Overcoming Drug-Resistant Cancer by a Newly Developed Copper Chelate through Host-Protective Cytokine-Mediated Apoptosis

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Abstract

Purpose: Previously, we have synthesized and characterized a novel Cu(II) complex, copper N-(2-hydroxy acetonaphone) glycinate (CuNG). Herein, we have determined the efficacy of CuNG in overcoming multidrug-resistant cancer using drug-resistant murine and human cancer cell lines.

Experimental Design: Action of CuNG following single i.m. administration (5 mg/kg body weight) was tested in vivo on doxorubicin-resistant Ehrlich ascites carcinoma (EAC/Dox) – bearing mice and doxorubicin-resistant sarcoma 180 – bearing mice. Tumor size, ascitic load, and survival rates were monitored at regular intervals. Apoptosis of cancer cells was determined by cell cycle analysis, confocal microscopy, Annexin V binding, and terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling assay ex vivo. IFN-γ and tumor necrosis factor-α were assayed in the culture supernatants of in vivo and in vitro CuNG-treated splenic mononuclear cells from EAC/Dox-bearing mice and their apoptogenic effect was determined. Source of IFN-γ and changes in number of T regulatory marker-bearing cells in the tumor site following CuNG treatment were investigated by flow cytometry. Supernatants of in vitro CuNG-treated cultures of peripheral blood mononuclear cells from different drug-insensitive cancer patients were tested for presence of the apoptogenic cytokine IFN-γ and its involvement in induction of apoptosis of doxorubicin-resistant CEM/ADR5000 cells.

Results: CuNG treatment could resolve drug-resistant cancers through induction of apoptogenic cytokines, such as IFN-γ and/or tumor necrosis factor-α, from splenic mononuclear cells or patient peripheral blood mononuclear cells and reduce the number of T regulatory marker-bearing cells while increase infiltration of IFN-γ-producing T cells in the ascitic tumor site.

Conclusion: Our results show the potential usefulness of CuNG in immunotherapy of drug-resistant cancers irrespective of multidrug resistance phenotype.

Multidrug resistance (MDR) is a complex phenomenon where malignant cells manifest resistance toward a range of unrelated chemotherapeutic agents and is a major obstacle to the successful pharmacologic treatment of cancers (1, 2). Many different mechanisms have been suggested to explain the development of MDR phenotype in cancer cells, such as a change in the specific target of a drug (1), the reduced uptake or increased efflux of a drug (3, 4), a differential compartmentalization (5, 6), an increased rate of detoxification of a drug (4, 7), an increased ability to repair DNA damage (4, 8), gene amplification (9, 10), and an increase in the activity of survival mechanisms has been the overexpression of several energy-dependent drug efflux pumps belonging to the ATP-binding cassette family of transporter proteins, such as the P-glycoprotein, MDR-associated proteins, and breast cancer resistance protein/ABCG2 (13–15). Overexpression of these integral membrane proteins causes cancer cells to become resistant to a variety of drugs, such as anthracyclines, Vinca alkaloids, taxanes, epipodophyllotoxins, etc. (1, 2, 16–18).

Doxorubicin is the most commonly used drug in the therapy for solid tumors, many ascitic tumors, and some leukemia. Involvement of ATP-binding cassette transporters and differential compartmentalization of drugs have been reported to cause resistance to doxorubicin and other drugs in different cell lines.
(5, 19, 20). Therefore, to overcome doxorubicin resistance efficacy of glutathione, depletors (21) and inhibitors (22, 23) of efflux pumps were studied as resistance-modifying agents for induction of apoptosis of resistant cells with doxorubicin. Because no resistance-modifying agent that has been highly successful clinically has emerged thus far, recently, immunomodulators and cytokines are being tested in vivo and in vitro against various drug-resistant cancers (24–28).

Earlier, we have synthesized a novel Schiff’s base chelate of Cu(II), copper N-(2-hydroxy acetophenone) glycinate (CuNG) and studied its chemical nature as well as its toxicity (29). Later, we have shown that i.p. administration of CuNG at a dose of 10 mg/kg body weight in doxorubicin-resistant Ehrlich ascites carcinoma (EAC/Dox)–bearing mice could reverse doxorubicin resistance and allowed doxorubicin to induce apoptosis in vivo and in vitro (30). Interestingly, single i.m. administration of CuNG alone at a lower dose (5 mg/kg body weight) disclosed that CuNG possessed immunomodulatory activity. Herein, we report that CuNG alone could resolve doxorubicin-resistant cancers through induction of host protective cytokines, such as IFN-γ and tumor necrosis factor-α (TNF-α), which are reported to have anticancer properties (27, 31). Moreover, CuNG was found to induce peripheral blood mononuclear cells (PBMC) from different drug-insensitive and radiation-insensitive patients to secrete protective cytokines that caused apoptosis of the doxorubicin-resistant human T lymphoblastic leukemia cell line, CEM/ADR5000. Because no highly effective resistance-modifying agent is available clinically, this immunomodulator holds immense promise for treatment of drug-resistant cancer.

**Materials and Methods**

**Materials.** TNF-α-neutralizing monoclonal antibodies, IFN-γ-neutralizing monoclonal antibodies (murine and human), murine recombinant IFN-γ (rIFN-γ), murine recombinant TNF-α (rTNF-α), Opt EIA kits for assay of murine and human IFN-γ and TNF-α, anti-CD4 peridinin-chlorophyll-protein complex–conjugated monomolecular antibody, anti-CD8 phycoerythrin-conjugated monomolecular antibody, anti-IFN-γ FITC-conjugated antibody, and anti-CD25 FITC-conjugated antibody were obtained from BD Biosciences (San Diego, CA). Anti-Foxp3 phycoerythrin-conjugated monoclonal antibody (murine) was obtained from eBioscience (San Diego, CA). Penicillin, streptomycin, RPMI 1640, trypan blue, propidium iodide (PI), brefeldin A, concanavalin A, phorbol 12-myristate 13-acetate (PMA), and ionomycin were obtained from Sigma (St. Louis, MO). All radioactive chemicals were purchased from New England Nucleotide (Boston, MA) unless otherwise mentioned. Annexin V-FITC and Apo-Direct kit were procured from Becton Dickinson immunocytometry system (San Jose, CA).

**Animals and cell lines.** Swiss albino mice, originally obtained from National Institute of Nutrition (Hyderabad, India) and reared in the institute animal facilities, were used for experimental purposes with prior approval of the institutional animal ethics committee. EAC/Dox, which is also resistant against cisplatin, cyclophosphamide, and vinblastine (32), and doxorubicin-resistant sarcoma 180 (S180/Dox) were developed and maintained according to the methods described previously (32). Doxorubicin-resistant human acute T lymphoblastic leukemia cell line CEM/ADR5000 (33), derived from the parental CCRF-CEM cell line (34), was provided by T. Efferth. This slow-growing cell line displayed >800-fold resistance to doxorubicin and overexpressed ABCB1/MDR1 (35).

**Peripheral blood samples of patients.** Leftover excesses of blood drawn for routine examinations of terminal cancer patients insensitive to various chemotherapeutics as well as toward radiation therapy in some cases (certified by the Department of Surgical Oncology and Medical Oncology, Hospital Unit, Chittaranjan National Cancer Institute) were collected as samples from the Department of Clinical Biochemistry, Hospital Unit, Chittaranjan National Cancer Institute. The patient profile in brief is presented in Table 1.

**Treatments.** EAC/Dox-bearing mice were either kept untreated or treated with a single dose of CuNG (5 mg/kg body weight) 7 days following peritoneal inoculation with 1 × 106 EAC/Dox cells derived from EAC/Dox-bearing mice treated with doxorubicin (48 hours before acquisition of cells). S180/Dox cells were maintained in peritoneal cavity of doxorubicin-treated mice. These mice were again treated with doxorubicin 48 hours before acquisition of cells from their peritoneal cavity for inoculation. Animals inoculated with 5 × 106 S180/Dox cells in right hind leg were either kept untreated or treated with CuNG (i.m., single administration) 5 mg/kg body weight in left hind leg after 15 days.

In some experiments, EAC/Dox cells were cultured in the presence of 2.5 μg/mL CuNG or 100 units/ml rIFN-γ (36) or 20 ng/ml rTNF-α (37) or nonadherent population of splenic mononuclear cells (SPMC) from CuNG-treated EAC/Dox-bearing mice (2 × 106 EAC/Dox cells with 1 × 105 nonadherent SPMC in 1 ml). In some other experiments, EAC/Dox cells were cultured in the presence of SPMC culture supernatants derived from CuNG-treated EAC/Dox-bearing mice (100 μL/ml) and/or neutralizing anti-IFN-γ (10 μg/ml) and/or anti-TNF-α (10 μg/ml). In some experiments, CEM/ADR5000 cells were cultured in the presence of culture supernatants (200 μL/ml) of in vitro CuNG-treated (or untreated) PBMC derived from patients and in the presence or absence of neutralizing anti-IFN-γ (10 μg/ml).

**Isolation of EAC/Dox cells from peritoneal cavity of mice.** The EAC/Dox cells were isolated from the peritoneal cavity of EAC/Dox-bearing mice (control or treated). Sterile PBS (2-3 ml) was injected into the peritoneal cavity of the mice and the peritoneal fluid containing the tumor cells was withdrawn, collected in sterile Petri dishes, and

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Type of cancer</th>
<th>Insensitive to</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Prostate cancer, prostate (prostate-specific antigen: 879.45 ng/mL; osteoblastic metastasis in pelvis)</td>
<td>Chemotherapy (cyclophosphamide) and antiandrogen therapy</td>
</tr>
<tr>
<td>2</td>
<td>Breast cancer (metastatic)</td>
<td>Chemotherapy (5-fluorouracil + doxorubicin + cyclophosphamide)</td>
</tr>
<tr>
<td>3</td>
<td>Rectal cancer (metastatic)</td>
<td>Chemotherapy (5-fluorouracil) and radiation</td>
</tr>
<tr>
<td>4</td>
<td>Breast cancer (metastatic)</td>
<td>Chemotherapy (5-fluorouracil + cyclophosphamide) and radiation</td>
</tr>
<tr>
<td>5</td>
<td>Nasopharyngeal cancer (metastatic)</td>
<td>Chemotherapy (cisplatin + 5-fluorouracil) and radiation</td>
</tr>
<tr>
<td>6</td>
<td>Seminoma of testis (metastasis to other tissues)</td>
<td>Chemotherapy (cisplatin + etoposide)</td>
</tr>
<tr>
<td>7</td>
<td>Breast cancer (metastatic)</td>
<td>Chemotherapy (5-fluorouracil + doxorubicin + cyclophosphamide)</td>
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incubated at 37°C for 2 hours. The cells of macrophage lineage adhered to the bottom of the Petri dishes. The nonadherent population was aspirated out gently and washed repeatedly with PBS. EAC/Dox cells were then separated from other nonadherent contaminating cells by fluorescence-activated cell sorting. More than 98% of this separated cell population was CD3 (T cell)/CD14 (macrophage)/CD19 (B cell)/CD56 (natural killer cell) negative as was determined by flow cytometer. Moreover, these cells were morphologically characterized as EAC by Wright staining (38) and viability was assessed to be >95% by trypan blue dye exclusion. The viable EAC/Dox cells were processed for further experiments.

**Derivation of mononuclear cells from spleen and lymph node of mice and blood of patients.** Mice [normal or EAC/Dox-bearing (untreated or CuNG treated)] were euthanized, and their spleen and lymph nodes (auxiliary, inguinal, and cervical) were removed. Spleens were homogenized separately in ice-cold RPMI 1640. Heparinized peripheral blood of patients was taken and diluted with equal volume of RPMI 1640. Lymphocyte-enriched mononuclear cells were isolated by Histopaque 1077 (Sigma) density gradient centrifugation of murine spleen cell suspension and diluted blood samples of patients, washed, and finally resuspended in cold RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum (RPMI-FBS). Lymph nodes from mice were teased over no. 80 steel screen (Sigma) to obtain lymph node cell suspension in RPMI-FBS (39). Cell viability (>95%) was checked by the trypan blue dye exclusion method. For certain experiments, the SPMC suspension, thus obtained, was kept in 35-mm-diameter plastic tissue culture plates for ~4 hours at 37°C under a 5% CO2-95% air atmosphere to allow attachment of adherent cells. Nonadherent cells (95% lymphocytes) were subsequently removed by aspiration, harvested by centrifugation, and resuspended in RPMI-FBS.

**Preparation of SPMC culture supernatant.** SPMC (4 × 10⁶) from EAC/Dox-bearing mice either untreated or treated with CuNG in vivo were cultured in RPMI-FBS for 24 or 60 hours. In some cases, 4 × 10⁶ SPMC from CuNG untreated EAC/Dox-bearing mice were cultured in the presence of 2.5 μg/mL CuNG in RPMI-FBS for 24 or 60 hours. Supernatants were collected by centrifugation at 500 × g.

**Preparation of PBMC culture supernatant.** PBMC (4 × 10⁶) from each patient were either kept untreated or treated in vitro with 1 μg/mL CuNG and maintained in RPMI-FBS for 48 hours. Supernatants were collected by centrifugation at 500 × g.

**Lymphocyte proliferation assay.** Lymphocyte proliferation experiments were carried out in vitro in 96-well tissue culture plates, each well of which contained 2 × 10⁵ cells in 200 μL culture. Cells were stimulated with concanavalin A (2.5 μg/mL) or a combination of PMA (20 ng/mL) and ionomycin (500 ng/mL) for 48 hours at 37°C under 5% CO2-95% air. Unstimulated (control) cultures did not receive any CuNG. Next, cell suspensions were pulsed with [³H]thymidine (0.5 μCi/well) for another 20 hours. Cells were harvested on glass fiber filter papers (Whatman, Maidstone, United Kingdom) by using a cell harvester (Nunc, Roskilde, Denmark), and incorporation of [³H]thymidine was measured by liquid scintillation counter (Wallac 1409, Gaithersburg, MD; ref. 39).

**Detection of apoptosis by flow cytometry.** For the determination of cell cycle phase distribution, EAC/Dox cells harvested from tumor-bearing mice or CEM/ADR5000 cells were permeabilized and nuclear DNA was labeled with PI. Cell cycle phase distribution of nuclear DNA was determined on fluorescence-activated cell sorting, fluorescence detector equipped with 488 nm argon laser light source and FITC-Br-dUTP. Cells were washed, incubated with PI/RNase solution, and analyzed on fluorescence-activated cell sorting. Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. A total of 10,000 events were acquired and the cells were properly gated for analysis. By this technique, we could distinguish between apoptotic and necrotic cells. Unfixed apoptotic cells are impermeable to PI, but Annexin V binds specifically to phosphatidylserine that is translocated to the outer leaflet of the membrane of apoptotic cells, whereas necrotic cells are permeable to both the fluorochromes.

To confirm the nature killing of EAC/Dox by CuNG treatment, EAC/Dox cells were fixed, permeabilized, and incubated with terminal deoxynucleotidyl transferase enzyme and FITC-Br-dUTP. Cells were washed, incubated with PI/RNase solution, and analyzed on fluorescence-activated cell sorting. Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. A dot plot of PI fluorescence (X axis) versus FITC fluorescence (Y axis) has been displayed (40).

**Oligonucleosomal fragmentation.** For the assessment of chromatin condensation and nuclear blebbing, EAC/Dox and CEM/ADR5000 cells were fixed and nuclear DNA was stained with PI (10 μg/mL) for 15 minutes at room temperature. A Leica model DM 900 (Wetzlar, Germany) fluorescent microscope was used to visualize apoptotic cells. Digital images were captured with cool (−25°C) CCD camera controlled with MetaMorph software (Universal Imaging, Downingtown, PA; ref. 40).

**Detection of infiltration of CD4⁺, CD8⁺, and T regulatory cells and intracellular IFN-γ by flow cytometry.** Ascitic fluids from five untreated and five in vivo CuNG-treated (15 days after treatment) EAC/Dox-bearing mice were drawn. Ascitic fluids from mice of each group were pooled and centrifuged at 100 × g for 5 minutes and supernatants were collected. Supernatants were then centrifuged at 400 × g for 10 minutes. Each pellet was resuspended in 5 mL RPMI-FCS, plated in FCS preoccupied tissue culture Petri dish, and incubated at 37°C in 5% CO2-95% air for 3 hours for adherence. Nonadherent cells were collected, washed twice with HBSS, and finally resuspended in 5 mL...
HBSS. This was then divided into two equal parts. One part was incubated with brefeldin A. Cells of these parts were incubated with anti-CD4 peridinin-chlorophyll-protein complex–conjugated monoclonal antibody and anti-CD8 phycoerythrin-conjugated monoclonal antibody for 45 minutes following blocking with 2.5% (v/v) normal mouse serum. Cells were next washed, fixed with 4% paraformaldehyde for 30 minutes, and then washed with 0.1% saponin in FACScan buffer (0.2% bovine serum albumin, 0.02% NaN3 in PBS). Cells were then incubated with anti-IFN-γ FITC-conjugated antibody or isotype control monoclonal antibodies. Cells were resuspended in FACScan buffer and used for flow cytometry. Cells from another part were incubated with anti-CD4 peridinin-chlorophyll-protein complex–conjugated monoclonal antibody and anti-CD25 FITC-conjugated antibody following blocking with 2.5% (v/v) normal mouse serum. Next, cells were washed, fixed with 4% paraformaldehyde, and then washed with 0.1% saponin in FACScan buffer as before. Cells were then incubated with anti-Foxp3 phycoerythrin-conjugated monoclonal antibody or isotype control monoclonal antibodies. Cells were resuspended in FACScan buffer and used for flow cytometry as before.

**Cytotoxicity assay.** Cytotoxicity was measured in terms of 51Cr released (41). Target cells (1 × 10^6) were labeled with 100 μCi Na2CrO4 for 1 hour at 37°C in 5% CO2 incubator and washed several times until no γ-irradiation count was detected in the supernatant. Nonadherent splenocytes from different experimental groups were incubated with CuNG treatment started 7 days following inoculation with EAC/Dox and 15 days following inoculation with S180/Dox. Ascitic load was studied 21 days following treatment, whereas i.m. tumor with S180/Dox was studied 45 days following treatment. Median survival of untreated S180/Dox-bearing mice was 75 ± 5, whereas no mortality was recorded even at 120 days after treatment in CuNG-treated group of S180/Dox-bearing mice (data not shown).

| Table 2. Decrease of ascites load and solid tumor size following CuNG treatment |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group           | EAC/Dox         | S180/Dox        |                 |                 |
|                 | Cell count (×10^6) | Ascitic fluid volume (mL) | Tumor size (mm³) | Secondary tumor |
| Untreated       | 2.250 ± 300     | 15 ± 2          | 105 ± 5         | +++              |
| CuNG treated    | 4 ± 0.1         | 0.5 ± 0.05      | 4.2 ± 1.5       | –                |

NOTE: Volumes (in mL) indicate the amounts of ascitic fluid in the peritoneal cavity. CuNG treatment started 7 days following inoculation with EAC/Dox and 15 days following inoculation with S180/Dox. Ascitic load was studied 21 days following treatment, whereas i.m. tumor with S180/Dox was studied 45 days following treatment. Median survival of untreated S180/Dox-bearing mice was 75 ± 5, whereas no mortality was recorded even at 120 days after treatment in CuNG-treated group of S180/Dox-bearing mice (data not shown).

![Fig. 2](image-url)
Resolution of Drug-Resistant Cancer by Copper Chelate

Fig. 3. A, effect of in vivo CuNG treatment on proliferation of splenic mononuclear cells and lymph node cells derived from EAC/Dox-bearing mice. Cells from CuNG-treated EAC/Dox-bearing mice were either kept untreated or treated in vitro with PMA + ionomycin (1 μl) or concanavalin A (Con A). Results were compared with SPMC or lymph node cells obtained from normal (N) and EAC/Dox-bearing mice untreated in vivo. B, effect of coculture of EAC/Dox cells with nonadherent SPMC from CuNG-treated EAC/Dox-bearing mice. Extensive nuclear fragmentation of EAC/Dox cells represented by sub-G0-G1 population (M1) was observed following their coculture with nonadherent splenocytes from CuNG-treated EAC/Dox-bearing animals. M1, sub-G0-G1; M2, G0-G1; M3, S, M4, G2-M. C, extent of cell-mediated cytotoxicity of nonadherent splenocytes from CuNG-treated EAC/Dox-bearing mice. Nonadherent SPMC derived 14 days following in vivo CuNG treatment of EAC/Dox-bearing animals showed no significant rapid cytotoxicity in vitro compared with nonadherent (NA) splenocytes of untreated EAC/Dox-bearing animals, with EAC/Dox cells from untreated animals as target.

$^{51}$Cr-labeled targets (EAC/Dox cells from untreated or doxorubicin-treated mice) in round-bottomed 96-well plates at a different E:T (12:1, 25:1, and 50:1) for 4 hours. After 4 hours of incubation with effectors, 100 μl cell-free culture supernatant was collected and counted in triplicates in liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument, Meridien, CT). Specific lysis was calculated according to the formula: % specific lysis = [([spontaneous release] - [maximum release - spontaneous release]) / (maximum release)] × 100, where spontaneous release represents basal count of cell-free culture supernatant of target cells in the absence of effector cells and maximum release (i.e., complete lysis) represents count of culture supernatant of target cells following their lysis with 10% (v/v) Triton X-100.

Statistical analysis. Each experiment was done three to five times and results are expressed as mean ± SE or Student's t test for significance was done and P < 0.01 was considered significant. Flow cytometric and fluorescence microscopic data show representative data of at least three independent experiments.

Results

CuNG treatment resolves both EAC/Dox and S180/Dox. It has been reported earlier that serum copper concentration increases, whereas tissue copper concentration, especially that of liver, decreases in mice bearing drug-resistant cancers (42). Hence, we decided to administer a chelate of Cu(II) [i.e., Cu(II) chelated to an organic backbone] through i.m. route, which can release copper in tissues of mice bearing drug-resistant cancers. The i.m. route was found to be more effective than i.v. or i.p. routes (data not shown).

Intramuscular administration of CuNG alone elevated tissue copper concentration (data not shown) and interestingly enough was observed to increase the survivability of peritoneal EAC/Dox-carrying mice (Fig. 1). It also increased the longevity of S180/Dox-bearing mice beyond 6 months (data not shown). Moreover, CuNG could reduce the loads of EAC/Dox (Table 2) by >99% (P < 0.001) by 21 days after treatment as well as the size of muscular S180/Dox tumor (Table 2). Actually, CuNG could resolve both doxorubicin-resistant carcinoma and sarcoma. Interestingly, CuNG increased urea, serum alanine aminotransferase, and aspartate aminotransferase (which are alarmingly lowered in EAC/Dox-bearing mice) to near normal level (data not shown) at this dose, and within 24 hours, the serum level of copper was ≤0.5 μg/mL (data not shown).

In vivo but not in vitro treatment of CuNG induced apoptosis of EAC/Dox cells. Because apoptosis is usually the preferred mode of elimination of cancer cells to avoid toxicity (43), we investigated whether the mechanism of killing of EAC/Dox cells by in vivo administration of CuNG was due to apoptosis. CuNG treatment in vivo increased the sub-G0-G1 population as observed by flow cytometric analysis of cell cycle (Fig. 2A, a). To better understand the nature of cell death, we used a double-labeling technique involving Annexin V-Fluos and PI. Our flow cytometric data revealed that in comparison with EAC/Dox cells derived from untreated mice, unfixed EAC/Dox cells from CuNG-treated mice showed Annexin V-FITC binding but negligible (<0.001%) PI staining (Fig. 2A, b). Nuclear fragmentation was observed with confocal microscopy (Fig. 2A, c). For further confirmation of apoptosis, we did terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. Appreciable increase in number of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–positive cells were observed in EAC/Dox cell population from CuNG-treated mice compared with that from untreated mice (Fig. 2A, d). These results together indicated that CuNG-induced death of cancerous cells was through apoptosis. Moreover, the sub-G0-G1
peaks of cell cycle analyses of EAC/Dox cells from untreated or CuNG-treated mice (Fig. 2A, a) well represented the extents of apoptosis in these cell populations. Interestingly, administration of CuNG (2.5 μg Cu/mL; i.e., 0.5 μg Cu/mL) in culture of EAC/Dox cells failed to inhibit their proliferation (Fig. 2B) or increase significantly their sub-G₀-G₁ population (Fig. 2C), indicating that CuNG could not directly kill doxorubicin-resistant cells in vitro.

CuNG treatment partially reversed cancer-induced immuno-suppression. To test the possibility of immune involvement in CuNG action, we undertook studies on lymphoproliferation in response to nonspecific mitogenic stimulations in vitro following CuNG treatment. EAC/Dox-bearing mice manifested severe suppression of lymphoproliferative response toward stimulation with a combination of PMA and ionomycin (PMA + ionomycin) or concanavalin A. Treatment with CuNG (i.m.) gradually restored the lymphoproliferative response of both SPMC (85.2% compared with normal values; \( P < 0.001 \)) and that of lymph node cells to a large extent toward stimulation with PMA plus ionomycin, which are membrane permeable and can directly activate the protein kinase C pathway. However, restoration of the lymphoproliferative response of SPMC and lymph node cells toward concanavalin A stimulation was only partial but significant (\( P < 0.005 \); Fig. 3A).

To address the issue of involvement of immune cells in CuNG-mediated apoptotic death of EAC/Dox cells, we cultured nonadherent SPMC from CuNG-treated mice with EAC/Dox cells. Coculture of nonadherent splenocytes from CuNG-treated EAC/Dox-bearing mice with EAC/Dox cells from untreated animals induced extensive increase in sub-G₀-G₁ population of the cancer cells (Fig. 3B).

To determine whether CuNG could activate cytotoxic cells, in vivo cytotoxicity assay with nonadherent SPMC derived from CuNG-treated (i.m.) EAC/Dox-bearing mice was done. Interestingly, our results showed that CuNG could not elicit appreciable levels of cell-mediated cytotoxicity against EAC/Dox cells for their rapid lysis (Fig. 3C).

CuNG treatment in vivo or in vitro could induce release of proapoptotic factors by SPMC. Because CuNG failed to induce cell-mediated cytotoxicity, we investigated whether CuNG could induce SPMC to secrete any proapoptotic factors. Cell-free culture supernatant of SPMC derived from CuNG-treated EAC/Dox-bearing mice was observed to induce apoptosis of EAC/Dox cells in vitro (Fig. 4A). This prompted us to investigate whether CuNG treatment of SPMC in vitro could also induce similar effect.

It was observed that culture supernatant of SPMC derived from EAC/Dox-bearing mice treated with CuNG in vitro inhibited proliferation of EAC/Dox cells obtained from untreated mice (Fig. 4B) and induced apoptosis of those cancer cells as evident from increased sub-G₀-G₁ population and nuclear fragmentation (Fig. 4B, insets).

CuNG treatment induced generation of the apoptogenic cytokines IFN-γ and TNF-α. Earlier studies showed that IFN-γ and TNF-α could induce apoptosis of different cancer cells (27, 31). Because CuNG treatment in vivo (or in vitro) could induce generation and release of proapoptotic factors from SPMC, we have checked for the presence of the above cytokines in culture supernatant of SPMC treated with CuNG in vivo or in vitro.

CuNG treatment in vivo of EAC/Dox-bearing mice was found to induce their SPMC to release high levels of IFN-γ (\( \sim 4.5 \)-fold compared with untreated control) and moderate levels of TNF-α even after 21 days following treatment (Fig. 5A). SPMC from untreated EAC/Dox-bearing mice also released appreciable levels of IFN-γ (\( \sim 7.5 \)-fold compared with untreated control) and moderate levels of TNF-α 60 hours following in vitro treatment with CuNG compared with those released by their in vitro untreated counterparts (Fig. 5A).

As CuNG treatment in vivo or in vitro induced SPMC from EAC/Dox-bearing mice to release IFN-γ and TNF-α, we investigated whether neutralization of these cytokines could
inhibit CuNG-induced apoptosis of EAC/Dox cells. Results presented in Fig. 5B show that rIFN-γ and/or rTNF-α could induce nuclear fragmentation and apoptosis of EAC/Dox cells. On the other hand, treatment of culture supernatant of SPMC from CuNG-treated EAC/Dox-bearing mice (21 days after treatment) with neutralizing concentrations of anti-IFN-γ and/or anti-TNF-α drastically reduced its ability to induce nuclear fragmentation and apoptosis of EAC/Dox cells from untreated animals.

CuNG treatment reduced T regulatory population and increased IFN-γ-producing CD4+ and CD8+ population. Because CuNG treatment induced SPMC to produce IFN-γ (Fig. 5A)

**Fig. 5.** A, estimation of IFN-γ and TNF-α released in supernatants of 60-hour cultures of SPMC obtained from EAC/Dox-bearing mice treated either *in vivo* (21 days after treatment) or *in vitro* with CuNG. In case of *in vitro* CuNG treatment, SPMC was derived from untreated EAC/Dox-bearing mice. Results were compared with IFN-γ and TNF-α released by SPMC from untreated EAC/Dox-bearing mice. B, effect of IFN-γ and TNF-α on induction of nuclear fragmentation. Induction of nuclear fragmentation by rIFN-γ and/or rTNF-α treatment *in vitro* of EAC/Dox cells derived from untreated mice and suppressive effect of anti-IFN-γ and/or anti-TNF-α treatment on induction of nuclear fragmentation *in vitro* of EAC/Dox cells obtained from untreated mice by culture supernatants of SPMC from CuNG-treated EAC/Dox-bearing mice. Left, flow cytometric data of cells in sub-G0-G1 phase (M1) of cell cycle. M2, G0-G1 (i.e., 2N) phase. Right, corresponding fluorescence microscopic data.
and because IFN-γ level was also observed to be increased in the ascitic fluid of CuNG-treated EAC/Dox-bearing mice (data not shown), we were interested to see whether CuNG treatment increased infiltration of IFN-γ-producing CD4+ and/or CD8+ cells in the tumor site. Results presented in Fig. 6A show that presence of much higher number of CD4+ (~2.5-fold with respect to untreated control) and CD8+ (~3.5-fold with respect to untreated control) cells in ascitic fluid of in vivo CuNG-treated EAC/Dox-bearing mice compared with that in ascitic fluid from their untreated counterparts. Moreover, these cells in ascitic fluid of in vivo CuNG-treated EAC/Dox-bearing mice were observed to produce IFN-γ even without any in vitro stimulation (e.g., with anti-CD3; Fig. 6A). In fact, the number of CD4+ and CD8+ cells decreased drastically in nonadherent population of SPMC and PBMC from untreated EAC/Dox-bearing mice compared with that in nonadherent SPMC and PBMC from their normal counterparts. Following CuNG treatment in vivo, this number not only increased substantially, but the cells were also found to produce IFN-γ even without in vitro stimulation (e.g., with anti-CD3) (data not shown). The number of IFN-γ-producing CD4+ and CD8+ cells was also observed to increase in draining lymph nodes (mesenteric) following CuNG treatment in vivo (data not shown). Interestingly, a sizable proportion of CD4+ cells of ascitic fluid of untreated EAC/Dox-bearing mice exhibited T regulatory markers (CD25 and Foxp3), whereas only a few CD25+/Foxp3+ cells were observed in ascitic fluid of in vivo CuNG-treated EAC/Dox-bearing mice (Fig. 6B).

Apoptosis of CEM/ADR5000 cells by cytokines released by in vitro CuNG-treated PBMC from patients could be blocked by anti-IFN-γ. Because CuNG treatment in vitro could induce SPMC from EAC/Dox-bearing mice to release the apoptogenic cytokines, IFN-γ and TNF-α, we wanted to see whether CuNG treatment could also activate PBMC from patients to release similar proapoptotic factors.

Results presented in Fig. 7 show that cell-free supernatants of 48-hour cultures of CuNG-treated PBMC from patients contained high levels of IFN-γ compared with their CuNG untreated counterparts. Cell-free culture supernatant of CuNG-treated PBMC could induce extensive apoptosis, marked by increase in sub-G0-G1 population as well as nuclear fragmentation of doxorubicin-resistant CEM/ADR5000 cells in 48 hours, whereas those from corresponding untreated cultures could not (Fig. 8). Interestingly enough, anti-IFN-γ treatment could almost completely inhibit the apoptogenic effect of culture supernatants of CuNG-treated PBMC from patients (Fig. 8).
Discussion

Generally, there are two ways to combat drug resistance. One is to develop newer drugs and the other is to use a resistance modifier along with existing drugs. However, neither development of a new drug ensures that resistance against it shall not be achieved quickly nor characterization of resistance markers is always possible in clinical cases for determination of an effective resistance modifier often due to involvement of heterogeneous factors. Moreover, no resistance modifier has yet emerged that is proven to be highly effective clinically. Because the immune system plays an immense role in the progression as well as resolution of cancer, immunotherapy is now being considered to be the most promising strategy. The beauty of immune-based therapies lies in the fact that these represent a non-cross-resistant treatment strategy with high specificity (44).
Schiff’s base-forming compounds have been reported to be good immunopotentiators that expand bystander T cells in the absence of antigen-presenting cells (45), whereas copper coordination complexes have been found to have excellent antiviral properties with negligible toxicity (46). Because copper homeostasis is altered in drug-resistant cancers (42), we had developed the Cu(II) chelate of Schiff’s base (CuNG; refs. 29, 30, 47). In the current study, we have shown that single i.m. administration of CuNG alone could resolve doxorubicin-resistant cancers via induction of host protective cytokine-mediated apoptosis.

Most of the available drugs induce apoptosis of cancer cells both in vitro and in vivo. Interestingly, CuNG failed to induce apoptosis of doxorubicin-resistant cancer cells in vitro but could do so in vivo. This indicated that CuNG might use immune system to induce apoptosis of drug-resistant cancer cells in vivo. Cancer-mediated immunotolerance and immunosuppression is a common phenomenon related to cancer progression (48, 49). Interestingly, CuNG was found to induce gradual reversal of immunosuppression as evidenced by restoration of lymphoproliferative response. Although the involvement of cytotoxic T cells and natural killer cells in induction of apoptosis of cancer cells is well documented (50–52), no perceptible direct cell-mediated cytotoxic response against EAC/Dox in CuNG-treated mice was observed. However, CuNG treatment in vitro or in vivo was found to stimulate SPMC from EAC/Dox-bearing mice to secrete IFN-γ and TNF-α, which are well reported to induce apoptosis of cancer cells (27, 53, 54). Further, it has been shown that these two cytokines were involved in induction of apoptosis of EAC/Dox cells as evidenced by almost complete suppression of the apoptogenic property of the culture supernatants of SPMC from in vitro CuNG-treated EAC/Dox-bearing mice by neutralization of these cytokines with their respective neutralizing antibodies. CuNG treatment in vitro was also found to induce generation of IFN-γ from PBMC of patients resistant to various chemotherapeutics as well as radiotherapy. It was further shown that this IFN-γ could induce apoptosis of MDR1-overexpressing CEM/ARD5000 cells as evidenced by almost complete suppression of the apoptogenic property of the culture supernatants of in vitro CuNG-treated PBMC derived from patients by neutralizing antibody against IFN-γ.

Immunotolerance of T cells, as well as immunosuppression, especially inhibition of IFN-γ production and type 1 response, has been shown to occur in the tumor microenvironment (55, 56). IFN-γ has been shown to reverse T-cell tolerance (56, 57) and administration of IFN-γ helps to sensitize tumors toward radiation therapy (58). Infiltration of T regulatory cells at the tumor site have been shown to cause T-cell tolerance and inhibition of secretion of apoptogenic cytokines, such as IFN-γ and TNF-α (59). Interestingly, we also found a sizeable number of T cells expressing T regulatory markers and very low percentage IFN-γ-producing T cells in ascitic tumor site of untreated mice. CuNG treatment in vivo increased the number of IFN-γ-producing CD4+ and CD8+ cells but decreased the number T regulatory marker-expressing T cells in ascitic tumor site. Again, TNF-α has been reported to help in elimination of malignant glioma cells (60) and combination of IFN-γ and TNF-α has been reported to synergistically reduce suppressor cell activity during metastatic Lewis lung carcinoma (61), which might also help in reducing tolerance. Because a more or less steady-state copper level was maintained in sera of animals for several days following single administration with CuNG (i.m.; data not shown), it seems that the drug remained in circulation for longer period. This perhaps ensured a high level of IFN-γ release at least up to 21 days after administration through increase in IFN-γ-producing CD4+ and CD8+ cells and a moderate level of TNF-α release. These cytokines were mainly responsible for the elimination of drug-resistant cancer cells. Interestingly, apoptogenic cytokines induced by CuNG treatment could cause apoptosis of MRP1-overexpressing (EAC/Dox) as well as MDR1-overexpressing (CEM/ADR5000) cancer cells. Thus, CuNG-mediated up-regulation of IFN-γ and subsequent apoptosis of tumor cells bypasses MDR phenotype, which indicates that this novel copper chelate can be used clinically for immunotherapy of different types of drug-resistant cancers.

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