-dichlorophenoxyacetate, technical grade) was purchased from BASF, Germany, and 2,4-D (99.7%) from Dr S. Ehrenstorfer, Augsburg, Germany. Additionally dimethylammonium 2,4-dichlorophenoxyacetate was prepared from 2,4-D (99.7%) and dimethylamine hydrochloride (> 98%). Contaminations of 2,4-D by 2,4-dichlorophenol were not detectable by GC-MS. $CuCl_2 \times 2H_2O$ p.a. was obtained from Merck, Darmstadt, Germany. PM2 DNA and catalase (from bovine liver, activity: 65 000 units per mg protein) were purchased from Boehringer, Mannheim, Germany. Neocuproine, ethidium bromide and superoxide dismutase (SOD; from bovine erythrocytes, specific activity: approx. 3000 units per mg protein) were obtained from Sigma, Deisenhofen, Germany.

Determination of Strand Breaks in PM2 DNA

PM2 DNA (0.2 μg in 2.5 μl 10 mM Tris · HCl, pH 7.4) was incubated with 10 μl $CuCl_2$ (0.25–2 mM, final concentration, in 10 mM Tris · HCl, pH 7.4) or with 10 μl 2,4-D or 10 μl 2,4-D · DMA for two hours at 37°C. Measuring the combination effect, PM2 DNA was pretreated with 0.5 mM $CuCl_2$ for one hour and subsequently 12.5 μl 2,4-D · DMA (U46 D Fluid) (diluted with 10 mM Tris · HCl, pH 7.4 to the desired concentration) were added to the incubation mixture for an additional hour. The final concentration of $CuCl_2$ in the incubation mixture was 0.25 mM. In experiments using scavengers or enzymes, 5 μl DMSO, neocuproine, catalase, or SOD were added together with 2,4-D · DMA (U46 D Fluid) to the incubation mixture after pretreatment of PM2 DNA with 0.5 mM $CuCl_2$. Final concentrations were 33 μg/ml catalase, 100 μg/ml SOD, 1% DMSO, or 1 mM neocuproine. In experiments investigating the time course of strand break formation 100 μl $CuCl_2$ were incubated with 25 μl PM2 DNA (80 μg/ml) for one hour at 37°C and subsequently 125 μl 2,4-D · DMA (U46 D Fluid) were added (final concentrations 12.5 mM or 25 mM U46 D Fluid, 0.25 mM $CuCl_2$, 10 mM Tris · HCl, pH 7.4). At desired times aliquots of 25 μl were taken from the reaction

mixture. Strand break formation was stopped by addition of 25% DMSO, 0.25% bromophenol blue and 10% Ficoll and chilling on ice.

Superhelical and nicked forms of PM2 DNA were separated by agarose gel electrophoresis (0.8% agarose, 4 V/cm, 6 h). The DNA was made visible by staining with ethidium bromide (1 mg/l) and UV illumination. The gels were photographed with a Polaroid camera (film No. 667). Photographs were densitometrically evaluated. Strand breaks per PM2 DNA molecule (N) were calculated from $N = -\ln \alpha$, where α is the fraction of superhelical DNA molecules.

Determination of the Reaction Products of $CuCl_2$ and 2,-4-D · DMA

For determination of 2,4-dichlorophenol 0.5 mM $CuCl_2$ were incubated in 10 mM Tris · HCl, pH 7.4 at 37°C. After one hour the same volume 2,4-D · DMA was added for an additional hour (final concentration 0.25 mM $CuCl_2$, 100 mM 2,4-D · DMA). Subsequently the reaction mixture was extracted three times by ether. The combined extracts were evaporated *in vacuo*. The residue was dissolved in dichloromethane and analyzed by GC-MS [Finnegan-MAT 212(70 eV) with GC Varian 3710 and Data System SS300; fused-silica capillary column Macherey-Nagel OV101 (length 25 m, i.d. 0.25 mm, 0.23 μm thickness of the film); temperature program 80°/5 min, 10°/min up to 280°C]. For determination of carbon dioxide $CuCl_2$ was added in solid form to 4 ml 2,4-D solution in a closed vial (final concentration 0.25 mM $CuCl_2$, 100 mM 2,4-D, 10 mM Tris · HCl, pH 7.4). The gas phase over the reaction mixture was analyzed by GC-MS to detect CO_2. For that an aliquot of the gas phase was removed by a gas-tight syringe and injected in the mass spectrometer. The intensity of the molecular ion of CO_2 ($m/z = 44$) was determined relative to the intensity of argon ($m/z = 40$) and compared with the relative concentration of CO_2 in the gas phase over the 2,4-D solution alone and the $CuCl_2$ solution, respectively. In the same reaction mixture formaldehyde was chemically determined by the acetylacetone method according EN 120 (European norm).

RESULTS

DNA Strand Break Formation

$CuCl_2$ alone induced maximally 0.2 single strand breaks (SSB's)/PM2 DNA molecule during a two hour incubation. 2,4-D · DMA as well as 2,4-D alone had no effect on PM2 DNA up to 500 mM (data not shown). If PM2 DNA was incubated for one hour with $CuCl_2$ followed by addition of 25–200 mM 2,4-D · DMA (final concentration) for an additional hour, up to 3.6 SSB's/PM2 DNA molecule were formed (Figure 1). By using 2,4-D instead of 2,4-D · DMA, a comparable number of DNA strand breaks was induced in combination experiments (data not shown). The number of SSB's by $CuCl_2$/2,4-D · DMA increased linearly during a period of 14 h (Figure 2). At concentrations more than 75 mM 2,4-D · DMA double strand breaks (DSB's) were formed in a small number, recognizable by appearance of the linear form of PM2 DNA after electrophoresis. At 150 mM 2,4-D · DMA 2.5% of the incised DNA was found as linear molecules.

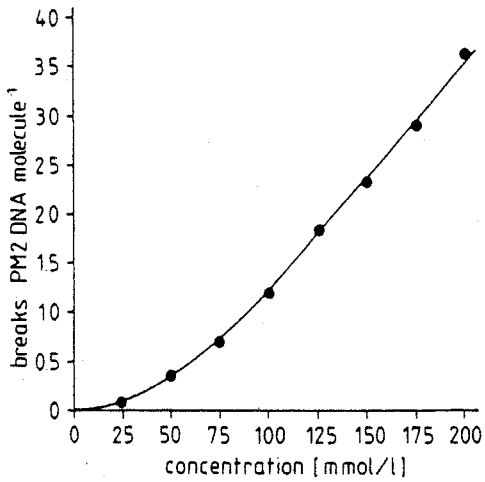

FIGURE 1 Single strand breaks induced in PM2 DNA by $CuCl_2$/2,4-D · DMA as a function of 2,4-D · DMA concentration. DNA was pretreated with 0.5 mM $CuCl_2$ for one hour and subsequently 2,4-D · DMA was added to the incubation mixture for an additional hour. Results indicate the mean of triplicate samples (SD < 5%).

Inhibition of DNA Strand Break Formation by DMSO, Neocuproine, SOD and Catalase

To examine the involvement of reactive oxygen species (ROS) in strand break formation, PM2 DNA was pretreated with 0.5 mM $CuCl_2$ for one hour and subsequently DMSO, SOD, catalase or neocuproine was added together with 2,4-D · DMA. DMSO, a ·OH radical scavenger, decreased strand break formation by about 30% at all 2,4-D · DMA concentrations tested (Figure 3). SOD did not show an inhibitory effect on DNA cleavage. Catalase totally suppressed the formation of DNA strand

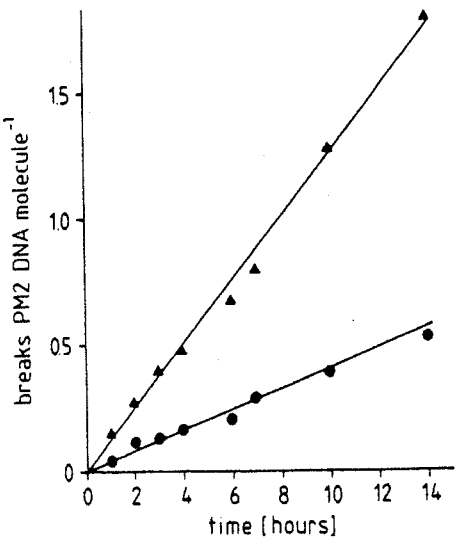

FIGURE 2 Single strand breaks induced in PM2 DNA by $CuCl_2$/2,4-D · DMA as a function of incubation time. PM2 DNA was pretreated with 0.5 mM $CuCl_2$ for one hour and subsequently 2,4-D · DMA was added and incubated for 1–14 h. Results indicate the mean of triplicate samples, (SD < 5%). —●— 12.5 mM 2,4-D · DMA. —▲— 25.0 mM 2,4-D · DMA.

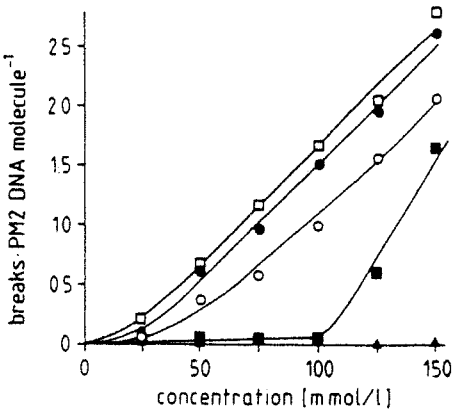

FIGURE 3 Effect of catalase, SOD, DMSO and neocuproine on DNA stand break formation by $CuCl_2$/2,4-D · DMA. PM2 DNA was pretreated with 0.5 mM $CuCl_2$ and subsequently 2,4-D · DMA (final concentration 25 mM–150 mM) was added to the incubation mixture. —●— without additives; —■— in the presence of 33 μg/ml catalase; —□—in the presence of 100 μg/ml SOD; —○— in the presence of 1% DMSO; —▲— in the presence of 1 mM neocuproine; Results indicate the mean of triplicate samples, (SD < 5%).

breaks up to 100 mM 2,4-D · DMA. At higher concentrations of 2,4-D · DMA the protective effect of catalase rapidly decreased. At these concentrations the enzyme activity was strongly inhibited as assayed by the method of Aebi.[12] Heat-inactivated catalase did not diminish DNA strand breaks produced by $CuCl_2$/2,4-D · DMA. This implies that prevention of DNA strand break formation was due to the enzymatic activity of catalase. Neocuproine, a specific Cu(I) chelator, which prevents the involvement of copper in redox reactions,[13] completely provided protection towards strand break formation at all 2,4-D · DMA concentrations.

Identification of Reaction Products of $CuCl_2$ and 2,4-D · DMA

For identification of reaction products of $CuCl_2$ and 2,4-D · DMA, we incubated the two agents under the same conditions as for the determination of strand break formation but omitting the DNA.

As reaction products 2,4-dichlorophenol, CO_2 and formaldehyde were detected. 2,4-Dichlorophenol was found in the ether extract by comparison of the EI-mass spectrum and the GC-retention time with an authentical sample. CO_2 was detected by analyzing the gaseous phase over the reaction mixture by GC/MS. Formaldehyde was photometrically determined in the reaction mixture by applying the acetylacetone method.

DISCUSSION

The combination of $CuCl_2$ and 2,4-D · DMA induced SSB's in PM2 DNA in a time and concentration dependent manner, whereas 2,4-D · DMA, 2,4-D or $CuCl_2$ alone did not show any or only a negligible effect on DNA. A comparable number of strand breaks was induced by using 2,4-D instead of 2,4-D · DMA. Therefore, we conclude that DNA cleavage resulted from reaction of 2,4-D and $CuCl_2$. The total suppression of DNA cleavage by the specific Cu(I) chelator neocuproine suggests the involvement of Cu(I) in DNA damage. Reoxidation of Cu(I) is known to generate superoxide

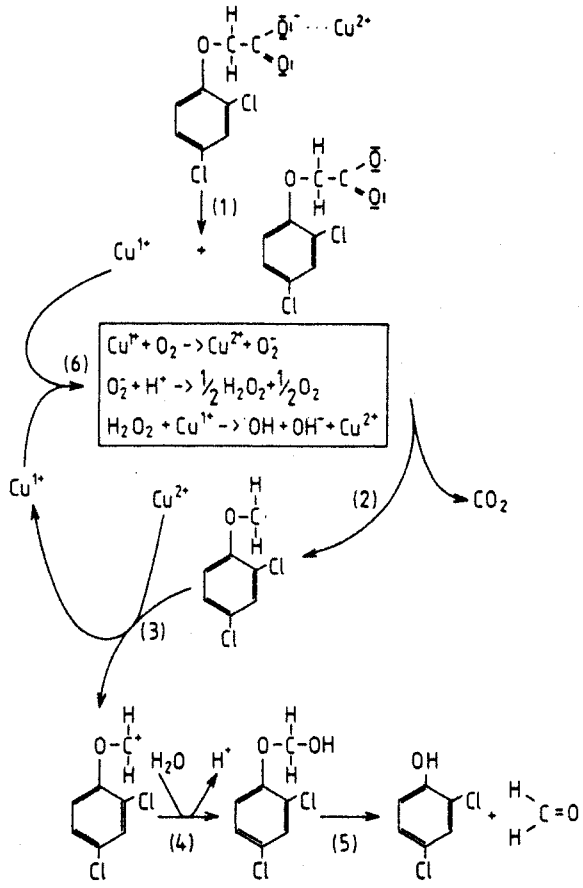

FIGURE 4 Proposed reaction of Cu(II) and 2,4-D. (1) Reduction of Cu(II) by 2,4-D via homolytic cleavage of the metalcarboxylate. (2) Formation of the alkyl radical from the acyloxyl radical by decarboxylation. (3) Oxidation of the alkyl radical by Cu(II) and formation of the carbenium ion. (4) Conversion of the carbenium ion to a hemiacetal by nucleophilic attack of water. (5) Formation of 2,4-dichlorophenol and formaldehyde by decomposition of the hemiacetal. (6) Generation of $^{\cdot}OH$ radicals by reoxidation of Cu(I).

radicals (O_2^-).[14] It is generally assumed that superoxide radicals are of low reactivity[9] and are probably not the ultimate DNA damaging species.[15] Superoxide radicals may be the precursors of radicals of higher toxicity such as the reactive $^{\cdot}OH$ radicals. The reaction of $^{\cdot}OH$ radicals may lead to a variety of DNA damages including altered bases, base loss and DNA strand breakage[11] and to mutagenic effects in mammalian cells.[10] The assumption that DNA strand breaks are due to the action of $^{\cdot}OH$ radicals is substantiated by the fact that catalase stopped DNA cleavage. The effectivity of neocuproine and catalase to prevent DNA cleavage suggests that $^{\cdot}OH$ radicals were formed by a Fenton-type reaction of H_2O_2 and Cu(I). The inability of SOD in suppressing DNA cleaving indicates that superoxide radicals are not of importance for the reduction of H_2O_2 via the Haber–Weiss reaction.

The $^{\cdot}OH$ radical scavenger DMSO showed only a small protective effect. A low efficiency of $^{\cdot}OH$ radical scavengers is described for $^{\cdot}OH$ radicals which are formed within the DNA molecule. Within the DNA molecule Cu(II) forms complexes with guanine-cytosine base pairs[2–4] and reduction of Cu(II) as well as formation of reactive $^{\cdot}OH$-radicals takes place within the DNA molecule. Thus the slight inhibitory effect

of DMSO could be explained by a Fenton driven site-specific reaction at DNA bound copper.

An additional argument for site-specific DNA strand breakage is the formation of double strand breaks (DSB's) in the DNA molecule by $CuCl_2$ in combination with 2,4-D · DMA concentrations higher than 75 mM. Repeated redox reactions of DNA bound copper lead to repeated formation of ·OH radicals at the specific binding site within the DNA resulting in the formation of double strand breaks.[15]

Since copper forms complexes also with proteins, the inactivation of catalase by $CuCl_2$/2,4-D · DMA at high 2,4-D · DMA concentrations probably resulted from a similar site-specific formation of ·OH radicals at the catalase molecule. Comparable effects on catalase were described for the combination Cu(II)/ascorbate.[16,17]

The reduction of metal ions i.e., Co(III), Ce(IV), Ag(II) by carboxylic acids via homolytic cleavage of its metal carboxylate is described.[18] An unstable acyloxyl radical is formed which leads after decarboxylation to an alkyl radical.[18,19] We suppose that Cu(II) is able to oxidize 2,4-D in a comparable manner. The hypothetical mechanism for the redox reaction resulting in the formation of Cu(I), CO_2, formaldehyde and 2,4-dichlorophenol is summarized in Figure 4. This scheme is supported by detection of the postulated reaction products.

Beside the reactive Cu(I), which leads over a cascade of reactions to DNA strand breaks, other electrophilic reactive species may be formed during the described oxidation process of 2,4-D. Carbenium ions and alkyl radicals are also reactive electrophilic species which could be candidates for DNA damages. These may not result in strand break formation *per se* and would therefore not be detectable with the used method. Experiments with human fibroblasts have also shown DNA damaging properties of the substance combination. The induction of DNA repair and the synergistic inhibition of DNA synthesis by $CuCl_2$/U46 D Fluid may be explained by the described redox reaction of Cu(II)/2,4-D.

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