Inhibition of Phorbol Ester Stimulated Interleukin 2 Production by Copper(II) Complexes

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ABSTRACT

Superoxide dismutase mimetic copper(II) complexes, such as copper(II)(3,5-diisopropylsalicylate), (CuDIPS), inhibit phorbol ester stimulated tumor promotion in mouse skin. Therefore, CuDIPS was tested as a potential inhibitor of another effect of phorbol esters, induction of interleukin 2 (IL2) synthesis, in the mouse thymoma cell line EL4. CuDIPS inhibited phorbol ester induced IL2 production in a concentration dependent manner with a 50% inhibitory concentration of about 10 μM. However, the ligand 3,5-diisopropylsalicylic acid also inhibited the induction of IL2 by phorbol esters (50% inhibitory concentration, 15 μM). Since the superoxide dismutase mimetic activity of CuDIPS is not stable in the presence of ethylene diamine tetraacetic acid, the effects of CuDIPS could be due to the free ligand and not to the intact metallocomplex. Consequently, a series of extremely stable copper(II) macrocyclic compounds was synthesized, and the reduction potential, superoxide dismutase mimetic activity, and ability to inhibit phorbol ester induced IL2 production were determined for each. Of the copper(II) macrocyclic complexes studied, only the most potent superoxide dismutase mimetic copper complex was found to inhibit phorbol ester induced IL2 production. Copper(II) complexes had to be added no later than 4 h following phorbol ester administration to be effective inhibitors of the IL2 response, suggesting that these compounds act subsequent to the binding of phorbol esters but prior to the transcription of IL2 messenger RNA. Adherence of EL4 cells to substrate in response to phorbol esters was unaffected by copper(II) compounds. In summary, copper(II) compounds with appropriate reduction potentials can act within a defined time period to inhibit some, but not all, of the effects of phorbol esters on EL4 cells.

INTRODUCTION

Several lines of evidence suggest that a reactive oxygen species such as superoxide anion, hydrogen peroxide, or the hydroxyl radical may mediate some of the effects of the tumor promoting phorbol esters (for reviews see Refs. 1 and 2): (a) free radical generating compounds such as benzoyl peroxide or the xanthine/xanthine oxidase couple have been shown to function as tumor promoters (3, 4). (b) the SOD mimetic compounds were tested as possible inhibitors of this induction. Lipophilic Cu(II) complexes, such as CuDIPS, were used instead of the native enzyme since SOD does not enter cells easily (10). However, the SOD mimetic activity of CuDIPS was not stable in the presence of EDTA, suggesting that cupric ion does not form a strong complex with DIPS. Consequently, some researchers have argued that the SOD mimetic activity of various copper complexes, including CuDIPS, may be due to small amounts of cupric ion in equilibrium with complexes (11). Since cells may contain substances which chelate copper very tightly, the CuDIPS complex could be broken, releasing free ligand and/or metal ion. Under these circumstances, effects due to the intact complex, effects due to the complex acting as a copper donor to some intracellular component, and effects due to the ligand cannot be distinguished. The synthesis of stable Cu(II) macrocyclic compounds eliminated some of these problems and allowed investigation of structure-activity relationships.

MATERIALS AND METHODS

All chemicals used in the investigation and preparation were reagent grade, unless otherwise noted.

Materials. CuCl2 and DIPS were purchased from Aldrich (Milwaukee, WI). Biacetyl, DMSO, CuCl2·2H2O, H2O, and Na2HPO4 were obtained from Fisher Scientific (Richmond, VA). NBT, KO2-, PDB, canine albumin, SOD, catalase, and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). The source of 1,5,8,12-tetraazadecane was Strem Chemical (Newburyport, MA). Tetraethylammonium perchlorate was purchased from Southwestern Analytical (Austin, TX). It was dried overnight at 70°C under vacuum prior to use. Anhydrous NaClO4 was purchased from G. F. Smith (Columbus, OH). 3H labeled L-amino acid mixture (250 mCi/mg) and 3H]thymidine (2.0 Ci/mmol) were obtained from ICN (Irvine, CA). EL4 cells (12) were recloned by Dr. William Young (Department of Pathology, University of Virginia) and grown in RPMI 1640 supplemented with 5% fetal calf serum (Gibco, Gaithersburg, MD) as described previously.

Synthesis of Metallocomplexes. CuDIPS was synthesized according to the method of Soronen (13) with the exception that the DIPS was first dissolved in concentrated ammonium hydroxide and then evaporated to dryness to form the ammonium salt. Ligand and CuCl2 were combined in a 2:1 ratio. CuTIM was prepared by the procedure of Ferraudi et al. (14). Cu4MeTIM and Zn4MeTim were prepared by the procedure of Coltrain and Jackels (15, 16). CuDIM was prepared by a modification of the method published by Fabbri and colleagues (17). A 40 μM solution of 1,5,8,12-tetraazadecane in methanol was cooled below 5°C.
Equimolar amounts of perchloric acid (70%), followed by biacetyl (1.3 m in methanol), were added, and the resulting solution was stirred at <5°C for 30 min and then warmed to room temperature. A slurry of 0.04 mol of copper(II) acetate monohydrate in 1 liter of methanol was slowly added, and the mixture was stirred for 1 h and reduced in volume on a rotary evaporator until an oil and tar remained. Upon addition of 40 ml of saturated aqueous sodium perchlorate to the oil, the compound precipitated as wine-red crystals. The product was recrystallized from ethanol:acetone (1:1) with a yield of 22%.

\[ \text{CuC}_6\text{H}_2\text{N}_4\text{Cl}_2\text{O}_4 \]

Calculated: C 29.63, H 4.97, N 11.51  
Found: C 29.63, H 5.45, N 11.46

The structures of these compounds are shown in Fig. 1.

**Electrochemical Measurements.** Electrochemical properties were determined in acetonitrile, DMF, and water. Both acetonitrile and DMF were dried over 4A molecular sieves before use. Solution concentrations of the metal complexes were 1 mM. For measurements in acetonitrile or DMF, the electrolyte concentration was 100 mM in tetraethylammonium perchlorate; for measurement in water, the electrolyte concentration was 100 mM in sodium perchlorate. Cyclic voltammograms were obtained with a PAR 173 potentiostat and the PAR 175 programmer and recorded with a Houston Omnigraphic Model 164 x-y recorder. The measurements were made at a Bioanalytical System platinum or glassy carbon working disc electrode versus a saturated sodium calomel electrode. Solutions were purged of O_2 with prepurified N_2 prior to electrochemical measurements.

**Determination of SOD Mimetic Activity of Cu(II) Complexes.** Inhibition of the reduction of the dye NBT by KO_2 in anhydrous DMSO was used as a measurement of the SOD mimetic activity. The assay was a variation of one originally devised by Beauchamp and Fridovich (18). The reaction mixture contained 10 mM Na_2HPO_4 (pH 7.5), 100 mM NBT, canine albumin (100 µg/ml), catalase (500 units/ml), 1 mM EDTA (when present), and the indicated concentrations of inhibitor in a total volume of 3 ml. The reaction was started with the addition of 0.1 ml of saturated KO_2 in anhydrous DMSO. The reduction of NBT was monitored at 560 nm, and the concentration of various inhibitors which yielded 50% inhibition was determined experimentally.

**Bioassay of Interleukin 2 Production.** A stock solution of PDB (1 mM) in absolute ethanol was diluted in RPMI 1640 before addition to cells. EL4 cells were incubated for 24–36 h at 37°C in the presence or absence of PDB (50 nM) and/or Cu(II) complexes. IL2 activity in the culture supernatants was assayed by the ability of the conditioned media to support growth of an IL2 dependent cell line as described previously (9).

**Inhibition of Protein Synthesis.** EL4 cells (10^5 cells/well) were suspended in amino acid free RPMI 1640 supplemented with 5% fetal calf serum in the presence or absence of PDB and/or Cu(II) complexes. Following a 10-h pulse with ^3H-L-amino acid mixture (specific activity, 250 mCi/mg), trichloroacetic acid was added to a final concentration of 10% to precipitate the proteins, and the plates were incubated at 4°C for 1 h. Labeled proteins were collected onto glass fiber filters and counted in a liquid scintillation counter.

**RESULTS**

**Reduction Potentials and SOD Mimetic Activity of Cu(II) Complexes.** Half-wave potentials determined by cyclic voltammetry are given in Table 1. The potentials for the Cu ligand I/II couple fall in the order: CuMeTIM > CuTIM > CuDIM, in both water and acetonitrile. The potentials are more negative in water than in acetonitrile, as expected based on the stronger coordinating properties of water in the axial positions of the copper complexes compared to acetonitrile.

Attempts were made to study reduction potential of CuDIPS in acetonitrile and DMF. In both solvents, the complex gave ill defined voltammograms. Reduction commenced at about +0.2 V and ceased at ~0.2 V. Reverse scanning indicated that elemental copper had formed at the electrode surface. The compound was not electrochemically stable, but it was possible to bracket its half-wave potential between +0.2 V and ~0.2 V. The sequence of activity for all of the complexes would be CuDIPS > CuMeTIM > CuTIM > CuDIM.

As shown in Table 1, CuDIPS was the most potent SOD mimetic compound tested, with an IC_50 of 8.9 µM for inhibition of reduction of NBT by KO_2 in anhydrous DMSO. The SOD mimetic activity of CuDIPS, however, was not stable in the presence of EDTA. Although CuMeTIM and CuTIM were less potent SOD mimetic compounds (IC_50 = 16 µM and 210 µM, respectively) than CuDIPS, the ability of these compounds to inhibit the reduction of NBT by KO_2 was not affected by EDTA. This result reflects the exceptional stability of these compounds. Copper cannot be released without breaking the ring structure of the ligand. DIPS and CuDIM have no SOD mimetic activity in the concentration range tested. The order of potency of the Cu(II) complexes as SOD mimetics was CuDIPS > CuMeTIM > CuDIM.

**Octanol/Water Partition Coefficients of the Cu(II) Complexes.** Octanol/water partition coefficients of the Cu(II) complexes would provide an estimate of the relative lipophilicity of these compounds. CuDIPS partitioned solely into the octanol phase;

<table>
<thead>
<tr>
<th>E_m (V)</th>
<th>NBT reduction (IC_50, µM)</th>
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<tbody>
<tr>
<td>Acetonitrile</td>
<td>Water</td>
</tr>
<tr>
<td>CuDIPS</td>
<td>0.2 (−0.2)</td>
</tr>
<tr>
<td>CuMeTIM</td>
<td>−0.22</td>
</tr>
<tr>
<td>CuTIM</td>
<td>−0.36</td>
</tr>
<tr>
<td>CuDIM</td>
<td>0.52</td>
</tr>
</tbody>
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*ND, not determined.  
*At high concentrations CuDIPS precipitates out of solution.  
During determinations.

Fig. 1. Structure of the copper(II) complexes.
COPPER(II) COMPOUNDS AND INTERLEUKIN 2 PRODUCTION

CuφMeTIM was only minimally soluble in either phase; and CuTIM, CuDIM, and CuCl2 entered the water phase exclusively. The only compound for which a partition coefficient could be calculated was the ligand DIPS (0.17). However, these data do allow a crude rank order of lipophilicity: CuDIPS > DIPS, CuφMeTIM > CuTIM, CuDIM, CuCl2.

Effects of Cu(II) Complexes on IL2 Production. CuDIPS inhibited phorbol ester induced IL2 production by mouse EL4 cells with an IC50 of 10 μM (Fig. 2). Phorbol ester induced IL2 production was not inhibited by 100 μM copper(II) chloride (data not shown). However, the ligand DIPS was an equally potent inhibitor (IC50 15 μM; copper chelates two DIPS molecules to form CuDIPS). Therefore, the inhibition of phorbol ester induced IL2 production by CuDIPS could be due to the ligand rather than to the intact complex. Thus, it was important to determine if other, more stable Cu(II) complexes with SOD mimetic activity could inhibit phorbol ester induced IL2 production. CuφMeTIM, which had the greatest SOD mimetic activity of the stable macrocyclic compounds, was an effective inhibitor. The weak SOD mimetic CuTIM (data not shown), the inactive CuDIM (Fig. 2), and the enzyme SOD (600 units/ml) in the presence or absence of catalase (2500 units/ml) (data not shown) failed to inhibit phorbol ester induced IL2 production. Addition of SOD, catalase, or any of the Cu(II) complexes to the CT6 cells used for the bioassay of IL2 had no effect on the ability of these cells to proliferate in response to a standard preparation of IL2.

For the above experiments, CuDIPS was added to EL4 cells in either ethanol or DMSO so that the final concentration of solvent was 1%. CuφMeTIM was added in either DMSO or DMF. CuTIM and CuDIPS were tested in both DMSO and an aqueous solution of 150 mM NaCl-20 mM NaH2PO4, pH 7.5. Both ethanol and DMF alone inhibited phorbol ester induced IL2 production [1% ethanol, 23 ± 8% (SD); 1% DMF, 12 ± 3% of control values]. DMSO had no significant effect (1% DMSO, 88 ± 10% of control). The NaCl solution did not affect IL2 production. The concentration dependent responses obtained for the Cu(II) complexes were identical in the different solvents when compared to the appropriate solvent controls, indicating that the inhibition of IL2 production by these compounds was independent of the solvent used.

Cell viability, estimated by trypan blue exclusion, was not altered by addition of solvents or Cu(II) complexes, with the exception of CuDIPS. High concentrations of CuDIPS or DIPS alone were toxic to EL4 cells, but 85% inhibition of IL2 production was achieved before cell viability was affected.

The effects of the inhibitors on protein synthesis were measured to determine if inhibition of general protein synthesis occurred before cytoxicity was seen. CuDIPS and, to a lesser extent, the ligand DIPS inhibited protein synthesis at concentrations similar to those which inhibited IL2 production (35–150 μM), but CuφMeTIM had no effect on general protein synthesis even at concentrations (200 μM) which completely inhibited IL2 productions (data not shown).

Although the Cu(II) macrocyclic complexes were shown to be stable in the presence of EDTA, ZnφMeTIM was synthesized in an attempt to demonstrate that the inhibition of IL2 production was due to the Cu(II) complex and not to the ligand alone. However, ZnφMeTIM proved to be unexpectedly toxic to EL4 cells, causing significant loss of viability at concentrations at which CuφMeTIM had no effect on viability as judged by trypan blue exclusion. ZnφMeTIM is a five-coordinate complex while CuφMeTIM is a four-coordinate complex, and this difference in geometry, the difference in net charge between the complexes, or the metal substitution must account for the different cytotoxicity observed.

To determine when the Cu(II) complexes were acting, the time of addition of inhibitor was varied relative to the time of stimulation by phorbol esters (Fig. 3). CuDIPS or CuφMeTIM could be added 16 h prior to or 4 h following administration of phorbol ester without altering the inhibition caused by these Cu(II) compounds. If the interval between administration of PDB and CuDIPS or CuφMeTIM was greater than 4 h, inhibition of phorbol ester induced IL2 production was diminished.

To determine whether the inhibition of phorbol ester induced IL2 production by Cu(II) complexes was reversible, EL4 cells were incubated with or without phorbol ester and/or Cu(II) complexes, washed twice, and resuspended in medium containing only phorbol ester. Cells incubated with Cu(II) complexes alone, then washed, and stimulated with phorbol ester produced as much IL2 as did cells that were not treated with Cu(II) complexes, presumably indicating that the copper compounds could be removed by washing. Cells incubated with Cu(II) complexes in the presence of phorbol ester and then washed produced diminished amounts of IL2. The presence of Cu(II) complexes for only the first 6 h of a 48-h incubation was sufficient to reduce IL2 production by 50%. When the Cu(II)
complexes were present for the first 12 h, IL2 production was reduced to the same level as if the inhibitor were present for the full 48 h (Fig. 4).

Effect of Cu(II) Complexes on Adherence. When stimulated by phorbol esters, EL4 cells not only produce IL2 but also adhere to substrate and become growth inhibited. The adherence response was not blocked by 35 μM CuDIPS or 150 μM CuMeTIM, concentrations which inhibited IL2 induction by greater than 85% (Table 2). Pretreatment of EL4 cells with either Cu(II) complex for 16 h prior to phorbol ester stimulation also failed to diminish the adherence response (data not shown).

DISCUSSION

The SOD mimetic compound CuDIPS has often been used to investigate the role of superoxide anion in biological processes, e.g., streptozotocin-induced diabetes in pancreatic islet cells (19), tumor initiation (20), tumor promotion (5), and chemiluminescence induced by phorbol ester in mouse epidermal cells (7). The CuDIPS used in this study exhibited an IC50 of 8.9 μM in the NBT reduction assay, a value which agrees well with the previously published value of 2.9 μM (21). The IC50 for inhibition of phorbol ester induced IL2 production by CuDIPS was 10 μM, slightly higher than the concentration of CuDIPS (5 μM) reported to inhibit the induction of anchorage independence in C141 cells, a promotable variant of JB6 mouse epidermal cells (22). In freshly isolated mouse epidermal cells treated with phorbol myristate acetate, 10 μM CuDIPS was reported to inhibit luminol-dependent chemiluminescence, a measure of the production of reactive oxygen species, by 65% (7).

The ligand, DIPS, has been shown to be less potent than CuDIPS in inhibiting tumor promotion by phorbol esters in mouse epidermis (5). However, DIPS was as potent an inhibitor of phorbol ester induced IL2 production as CuDIPS, raising the possibility that the inhibition caused by CuDIPS could be due to the ligand and not the intact complex. The inhibitory activity of the ligand and the lack of stability of CuDIPS made the synthesis of a more stable SOD mimetic complex, such as CuMeTIM, essential.

There have been only a few other reports of metal complexes that SOD mimetic activities of which were stable in the presence of EDTA, amongst them copper and nickel macrocyclic polyaamine complexes (23) and metalloporphyrins (24, 25). However, more than 50 macrocyclic polyaamine complexes investigated by Kimura et al., only two had IC50s of less than 200 μM in a NBT reduction assay. No information is available on their potency in any biological system. Metalloporphyrins, such as iron(III) tetrakis(4-n-methylpyridyl) porphine, appear to be irreversibly modified by hydrogen peroxide, the product of the dismutation of O2·- (24). None of the metalloporphyrins tested as potential antitumor agents had a significant effect on tumor growth or animal survival in mice implanted with Ehrlich carcinoma cells (26). Other than these studies, very little data have been reported correlating reduction potentials, SOD mimetic activity, and biological activity.

In addition to examining CuDIPS, we investigated a series of extremely stable Cu(II) complexes, CuDIM, CuTIM, and CuMeTIM. These three compounds differed in reduction potential, in solubility, in SOD mimetic activity, and in potency as inhibitors of phorbol ester induced IL2 production in EL4 cells. The ability of these three compounds to inhibit the reduction of NBT by KO2·- paralleled the reduction potential of the compounds. The SOD mimetic activity of both CuMeTIM and CuTIM was unaffected by the presence of EDTA and thus the SOD-like activity of these complexes cannot be attributed to free cupric ion in equilibrium with the complexes.

The differences between the rank order of potency of the compounds as SOD mimetics in vitro (CuDIPS > CuMeTIM > CuTIM > CuDIM, DIPS) and as inhibitors of phorbol ester stimulated IL2 production (CuDIPS = DIPS > CuMeTIM > CuTIM, CuDIM) suggest that factors other than SOD mimetic activity affect the ability of these compounds to function as inhibitors. The relative lipophilicity of the compounds (CuDIPS > DIPS, CuMeTIM > CuTIM, CuDIM, CuCl2) does correlate with their order of potency as inhibitors, and thus poor lipophilicity may contribute to ineffectiveness of some agents. The failure of CuTIM to inhibit the induction of IL2 by phorbol ester could be due to either the relatively weak SOD mimetic activity of the compound or to relatively poor lipophilicity of the compound which would impair the entry of CuTIM into the cell or requisite cellular compartment. Similarly, the inability of SOD to inhibit the effects of phorbol ester might be due to poor penetration of the enzyme into the cells.

Since CuDIPS and DIPS inhibited general protein synthesis at concentrations similar to those concentrations which inhibited IL2 production, the mechanism of action of these compounds remains in doubt. In vitro, DIPS has been shown to be a poor inhibitor of prostaglandin synthesis (21), but it is not known what action this compound might have in vivo. There are few studies investigating the effect of cyclooxygenase or lipoxygenase inhibitors in lymphocyte systems. One study suggested that the proliferation of a mixed population of murine spleen lymphocytes stimulated with concanavalin A could be reduced by the addition of lipoxygenase but not cyclooxygenase inhibitors (27). Therefore, the inhibition of IL2 production by DIPS, which lacked SOD mimetic activity, could be due to...
inhibition of general protein synthesis or to some other action of the compound. Similarly, inhibition of IL2 production by CuDIPS could be due to effects on protein synthesis rather than the SOD mimetic activity of the complex. However, the ability of Cu$_2$MeTIM to inhibit IL2 production cannot be attributed to effects on general protein synthesis. While it cannot be concluded that the effects of Cu$_2$MeTIM, while a less potent SOD mimetic than CuDIPS, is more stable and less cytotoxic than CuDIPS. Cu$_2$MeTIM is sufficiently lipophilic to enter cells and does not inhibit general protein synthesis.

Both time course and reversibility studies suggest that the event sensitive to inhibition by Cu(II) complexes occurred within the first 4 to 6 h following phorbol ester administration. This conclusion is also supported by the report that CuDIPS had to be present 2 h before or 2 h following phorbol ester stimulation to inhibit the induction of ornithine decarboxylase activity by phorbol ester in mouse epidermis (5). However, EL4 cells could be pretreated with Cu(II) complexes for up to 16 h with no loss of inhibition. The difference in the time course for EL4 cells as opposed to mouse epidermis may be the result of diffusion of CuDIPS from the site of action when CuDIPS was applied to mouse skin or to differences in the metabolism of CuDIPS between EL4 cells and mouse epidermis.

It is now generally accepted that the cellular receptor for phorbol esters is the Ca$^{2+}$, phosphorylated protein kinase C (28). In EL4 cells, phorbol ester treatment caused a rapid translocation of the phorbol ester receptor from the cytosol (100,000 $\times$ g supernatant) to the particulate fraction (12). Cu(II) complexes probably do not interfere with either the binding of phorbol esters to their receptor (C kinase) or the translocation of the receptor to membranes since Cu(II) complexes inhibited IL2 induction by phorbol ester even if added after binding and translocation had already occurred. The Cu(II) complexes could, however, interfere with the generation or transduction of an intracellular signal subsequent to phorbol ester binding. The adherence of EL4 cells to substrate in response to phorbol ester administration must then require a different signal, since the adherence response was not blocked by treatment with the Cu(II) complexes. IL2 synthesis requires the synthesis of mRNA. Presumably, the rapid adherence response does not require RNA synthesis. Investigations are currently under way to determine if the inhibition of phorbol ester induced IL2 production by Cu(II) complexes occurs prior to the synthesis of IL2 mRNA.

Acknowledgments

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