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Antiangiogenic and Antitumoral Activity of Phenyl-3-(2-Chloroethyl)Ureas: A Class of Soft Alkylating Agents Disrupting Microtubules That Are Unaffected by Cell Adhesion-Mediated Drug Resistance

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ABSTRACT

The development of new anticancer agents with lower toxicity, higher therapeutic index, and weaker tendency to induce resistant phenotypes in tumor cells is a continuous challenge for the scientific community. Toward that end, we showed previously that a new class of soft alkylating agents designed as phenyl-3-(2-chloroethyl)ureas (CEUs) inhibits tumor cell growth *in vitro* and that their efficiency is not altered by clinically relevant mechanisms of resistance such as overexpression of multidrug resistance proteins, increase in intracellular concentration of glutathione and/or glutathione *S*-transferase activity, alteration of topoisomerase II, and increased DNA repair. Mechanistic studies have showed recently that the cytotoxic activity of several CEUs was mainly related to the disruption of microtubules. Here, we present results supporting our assumption that 4-tert-butyl-[3-(2-chloroethyl)ureido]phenyl (tBCEU) (and its bioisosteric derivative 4-iodo-[3-(2-chloroethyl)ureido]phenyl (ICEU) are potent antimicrotubule agents both *in vitro* and *in vivo*. They covalently bind to β-tubulin, leading to a microtubule depolymerization phenotype, consequently disrupting the actin cytoskeleton and altering the nuclear morphology. Accordingly, tBCEU and ICEU also inhibited the migration and proliferation of endothelial and tumor cells *in vitro* in a dose-dependent manner. It is noteworthy that ICEU efficiently blocked angiogenesis and tumor growth in three distinct animal models: (*a*) the Matrigel plug angiogenesis assay; (*b*) the CT-26 tumor growth assay in mice; and (*c*) the chick chorioallantoic membrane tumor assay. In addition, we present evidence that CEU cytotoxicity is unaffected by additional resistance mechanisms impeding tumor response to DNA alkylating agents such as cisplatin, namely the cell adhesion mediated-drug resistance mechanism, which failed to influence the cytocidal activity of CEUs. On the basis of the apparent innocuousness of CEUs, on their ability to circumvent many classical and recently described tumor cell resistance mechanisms, and on their specific biodistribution to organs of the gastrointestinal tract, our results suggest that CEUs represent a promising new class of anticancer agents.

INTRODUCTION

Phenyl-3-(2-chloroethyl)ureas (CEU) are members of a class of therapeutic agents that we have developed and studied for the past 15 years. Structurally, CEUs are constituted of the aromatic moiety of nitrogen mustards, such as chlorambucil, and the non-nitrosated pharmacophore of aliphatic nitrosoureas, such as carmustine (1, 2). The cytotoxicity of many CEUs was demonstrated on several tumor cell lines (3) and was shown to be significantly higher than chlorambucil and carmustine. In addition, we also found that several CEUs were not mutagenic in the Ames test (2) and that 4-tert-butyl-[3-(2-chloroethyl) ureido]phenyl (tBCEU; Fig. 1*A*) did not display any differential cytotoxicity in a panel of cell lines that had acquired resistance to several conventional chemotherapeutic agents (4). It is noteworthy that several CEUs were shown to disrupt microtubule assembly leading to a profound effect on cell cycle and on cell death (5). Mechanistic studies using carbon-14-labeled tBCEU (6, 7) clearly established the irreversible binding of CEUs to cytosolic proteins through an alkylation mechanism (5). In these experiments, we identified that β-tubulin was one of the proteins putatively involved in the antimicrotubule effect observed when using tBCEU (5). Interestingly, tBCEU was shown to alkylate Cys^{239} of β-tubulin,[5] leading to inactivation of the protein function (5) without triggering the synthesis of new β-tubulin in MDA-MB-231 cells (8). Interestingly, biopharmaceutical evaluation in mice has shown that tBCEU was distributed preferentially into organs of the gastrointestinal tract, such as liver, colon, stomach, and duodenum (9), supporting a promising approach for the chemotherapy of these resilient cancers and their common sites of tumor metastasis.

In recent years, several other antimicrotubule agents, including paclitaxel and *Vinca* alkaloids such as vinblastine and vincristine, have been shown to act as potent antiangiogenic agents, impeding tumor growth (10, 11). Angiogenesis is recognized as an important step during carcinogenesis and occurs in many physiological processes. The growth and metastasis of solid tumors is dependent indeed on neovascularization spreading into target organs. The process of angiogenesis is complex and is regulated through the synergistic activities of intracellular signal transduction pathways, stimulated by many soluble angiogenic factors, including ligation of integrins and components of the extracellular matrix (ECM). For angiogenesis to occur, therefore, requires the migration and adhesion of endothelial and smooth muscle cells, paralleled with the degradation of ECM, undergoing tissue remodeling and new blood vessel formation.

Integrins are cell-membrane receptors that bind to ECM proteins. They are involved in the intracellular signaling of cell adhesion and migration (12) during angiogenic and metastatic processes. During tumorigenesis, it was confirmed that several components of the ECM accumulated in the vicinity of most tumors in their native but also proteolyzed forms, thus, potentially helping the tumor cell survival by mediating integrin signaling (13). Interestingly, the first author of this paper and others have demonstrated recently that ECM proteins could modulate integrin-mediated tumor cell motility and impede their ability to undergo cell death through either necrosis or apoptosis (13, 14). This prosurvival signal, initiated by the integrin family, was

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[5] Unpublished observations.

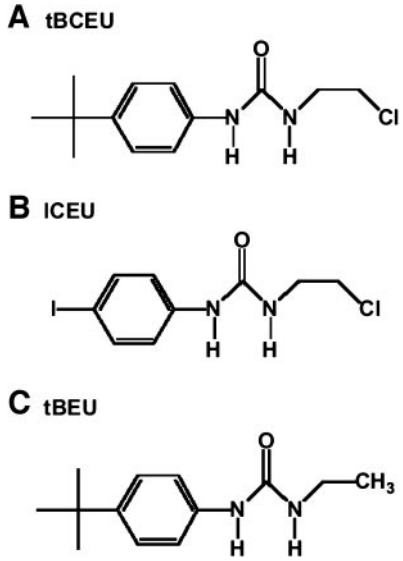

Fig. 1. Chemical structures of 4-tert-butyl-[3-(2-chloroethyl)ureido]phenyl (*A*), 4-iodo-[3-(2-chloroethyl)ureido]phenyl (*B*), and 4-tert-butyl-[3-(2-ethyl)ureido]phenyl (*C*). *tBCEU*, 4-tert-butyl-[3-(2-chloroethyl)ureido]phenyl; *ICEU*, 4-iodo-[3-(2-chloroethyl)ureido]phenyl; *tBEU*, 4-tert-butyl-[3-(2-ethyl)ureido]phenyl.

additionally shown to conduct to a cell cycle arrest in G_1-S phase (15). In an elegant series of studies, it has been suggested recently that this integrin-dependent prosurvival mechanism was critical in tumor cells that were challenged by cytotoxic agents, such as cisplatin (cDDP), thus, defining a new cell adhesion mediated-drug resistance (CAM-DR) mechanism (15–18).

In the present study, we first demonstrate the potency of different soft alkylating CEUs to affect cell growth and β-tubulin integrity by comparing their effect with well-known antimicrotubule agents such as *Vinca* alkaloids, colchicine, and paclitaxel. We also evaluated the antiangiogenic and antitumoral activity of prototypal CEUs, such as tBCEU, its bioisostere 4-iodo-[3-(2-ethyl)ureido]phenyl (ICEU; Fig. 1*B*), and their analogous but nonalkylating derivative, 4-tert-butyl-[3-(2-ethyl)ureido]phenyl (tBEU; Fig. 1*C*), in different *in vitro* and *in vivo* models. Using human umbilical vascular endothelial cells (HUVEC) and various tumor cell lines in a cell growth inhibition assay and migration chamber, we demonstrated a dose-dependent effect of tBCEU and ICEU on the migration and the proliferation of tumor and endothelial cells *in vitro*. Interestingly, the nonalkylating tBEU was noncytotoxic and also ineffective in all of these models even at high doses. We finally show that the ECM had no protective effect on the tumor cell death mediated by CEUs, although it protected the tumor cells against the cDDP cytotoxicity. Altogether, these results suggest that CEUs are interesting, new antiangiogenic and antitumor agents that may lead to more selective and less toxic antineoplastic therapy.

MATERIALS AND METHODS

Reagents and Chemicals. The cDDP and 4′,6-diamidino-2-phenylindole were purchased from Sigma (St. Louis, MO). The tBCEU and ICEU were prepared as published previously (1, 3, 4). The tBCEU, ICEU, and tBEU were kindly provided by Dr. Jean Rousseau from IMOTEP Inc. (Quebec City, Quebec, Canada). All of the drugs were dissolved in DMSO, and the final concentration of DMSO in the culture medium was maintained under 0.5% (v/v). Vinblastine sulfate, colchicine, and paclitaxel were purchased from Sigma.

Cell Lines and Tissue Culture. HUVECs were obtained from the institution of our neonatal unit and isolated as described previously (19). HUVECs were maintained in a gelatin-coated 75-cm^2 flask in M199 (Invitrogen, Burlington, Ontario, Canada), supplemented with 20% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml basic fibroblast growth factor (bFGF; Invitrogen), and 5 units/ml heparin (Invitrogen). Dr. David A. Cheresh (The Scripps Research Institute, San Diego, CA) and Dr. Caroline Damsky (University of California, San Francisco, CA) kindly provided M21 human melanoma cells and hamster CS1 melanoma cells, respectively. HT1080 human fibrosarcoma cells were purchased from American Type Culture Collection (Bethesda, MD) and MDA-MB-231 from the American Type Culture Collection (Manassas, VA). These cells were cultured in DMEM containing $NaHCO_3$ (2.2 g/liter), glucose (4.5 g/liter), streptomycin sulfate A (100 μg/ml), penicillin G (100 units), and glutamine (292 μg/ml), supplemented with 5% fetal bovine serum for M21 and MDA-MB-231 cells or with 10% fetal bovine serum for CS1 and HT1080 cells. Murine colon CT-26 tumor cells were kindly provided by Dr. Isaiah J. Fidler (MD Anderson Cancer Center, University of Texas, Houston, TX) and were maintained as a monolayer culture in MEM, 1% solution of vitamins, 1% sodium pyruvate, 1% nonessential amino acids, and 0.04% gentamicin base (MEM; Life Technologies, Inc., Paisley, Scotland), supplemented with 10% FCS (Sigma-Aldrich, France). All of the cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Growth Inhibition Assays. Tumor cell lines were inoculated into 96-well tissue culture plates in 100 μl containing 2×10^3 cells and incubated at 37°C. After 24 h, freshly solubilized drugs in DMSO were diluted in fresh medium. Aliquots of 100 μl containing escalating concentration of drugs (0.3 μM to 100 μM) were added to the appropriate microtiter wells already containing 100 μl of culture medium. The cells were incubated for different periods of time ranging from 3 h to 48 h. The supernatant was removed, and the cells were washed and incubated with fresh medium to complete the total incubation time to 48 h for each condition. Assays were stopped by addition of cold trichloracetic acid (TCA) to the wells (final concentration, 10%) followed by their incubation for 60 min at 4°C. The supernatant was discarded, and the plates were washed five times with tap water and air-dried. Sulforhodamine B solution (50 μl) at 0.1% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 15 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid, and the plates were air-dried. Bound stain was solubilized with 10 mM Trizma base, and the absorbance was read using a μQuant Universal Microplate Spectrophotometer (Biotek, Winooski, VT) at 585 nm. The results were compared with those of a control reference plate fixed on the treatment day, and the growth inhibition percentage was calculated for each drug contact period.

Morphological Analysis of Microtubules, Actin Cytoskeleton, and Nucleus by Immunocytochemistry. Cells were seeded at 1×10^5 cells in 35-mm Petri dishes and incubated for 16 h at 37°C. After treatments with either CEUs or classical antimicrotubule agents, the cells were washed twice with PBS (pH 7.4) and then fixed with 3.7% formaldehyde in PBS for 20 min. After two washes, the cells were permeabilized and blocked with 0.1% saponin and 3% (w/v) BSA in PBS during 1 h at 37°C. The cells were then additionally incubated during 1 h at 37°C with antitubulin [clone TUB2.1, which is specific to β-tubulin and does not cross-react with other tubulin isoforms (Sigma-Aldrich, St. Louis, MO); 1:200] in 0.1% saponin and 3% BSA in PBS. The cells were washed three times with PBS containing 0.05% of Tween 20 and incubated 1 h at 37°C in blocking buffer containing antimouse IgG Alexa-488 (1:1000), 4′,6-diamidino-2-phenylindole (2.5 μg/ml in PBS) to stain nuclei, and rhodamine-labeled phalloidin (1:600) to stain the actin cytoskeleton. The observations were made using a Nikon Eclipse E800 microscope (Tokyo, Japan) equipped with a $\times$40 objective. Images were captured as a 16-bit tagged image file format files with a Hamamatsu ORCA ER cooled (−20°C) digital camera (Photonics Management Management Corp., Bridgewater, NJ) driven by SimplePCI AIC software (Compix Inc. C Imaging Systems, Cranberry Township, PA).

Western Blot Analysis of β-Tubulin Monomer. Cells were seeded at 5×10^5 cells in six-well plates and incubated for 16 h. After treatments with tested drugs, floating and adherent M21 cells or MDA-MB-231 cells were washed in ice-cold PBS, pooled, and then solubilized in buffer containing 62.5 mM Tris (pH 6.8), 2% SDS, 6 M urea, 10% glycerol, 0.00125% bromphenol blue, and 720 mM β-mercaptoethanol. The cell extracts were boiled for 5 min, separated on 10% SDS-PAGE electrophoresis gel, and transferred onto nitrocellulose membrane. The membranes were blocked for 1 h at 37°C with 5% (w/v) milk in Tris-buffered saline containing 0.1% Tween 20 and then incu-

bated 1 h at 37°C with the appropriate antibody diluted in 5% milk in Tris-buffered saline containing 0.1% Tween 20. The apparition of an additional immunoreactive band of β-tubulin was evaluated with the monoclonal antitubulin antibody (1:500). Membranes were incubated with a horseradish peroxidase-conjugated goat antimouse IgG secondary antibody (1:2500; Amersham Canada, Oakville, Ontario, Canada), diluted in 5% milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature, followed by chemiluminescent detection using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Chemotaxis Assay. The chemotaxis motility of HT1080 was assessed using Boyden chambers. Briefly, the underside of transwell migration chamber membranes (8-μm pore size) were coated with collagen IV as described previously (20, 21) and as modified by Petitclerc *et al.* (14). The cells were preincubated or not for 16 h with escalating concentrations of different drugs, then they were added to the top of the transwell in DMEM containing 0.1% BSA in the presence of drugs. Soluble fibronectin (25 μg/ml) was added to the lower chamber to induce chemotaxis. The cells were allowed to migrate for 4–6 h at 37°C, fixed, and stained for quantification. The number of migrating cells per well was counted. The results expressed the mean ± SE of triplicates.

Matrigel Plug Angiogenesis Assay in Mice. The experimental protocol was conducted as described previously (22–24). Briefly, the liquid Matrigel (Becton Dickinson, Bedford, MA) was kept on ice until the injection to avoid gelling of the colloid. The Matrigel solution was either loaded with bFGF (250 ng/ml) to induce angiogenesis or left unloaded to be used as negative controls. Five hundred μl of cold Matrigel were injected s.c. on the ventral line of mice. After the injection, the Matrigel preparation was allowed to polymerize for 5 min. The drugs [50 and 100 mg/kg in dimethylacetamide (DMA), transcutol, chremophor, and Tween 20 (1:3:3:3)] were then administered i.p. according to the method described by Stefansson *et al.* (24). After 5–7 days, the mice were euthanized by CO_2 asphyxiation, the Matrigel plugs were excised, and only those that did not show any sign of hematoma were solubilized in PBS-Triton X-100 (0.1%). The hemoglobin content of the Matrigel plugs was compared with a hemoglobin standard curve (405 nm) to evaluate the level of vascularization (24).

Chorioallantoic Membrane (CAM) Tumor Assay. Human HT1080 fibrosarcoma and hamster CS1 melanoma cell lines were used to assess the antitumoral activity of CEUs in the chick chorioallantoic assay (14, 25, 26). In brief, day-0 fertilized chicken eggs were purchased from Couvoirs Victoriaville (Victoriaville, Quebec, Canada). The eggs were incubated for 10 days in a Pro-FI egg incubator fitted with an automatic egg turner before being transferred to a Roll-X static incubator for the rest of the incubation time (incubators were purchased from Lyon Electric, Chula Vista, San Diego, CA). The eggs were kept at 37°C in a 60% humidity atmosphere for the whole incubation period. Using a hobby drill (Dremel, Racine, WI), a hole was drilled on the side of the egg, and a negative pressure was applied to create a new air sac. A window was opened on this new air sac and was covered with transparent adhesive tape to prevent contamination. A freshly prepared cell suspension (40 μl) of either HT1080 (3.5×10^5 cells/egg) or CS1 (5×10^6 cells/egg) cells was applied directly on the freshly exposed CAM tissue through the window. On day 11, the tested drugs were injected i.v. in 10–12 eggs in a small volume (100 μl). The eggs were incubated until day 17, at which time the embryos were euthanized at 4°C followed by decapitation. Tumors were collected, pictures were taken to illustrate the different groups, and the tumor-wet weights were recorded. In all of the experiments, the number of dead embryos from the different groups was monitored for any sign of toxicity.

Harvesting and Grafting of CT-26 Tumor Cells onto BALB/c Mice. Growing CT-26 cells were harvested from subconfluent cultures by a 2-min trypsinization treatment using trypsin-EDTA (Life Technologies, Inc.). After trypsinization, which was stopped by MEM addition, the cells were centrifuged, washed once, and resuspended in PBS. The tumor cells were grafted onto mice by s.c. injection into the right flank of mice of a suspension of 2.5×10^5 cells in 100 μl of PBS. The mice were then randomized into various treated and untreated groups. The ICEU was dissolved 30 min in a mixture of Labrafils M1944 Cs (Gattefossé, France), dimethylacetamide, and Tween 80 (Sigma-Aldrich; 89:9:1%, v/v) before its i.p. injection (13 mg/kg) into mice at days 1, 5, and 9. The 5-fluorouracil (5-FU; Fluorouracile, TEVA) was dissolved in PBS to allow the administration of 20 mg/kg in 150 μl of PBS at days 7–11. BALB/c male mice 6 weeks old were purchased from the Charles River Company (Lyon, France). Mice were handled and cared for in accordance with the Guide for the Care's and Use of Laboratory Animals and the European Directive EEC/86/809. The experiments were carried out under the supervision of authorized investigators in accordance with the general guidelines established for the preclinical toxicology of new anticancer agents (27). The calculated tumor weight (CTW) of each tumor was estimated from two-dimensional measurements performed on days 7, 9, 11, and 15 after tumor inoculation with a slide caliper. The CTW was calculated as described by Bissery *et al.* (28) according to the following formula: CTW (mg) = $(L \times W^2)/2$ with L = length in mm and W = width in mm. For each treatment, a group of 18 (controls), 8 (5-FU), and 12 (ICEU) mice were used. The CTW values were averaged within each group. Differences in CTW between treated and untreated groups were analyzed for significance using the Student *t* test. Values of $P < 0.05$ were considered as statistically significant.

Clonogenic Survival of M21 Cells on ECMs. M21 skin melanoma cells were plated (1×10^6 cells in 100-mm Petri dishes) on different ECMs and challenged with CEUs or the strong alkylating agent cDDP as described previously (29). Briefly, native and heat-denatured type IV collagen (50 μg/ml), fibronectin (25 μg/ml), and fibrin (50 μg/ml) were used to coat nontissue culture plates (Nunc). After washes with serum-free DMEM, cells were plated in serum-containing or serum-free media on the different matrices for 16 h. Cells were then challenged with cDDP (50 μM) for 3 h or with CEUs (20 μM) for 24 h. Forthwith, the cells were washed, trypsinized, and plated at appropriate dilutions ranging from 10^2 to 10^5 cells per well. The experiments were conducted in triplicate to have approximately 50–200 viable cells per dish (30). Relative survival was calculated from the number of single cells that formed colonies of ≥50 cells within 12 days. The survival data were corrected for the plating efficiency of the appropriate controls.

RESULTS

CEUs Inhibit the Proliferation of Endothelial and Tumor Cell Lines. We first evaluated CEU cytotoxicity by assessing their effect on the growth of human M21 melanoma and HT1080 fibrosarcoma tumor cell lines and on HUVECs for 3, 6, 12, 24, and 48 h, respectively. Cells were treated with the soft alkylant tBCEU, its bioisosteric derivative, ICEU, and its nonalkylating counterpart, tBEU, with escalating concentrations ranging from 0.3 μM to 100 μM (Fig. 2). The cytotoxicity of these soft alkylating agents was compared with that of a classical and strong alkylating agent, namely cDDP. The antimicrotubule agents colchicine, vinblastin, and paclitaxel were also tested in this assay but were found not cytotoxic until they reach 48 h of exposure (data not shown). When they were in contact for <6 h with either cell lines, virtually none of the tested CEUs showed inhibition of tumor cell growth and proliferation. However, as the time of contact between CEUs and tumor cells was increased from 6 to 48 h, the comparable GI_{50} of tBCEU and ICEU shifted markedly to the left-hand side of the graph (Fig. 2, *A* and *B*), and this was shown in the low μM range for all of the tumor cell lines tested. These were strikingly different from the cDDP effect, which displayed cytotoxicity after only 3 h of treatment, with a GI_{50} that was essentially in the same range for all of the time of contact tested (Fig. 2*D*). Interestingly, at all of the concentrations tested, the nonalkylating tBEU showed no apparent cytotoxicity (Fig. 2*C*), suggesting that the chemical alkylating property of CEUs was essential for their cytotoxicity. Overall, soft alkylating CEUs were as cytotoxic as cDDP. However, they require a longer time of contact to display their proliferative inhibitory activity, which is compatible to the incubation time of other anti-antimicrotubule agents tested, such as colchicine and paclitaxel (data not shown).

Binding of CEUs to β-Tubulin Monomer, CEUs Induces Microtubule Depolymerization and Collapse of Actin Cytoskeleton in Endothelial and Tumor Cells. On the basis of our anterior observation that β-tubulin is the primary pharmacological target of tBCEU (5), we compared the effect of tBCEU, ICEU, and tBEU on the microtubule network by an immunofluorescence staining of β-

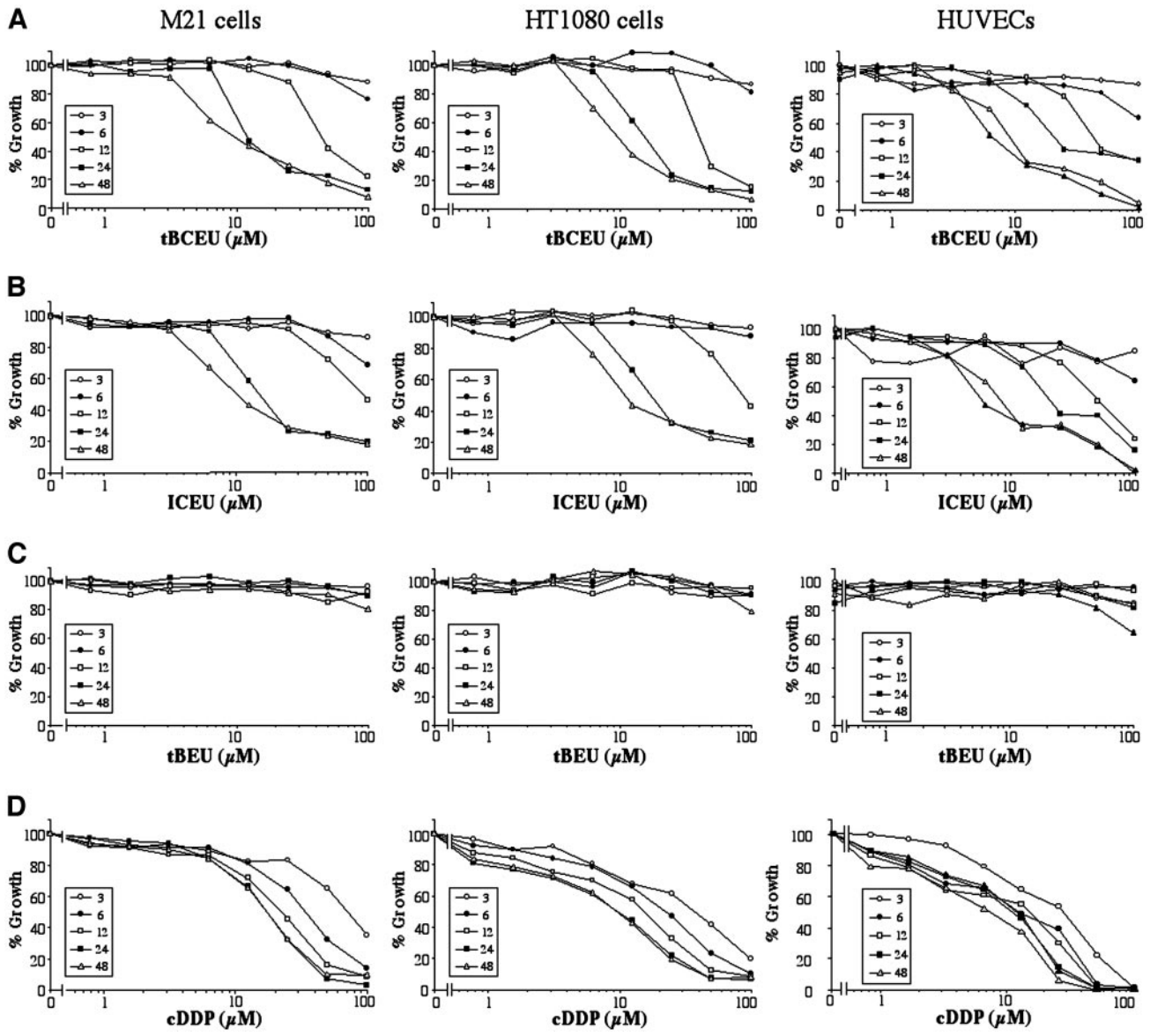

Fig. 2. Inhibition of human umbilical vascular endothelial cells (*HUVEC*) and tumor cell growth by phenyl-3-(2-chloroethyl)ureas in a dose- and time-dependent manner. HUVECs or M21 and HT1080 tumor cells plated in 96-well tissue culture plates were incubated for 3, 6, 12, 24, or 48 h with escalating concentration ranging from 0.3 μM to 100 μM of (*A*) 4-tert-butyl-[3-(2-chloroethyl) ureido] phenyl, (*B*) 4-iodo-[3-(2-chloroethyl)ureido]phenyl, (*C*) 4-tert-butyl-[3-(2-ethyl)ureido]phenyl, and (*D*) cisplatin. After a total incubation time of 48 h, the cells were fixed and stained with sulforhodamine B. The absorbance was read at 585 nm. The growth inhibition percentage is expressed as the mean of triplicates for each drug contact period, compared with those of a control reference plate fixed on the day of the treatment. *tBCEU,* 4-tert-butyl-[3-(2-chloroethyl) ureido] phenyl; *ICEU,* 4-iodo-[3-(2-chloroethyl)ureido]phenyl; *tBEU,* 4-tert-butyl-[3-(2-ethyl)ureido]phenyl; *cDDP,* cisplatin.

tubulin (Fig. 3). We also compared their effect on the apparition of a second immunoreactive band of β-tubulin on SDS-PAGE, reminiscent of β-tubulin alkylation, as described by Legault *et al.* (Ref. 5; Fig. 4). In comparison with untreated cells, 100 μM of tBCEU during 24 h considerably affected the β-tubulin fibers (Fig. 3, *A* and *B*) showing a punctated β-tubulin staining that leads to a microtubule depolymerization phenotype. This was established by a comparison with a 24-h cell exposure to paclitaxel (50 μM), colchicine (25 μM), or vinblastine (5 μM), whereas classical antimicrotubule agents were having an opposite effect on the microtubule network by their noncovalent binding to β-tubulin. Paclitaxel stabilizes the microtubules, thus, inhibiting their depolymerization, whereas the others were blocking their polymerization, therefore, inducing a depolymerization phenotype (Fig. 3, *A* and *B*). In fact, the tBCEU effect on β-tubulin was drastically different from what was observed after paclitaxel treatment; the effect was not as severe as vinblastine but rather similar to that observed after colchicine cell exposure. As expected, the bioisosteric derivative ICEU showed a similar microtubule dissolution activity as tBCEU. On the contrary, tBEU did not exhibit any effect on the microtubule network nor did it affect the filamentous structure of actin. However, tBCEU, ICEU, colchicine, and vinblastine decreased considerably the amounts of filamentous actin in both cell lines, presumably as an indirect consequence of β-tubulin depolymerization, showing a more punctate actin distribution. This collapse of the actin structure was not observed in response to paclitaxel. Interestingly, the toxic effect of cDDP on β-tubulin and on actin filaments seemed associated to its proapoptotic mechanism, actin, and microtubules being dissolved in cells only showing a typical apoptotic nuclear fragmentation phenotype (29, 31). Stress fibers were observed in cDDP-treated cells that are still nonapoptotic. This was still contrasting with CEUs that were inducing a nonclassical nuclear condensation phenotype consequently or in parallel to their microtubules

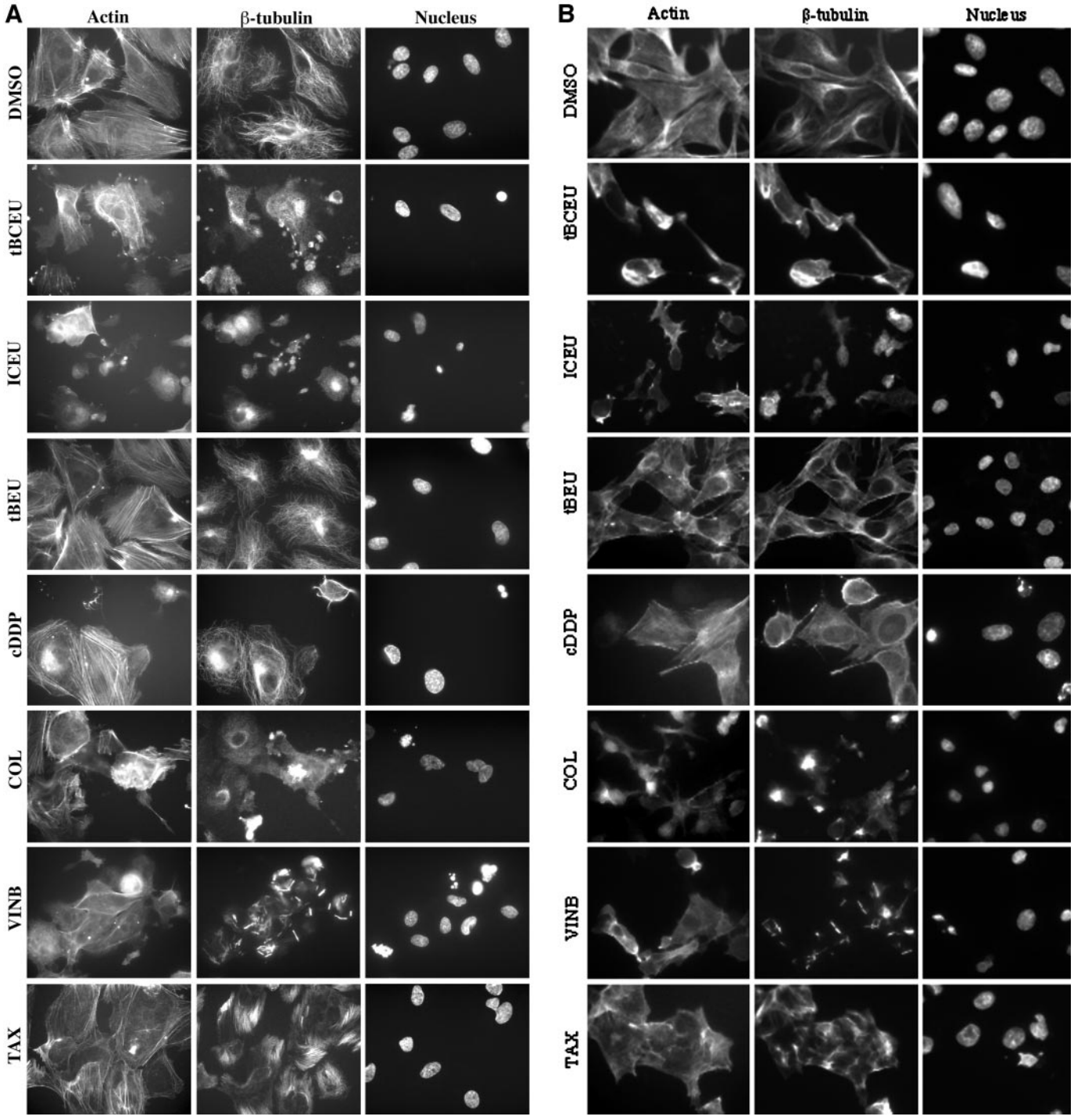

Fig. 3. Microtubule depolymerization and cytoskeleton disruption induced by phenyl-3-(2-chloroethyl)ureas. Human umbilical vascular endothelial cells (*A*) and M21 tumor cells (*B*) plated in 35-mm Petri dishes were treated with 100 μM of 4-tert-butyl-[3-(2-chloroethyl) ureido] phenyl, 4-iodo-[3-(2-chloroethyl)ureido]phenyl, or 4-tert-butyl-[3-(2-ethyl)ureido]phenyl and with 50 μM of cisplatin, 25 μM of colchicine, 5 μM of vinblastine, or 50 μM of paclitaxel for 24 h. β-Tubulin, actin, and nucleus were stained as described in "Materials and Methods" and examined by fluorescence microscopy. Representative fields are shown from three separate experiments. *tBCEU,* 4-tert-butyl-[3-(2-chloroethyl) ureido] phenyl; *ICEU,* 4-iodo-[3-(2-chloroethyl)ureido]phenyl; *tBEU,* 4-tert-butyl-[3-(2-ethyl)ureido]phenyl; *cDDP,* cisplatin; *COL,* colchicine; *VINB,* vinblastine; *TAX,* paclitaxel.

disruption effect. The precise mechanism of this nonclassical nuclear condensation is under investigation.

To confirm that ICEU was as active as tBCEU to bind to β-tubulin, Western blot analysis of β-tubulin was carried out as reported by Legault *et al.* (Ref. 5; see "Materials and Methods"). They initially reported that the cell incubation with [urea-^{14}C]-tBCEU revealed the apparition of a radioactive protein on SDS-PAGE that exactly coincided with a second immunoreactive band of β-tubulin monomer, which was detected by Western blotting, demonstrating the covalent binding of ^{14}C-tBCEU to β-tubulin. Here, we confirmed the time-dependent detection of this additional immunoreactive band in M21 (Fig. 4), HUVECs, and MDA-MB-231 cells (data not shown). In line with this previous study, we show that tBCEU and ICEU generate a band that is presumed to be the result of β-tubulin alkylation (Fig. 4). This second band was, however, not observed after cell exposure to tBEU and cDDP nor after treatment with paclitaxel, colchicine, or

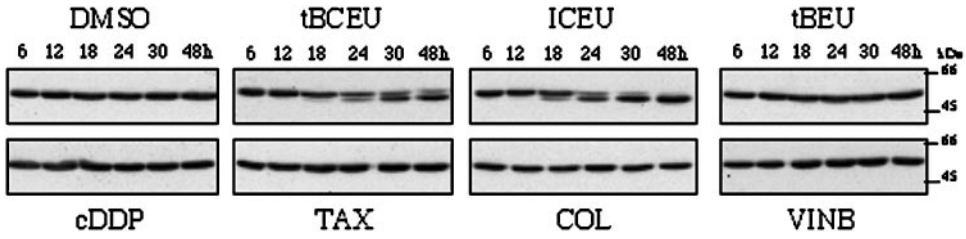

Fig. 4. Generation of an alkylated form of β-tubulin by 4-tert-butyl-[3-(2-chloroethyl) ureido] phenyl and 4-iodo-[3-(2-chloroethyl)ureido]phenyl. M21 cells plated in six-well plates were treated or untreated (DMSO; 0.25%) with 50 μM of 4-tert-butyl-[3-(2-chloroethyl) ureido] phenyl, 4-iodo-[3-(2-chloroethyl)ureido]phenyl, or 4-tert-butyl-[3-(2-ethyl)ureido]phenyl and 50 μM of cisplatin, 25 μM of colchicine, 5 μM of vinblastine, or 50 μM of paclitaxel for 6, 12, 24, 30, or 48 h. After treatment, adherent cells were pooled with floating cells, and proteins were extracted and separated on a 10% SDS-PAGE. The apparition of an additional immunoreactive band of β-tubulin was evaluated by Western blotting with a monoclonal antitubulin antibody as described in "Materials and Methods." The same results were obtained in duplicates using two different cell lines, notably M21 and MDA-MB-231 cells (data not shown). *tBCEU,* 4-tert-butyl-[3-(2-chloroethyl) ureido] phenyl; *ICEU,* 4-iodo-[3-(2-chloroethyl)ureido]phenyl; *tBEU,* 4-tert-butyl-[3-(2-ethyl)ureido]phenyl; *cDDP,* cisplatin; *COL,* colchicine; *VINB,* vinblastine; *TAX,* paclitaxel.

vinblastine, presumably because of their noncovalent binding to β-tubulin. Hence, our results confirmed that tBCEU and its bioisosteric derivative, ICEU, are both potent antimicrotubule and soft alkylating agents, which covalently bind to β-tubulin, inducing a microtubule depolymerization phenotype. The additional immunoreactive band is similar to the one observed with an impressive amount of tumor cell lines, including T-24 bladder carcinoma, LoVo, Caco-2, HT-29, and SW-1417 colon carcinoma cells.[5]

CEUs Abrogate the Motogenic Potential of Endothelial and Tumor Cells. To evaluate whether the CEU-induced microtubule depolymerization and actin cytoskeleton disruption could abrogate tumor cell migration, chemotaxis assays were performed using the highly motile HT1080 and HUVEC cells. The cells were seeded on collagen IV-coated transwell membranes, separating the lower and upper part of Boyden chambers, and chemotaxis was induced by addition of soluble fibronectin to the lower part of the chamber. In

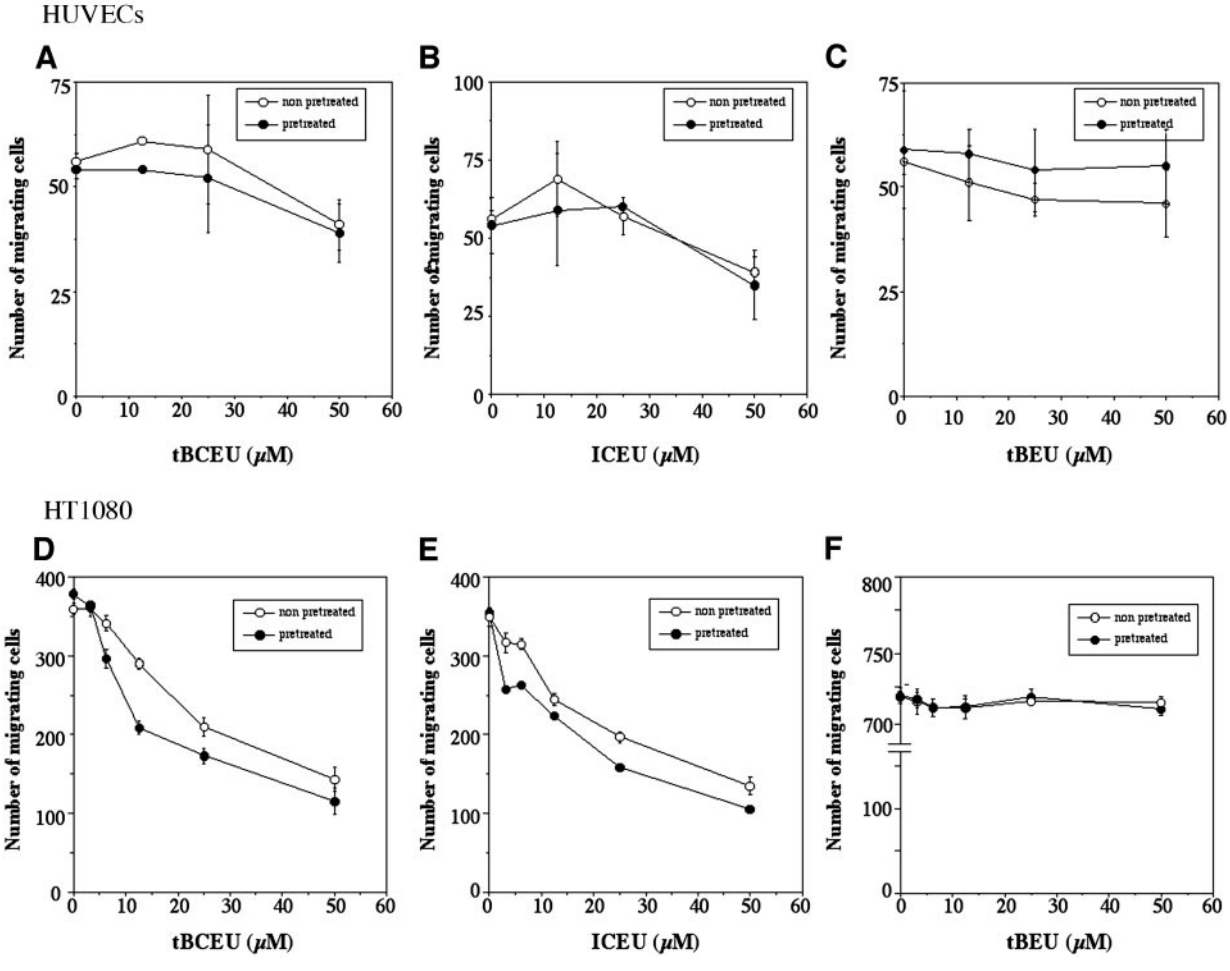

Fig. 5. Inhibition of endothelial and tumor cell migration by 4-tert-butyl-[3-(2-chloroethyl) ureido] phenyl and 4-iodo-[3-(2-chloroethyl)ureido]phenyl. Human umbilical vascular endothelial cells (*A–C*) and HT1080 (*D–F*) cells were pretreated (●) or not (○) for 24 h, with escalating concentrations of (*A* and *D*) 4-tert-butyl-[3-(2-chloroethyl) ureido] phenyl, (*B* and *E*) 4-iodo-[3-(2-chloroethyl)ureido]phenyl, or (*C* and *F*) tBEU 4-tert-butyl-[3-(2-ethyl)ureido]phenyl for 16 h. Cells were then plated on collagen IV-coated membranes (8.0-mm pore size) in a Boyden chamber in the presence of the same drugs. The cells were allowed to migrate for 4–6 h before being fixed and stained for quantification as described in "Materials and Methods." The results are expressed as mean ± SE of triplicates. *tBCEU,* 4-tert-butyl-[3-(2-chloroethyl) ureido] phenyl; *ICEU,* 4-iodo-[3-(2-chloroethyl)ureido]phenyl; *HUVECs,* human umbilical vascular endothelial cells; *tBEU,* 4-tert-butyl-[3-(2-ethyl)ureido]phenyl.

contrast to the effect of tBEU on M21 cells (Fig. 5*C*), 50 μM of tBCEU or ICEU (Fig. 5, *A* and *B*) induced a strong and reproducible inhibition of cell migration with an overall efficiency of 70% for either drug. This inhibition was observed to a lesser extent in HUVECs, which had an overall efficiency of 30–40%. Because the antiproliferative effect of CEUs depends on the time of contact, the migration assays were conducted with either 0 or 16 h pretreatments. The inhibitory effect of CEUs was essentially the same for both conditions for all of the drugs, indicating that the antimigrational action of CEUs is an early mechanism of action.

CEUs Inhibit Angiogenesis in the Matrigel Plug Assay. To additionally evaluate the antiangiogenic effect of CEUs, we s.c. injected mice with a Matrigel mixture containing or not containing bFGF as an angiogenic stimulating factor, then 50 and 100 mg/kg of CEUs were administered daily i.p. during 5–7 days. Matrigel plugs containing bFGF induced a significant 2-fold increase in hemoglobin content over Matrigel alone (Fig. 6). When tBCEU and ICEU were administrated at 100 mg/kg, they were potent inhibitors of bFGF-induced blood vessel formation (Fig. 6), causing a significant reduction of the hemoglobin content of 62% and 58%, respectively. At the same dose, tBEU clearly failed to reduce bFGF-induced vascularization, showing a hemoglobin content statistically similar to that from mice injected with the vehicle or not (untreated). In addition to their antimigrational property *in vitro*, our results revealed that CEUs have antiangiogenic action *in vivo*. CEU-injected mice showed no sign of toxicity.

CEUs Modulate the Growth of Solid Tumors in the Chick CAM Model. The growth of solid tumors on the surface of the CAM depends on the ability of injected-tumor cells to stimulate angiogenesis and to grow significantly within a 7-day period. The CS1 hamster melanoma and HT1080 human fibrosarcoma were used because they produce solid tumors that are sensitive to antiangiogenic and antitumoral therapy, including strong alkylating agents such as cDDP (14, 25, 32, 33). Because CEUs were shown to be strong growth inhibitors for primary endothelial cells and tumor cells *in vitro* (Fig. 2) and because they were showing antiangiogenic activity *in vivo* (Fig. 6), we evaluated their ability to inhibit the formation of solid tumors in the chick CAM assay. The cDDP was used as an internal positive control of a strong alkylating agent. Fig. 7, *A* and *B*, shows that incubation of CS1-derived tumors on the CAM with tBCEU or ICEU resulted in a significant dose-dependent reduction of the tumors size, as observed also with cDDP (Fig. 7*D*). Moreover, both CEUs also inhibited the formation of HT1080 tumor mass in the same concentration range (data not shown). It is noteworthy that the nonalkylating homologue tBEU failed to influence the growth of CS1 tumors (Fig. 7*C*), supporting the assumption that the antitumoral effect of tBCEU and ICEU was dependent on their alkylating activity. In the same experimental settings, only 10 μg/egg of cDDP was sufficient to inhibit tumor cell growth. However, a high level of chick embryo toxicity was observed at a higher concentration, because 95–100% of embryos died at a cDDP concentration reaching 50 μg/egg (Fig. 7). By contrast, the antitumoral effect of tBCEU and ICEU was shown at doses that were well tolerated by the chick embryo, as we monitored by chick necropsy (up to 150 μg/egg; data not shown).

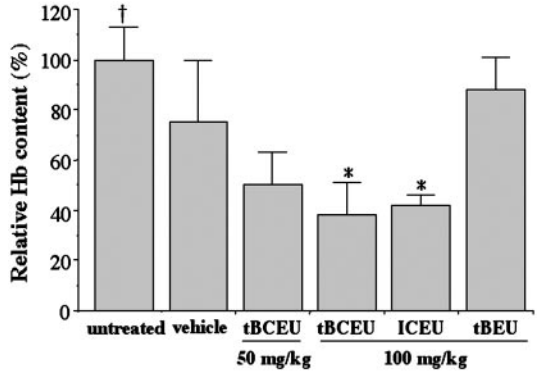

Fig. 6. Phenyl-3-(2-chloroethyl)ureas inhibit the basic fibroblast growth factor (bFGF)-induced angiogenesis in the mice Matrigel plug assay. Matrigel (0.5 ml), with or without bFGF (250 ng/ml), was injected on the ventral line of BALB/c mice. The vehicle alone, 4-tert-butyl-[3-(2-ethyl)ureido]phenyl (50 and 100 mg/kg), 4-iodo-[3-(2-chloroethyl)ureido]phenyl, or 4-tert-butyl-[3-(2-ethyl)ureido]phenyl (100 mg/kg) were injected i.p. in mice daily during 5–7 days. Mice were then euthanized, the Matrigel plugs were dissected out, and quantification of the hemoglobin content was performed. The results are normalized according to the Matrigel total dry weight. The results are representative of three independent experiments. The hemoglobin content of bFGF-containing Matrigel was used as the 100% reference. ANOVA test revealed a significant difference between the groups ($P < 0.05$), and the Dunnett test was performed (†, $P < 0.05$, when compared with Matrigel alone; and *, $P < 0.05$, when compared with bFGF-containing plugs). For the sake of clarity, the results corresponding to the Matrigel without bFGF are not shown but revealed that a significant vascularization was induced by bFGF, after its addition to the Matrigel plugs. *ICEU*, 4-iodo-[3-(2-chloroethyl)ureido]phenyl; *tBCEU*, 4-tert-butyl-[3-(2-ethyl)ureido]phenyl; *tBEU*, 4-tert-butyl-[3-(2-ethyl)ureido]phenyl; *bars*, ±SD.

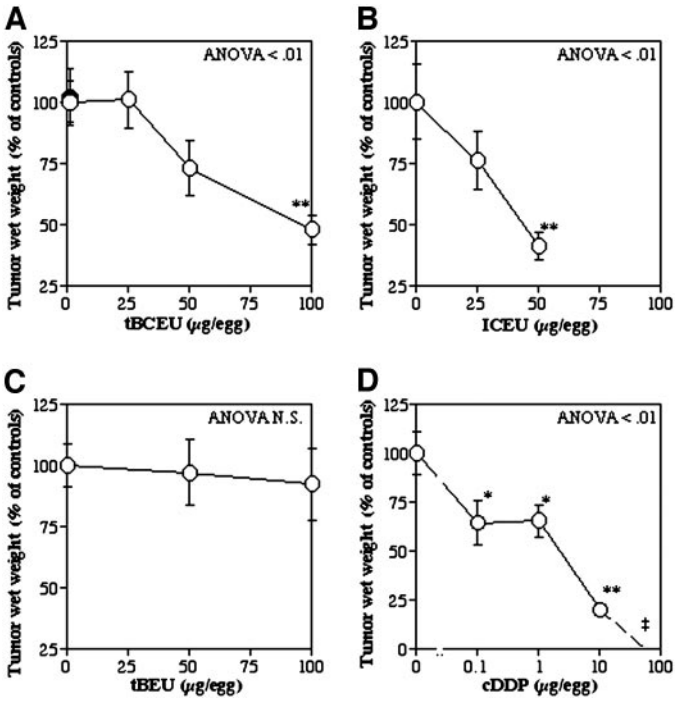

Fig. 7. Phenyl-3-(2-chloroethyl)ureas impede the growth of two unrelated tumor cell lines in the chick chorioallantoic membrane assay. Solid tumors were initiated on 10-day-old embryos by inoculation of hamster CS1 melanoma (3×10^5 cells/egg) or human HT1080 fibrosarcoma (5×10^6 cells/egg; data not shown) directly on the chick chorioallantoic membrane tissue. Escalating concentrations of (*A*) 4-tert-butyl-[3-(2-ethyl)ureido]phenyl, (*B*) 4-iodo-[3-(2-chloroethyl)ureido]phenyl, (*C*) 4-tert-butyl-[3-(2-ethyl)ureido]phenyl, and (*D*) cisplatin were then injected i.v. on day 11 in 10–12 eggs as described in "Materials and Methods." At day 17, embryos were euthanized, decapitated, and the tumor-wet weights were recorded. Cumulative results are shown from three independent experiments. ANOVA test showed a significant difference between doses ($P < 0.01$). Dunnett test was performed (*, $P < 0.05$; and * *, $P < 0.01$). ‡, indicates 95–100% chick embryos mortality in the group when using 25 μg/ml cisplatin. *A*, the ● corresponds to the injection of the solvent used to solubilize the drugs. *tBCEU*, 4-tert-butyl-[3-(2-ethyl)ureido]phenyl; *ICEU*, 4-iodo-[3-(2-chloroethyl)ureido]phenyl; *tBEU*, 4-tert-butyl-[3-(2-ethyl)ureido]phenyl; *cDDP*, cisplatin.

ICEU Prevents the Growth of CT-26 Colon Carcinoma Inoculated into BALB/c Mice. To confirm our *in vivo* data from the CAM assay, the antitumor activity of ICEU was assessed on the syngenic murine colon carcinoma CT-26, inoculated s.c. into BALB/c mice. The CT-26-bearing mice were treated or not treated with 13 mg/kg of ICEU administered i.p. on days 1, 5, and 9 or with 20 mg/kg 5-FU administered i.v. on days 7–11. Both treatments were well tolerated and did not cause side effects such as weight loss. The tumor sizes

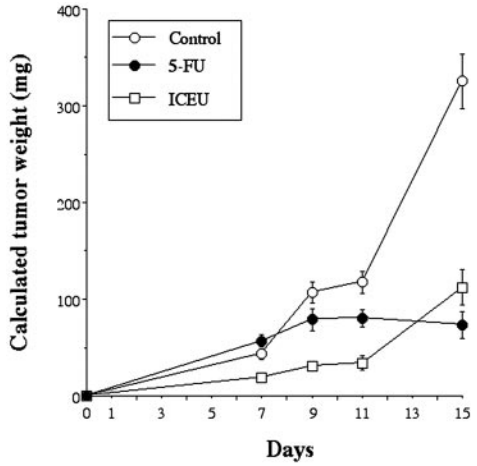

Fig. 8. Inhibition of CT-26 colon carcinoma cell growth into BALB/c mice. A suspension of 2.5×10^5 CT-26 cells was injected s.c. in the right flank of BALB/c mice on day zero. The 4-iodo-[3-(2-chloroethyl)ureido]phenyl (13 mg/kg) and 5-fluorouracil (20 mg/kg) were intraperitoneally injected at days 1, 5, and 9 or at days 7–11, respectively. The calculated tumor weight (CTW) of each tumor was estimated from two-dimensional measurements performed on day 7, 9, 11, and 15 after tumor inoculation. The CTW are the mean ± SE for each group of mice {Control, $n = 18$; 5-fluorouracil, $n = 8$; and 4-iodo-[3-(2-chloroethyl)ureido]phenyl, $n = 12$}. Analysis for significance with the Student t test confirms differences in CTW between treated and untreated groups. Values of $P < 0.05$ were considered as statistically significant. *ICEU*, 4-iodo-[3-(2-chloroethyl)ureido]phenyl; *5-FU*, 5-fluorouracil.

were evaluated on days 7, 9, 11, and 15 after CT-26 inoculation, and the tumor weights were calculated as described in "Material and Methods." The results presented in Fig. 8 show that a clinical anticancer agent used to treat colorectal cancer, the 5-FU, inhibits significantly the tumor growth (decreasing ~78% on day 15; $P < 0.05$) in comparison to the untreated group. Of outstanding interest, the treatment with ICEU was evenly very efficient to prevent the growth of CT-26 tumors. The results indicate that the CTW of ICEU-treated mice was significantly ($P < 0.05$) lower than the untreated group from day 7 until day 15, with reduced CTW ranging from 57% to 71%. On that specific tumor cell line, tBCEU was inactive (data not shown), and the *in vivo* testing of tBEU was cancelled, because this molecule failed to exhibit any biological activity in both cell cytotoxicity screening and β-tubulin alkylation potency assessment.

CEUs Overcome the Resistance of Tumor Cells Conferred by Extracellular Matrices. It was shown recently that the tumor microenvironment could modulate the ability of tumor cells to resist to chemotoxic agents such as cDDP, triggering a prosurvival signal through integrins (15, 34, 35). To evaluate whether this CAM-DR mechanism may impede the cytotoxic effect of CEUs, we verified whether purified ECMs could influence the outcome of CEU-treated tumor cells in clonogenic survival assay compared with the cytotoxic effect of cDDP. Despite its *in vitro* and *in vivo* cytotoxic effect, the cytotoxicity of cDDP was markedly decreased by the presence of fibronectin matrix protein, as shown by the relative survival increase, estimated from the number of viable colonies that had resisted treatments (Fig. 9, *left*). Unexpectedly, fibronectin conferred no protective effect against tBCEU- and ICEU-treated cells. Moreover, it was a sensitizing factor, thus, enhancing the toxicity of CEUs on tumor cells (Fig. 9, *center* and *right*). Furthermore, similar results were obtained with a number of ECM proteins, namely fibrinogen, fibrin, and heat-denatured type IV collagen (data not shown). Therefore, CEUs show no sensitivity to CAM-DR that may sensitize tumor cells to the effects of these alkylating agents.

DISCUSSION

CEUs such as tBCEU and ICEU are soft alkylating agents that have been shown to be potent cytotoxic agents in >40 different human and animal cancer cell lines. CEUs were also proven devoid of genotoxicity, unable to alkylate nucleophiles, and their cytotoxicity was unaffected in cells expressing various mechanisms of resistance (1, 4, 36). Mechanistic studies have demonstrated that [^{14}C-urea]-tBCEU covalently binds to β-tubulin by specifically alkylating its Cys^{239} residue (5, 8). The alkylation of the Cys^{239} residue abrogates the reentry of the alkylated β-tubulin into the α-, β-tubulin polymerization-depolymerization cycle, thereby promoting microtubules disruption and cell death.

In the present study, we compared the antimicrotubule activity of colchicine, vinblastine, and taxol to the soft alkylating aromatic ureas such as tBCEU, ICEU, and to a strong DNA alkylating agent such as cisplatin. At subcytotoxic concentrations, the proliferation, adhesion, and the level of migration of human endothelial cells have been shown to be reduced by antimicrotubule agents such as colchicine, *Vinca*, and *Taxus* alkaloids, and these drugs have also exhibited antiangiogenic properties in several *in vitro* models (10, 11, 37). Thus, chronical administration of low doses of these agents has been propounded as an optimal way of delivering antiangiogenic cancer therapy (37). CEUs expressed potent antiendothelial and antitumor cell activity *in vitro*, suggesting that they might be useful agents *in vivo*. The effects of CEUs on the vascular endothelium translate into the direct inhibition of angiogenesis in the Matrigel plug assay; i.p. injections of tBCEU and ICEU elicit a significant inhibition of the presence of hemoglobin in the Matrigel plug harvested from mice. These results suggest that the requirement of an interplay between microtubule and actin filaments in the angiogenic process is critical for the migration

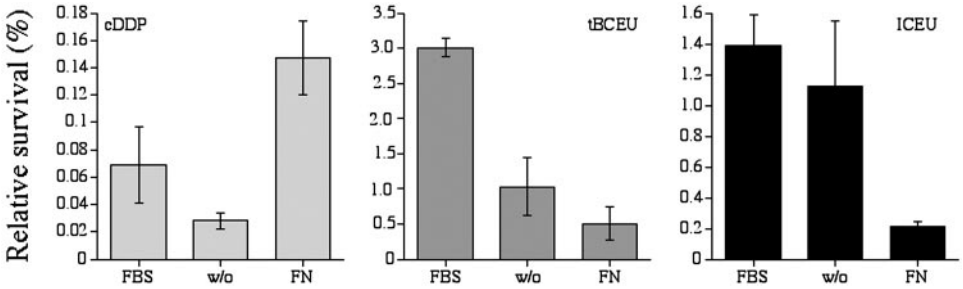

Fig. 9. Extracellular matrices and serum do not protect tumor cells against phenyl-3-(2-chloroethyl)ureas toxicity in clonogenic survival assay. M21 cells were plated in serum-free media on nontissue culture Petri dishes, coated with fibronectin (FN; 25 μg/ml) or not (w/o) for 16 h, or they were plated on tissue culture Petri dishes in the presence of serum (fetal bovine serum). Cells were then treated for 3 h with the strong alkylating agent cisplatin (50 μM) or for 24 h with the soft alkylating 4-tert-butyl-[3-(2-ethyl)ureido]phenyl and 4-iodo-[3-(2-chloroethyl)ureido]phenyl (20 μM). After treatments, survival was determined by colony formation as described in "Materials and Methods." One hundred percent is defined by the surviving colonies formed by untreated cells at the same dilution. Representative results from two independent experiments performed in quadruplicates are shown. *cDDP*, cisplatin; *tBCEU*, 4-tert-butyl-[3-(2-ethyl)ureido]phenyl; *ICEU*, 4-iodo-[3-(2-chloroethyl)ureido]phenyl; *bars*, ±SD.

and the differentiation of vessel sprouts, leading to the formation of functional blood vessels.

The precise mechanism underlying the role of microtubules during angiogenic and metastatic processes remains to be deciphered. During angiogenesis, the binding of integrins to the ECMs is embodied in the intracellular signaling pathways of cell adhesion and migration (12). Contact between integrins and ECM molecules is followed by a rapid association between actin stress fibers, microtubules, and focal adhesions structures. These structures are specialized contact sites involved in cell attachment to ECMs and are composed of specific molecules that assemble or disassemble, as cells migrate or enter into mitosis (38). Several specific proteins of these focal adhesion sites, such as focal adhesion kinase and paxillin (38), are also tyrosine phosphorylated during the association of actin cytoskeleton and focal adhesion structures. The intracellular role of microtubules has been associated for a long time to structural or transport functions. Nevertheless, evidence suggests that the microtubular network together with the actin cytoskeleton play critical roles in integrin-dependent transduction signals, triggering cell adhesion, spreading, and migration (39–41). For example, it was shown that cell spreading is closely correlated to microtubule integrity; cellular spreading was being impaired when microtubules were depolymerized (42). On the other hand, it was shown recently that microtubule depolymerization or stabilization leads to activation of cell adhesion, thus, establishing an opposite role normally played by microtubules in cell adhesion and cell spreading, as it has been described for actin filaments (41). In addition to activating cell-cell adhesion, microtubule-disrupting agents have been shown to increase the lateral mobility of β_2 integrins, allowing its subsequent clustering and activation. This was also accompanied by an increase in paxillin phosphorylation (41). Interestingly, an interaction between paxillin and γ- and α-tubulin was identified in human T lymphoblasts (43). A direct spatial interaction between microtubules and focal adhesion sites have also been identified during cell spreading and migration (44). The growing microtubule filaments were targeted toward the cell periphery, connecting directly to focal adhesion sites (44, 45). It was suggested that microtubules specifically modulated these contact sites by the delivery of a relaxing impulse, leading to an inhibition of actin-myosin contractility and resulting in the disassembly of focal adhesion structures and to cell detachment from the substratum (46). In that system, microtubule disruption additionally induced the enlargement of focal contacts, suggesting that the modulation of cell motility implies an important role for microtubules in the regulation of the turnover rate of focal contacts. Overall, a dynamic microtubule system seems to be required to regulate integrin-cytoskeleton interactions. The state of the microtubule network can greatly influence the organization of focal adhesion and actin stress fibers. Our preliminary unpublished data suggest that microtubule network disruption and alteration of the actin cytoskeleton observed in response to the soft alkylation of β-tubulin by CEUs may impede the ability of integrins to achieve their binding to the ECM. Thus, the mechanism of action of CEUs may involve the triggering of an inside-out signaling that leads to nonspecific inhibition of all integrins. That mechanotransduction process might be also involved in the antimigrational and antiangiogenic effects of CEUs. The evaluation of such signaling pathway is currently in progress.

The results on the antiproliferative, antimotility, and antiangiogenic properties of CEUs prompted us to evaluate their antitumor potential *in vivo*, using the chick embryo model first. The i.v. treatments with tBCEU and ICEU inhibited in a dose- dependent manner the formation of a solid tumor mass without any toxicity to the chick embryo. In the same assay, we report that cisplatin was highly toxic at similar concentrations. The antineoplastic activity of CEU was additionally assessed in mice, using another relevant model. The choice of the CT-26 colon carcinoma model was based on a previous biopharmaceutical evaluation using i.p. administration of [^{14}C]-tBCEU in mice, showing that tBCEU accumulates strongly into organs of the gastrointestinal tract such as liver, stomach, duodenum, colon, and kidney (7, 9). The affinity of the drug for these tissues suggested a potential usefulness of tBCEU against these resilient cancers. The experiments conducted on mice bearing CT-26 colon carcinoma confirmed, however, that tBCEU exhibited insignificant antineoplastic activity when administered i.p.[5] Unexpectedly, tBCEU had a significant antitumor activity, nevertheless, when administered intratumorally,[5] suggesting either an unsuitable drug biodistribution into the s.c. grafted tumors and/or a problem of extensive liver metabolism of the drug into inactive metabolites. The latter assumption was supported by studies in mice, showing that tBCEU was quickly absorbed and readily bioavailable orally (>82%) and i.p. Most importantly, tBCEU was largely inactivated by cytochrome P_{450} 1A2 and 2E1, being hydroxylated on its tert-butyl moiety into two main inactive metabolites, dithioic acid and an unstable glucuronide (9). To circumvent the hepatic inactivation of tBCEU, a "metabolically stabilized" analog was prepared. Therefore, the tert-butyl group of tBCEU was substituted by the nonhydroxylable iodine atom to generate ICEU. However, the chlorine atom present in the 2-chloroethylamino moiety of CEU was conserved, because it was shown essential to the alkylating and the cytotoxic activity of CEUs, based on our studies using tBEU, the nonhalogenated counterpart of tBCEU.

As expected, ICEU retained the same alkylating potency as tBCEU on β-tubulin, the same disruptive effect on both the microtubule network and the actin cytoskeleton, and the same cytotoxicidal activity on numerous tumor cell lines (Figs. 2 and 3). Therefore, ICEU was tested on mice bearing CT-26 colon carcinoma tumors. However, in our mouse model of tumor growth, the drug potency was not compared with those of classical antimicrotubules or to strong alkylating agent cDDP, because none of these drugs are used in clinical chemotherapy of human colon cancers, and these drugs are not efficiently biodistributed in these tissues. Instead, it was preferred to evaluate the antineoplastic potency of ICEU in regard of 5-FU, which has been in use for several decades in the first line of treatment of human colorectal cancers (47). Notably, 5-FU is also used in the treatment of other neoplastic diseases such as breast, aerodigestive tracts, and head/neck cancers (48). Therefore, 5-FU was a significant positive control to estimate the potential of ICEU for the treatment of cancer tumors related to the gastrointestinal tract. Accordingly, i.p. administration of 13 mg/kg of ICEU had the same potency to inhibit the growth of CT-26 colon carcinoma cells grafted in mice as 20 mg/kg of 5-FU administered i.v. We are currently evaluating the possible effects of CEUs on colon cancer cells that are implanted within the colonic tissue.

Our previous finding that CEUs overcome several classical mechanisms of chemoresistance (4) is of utmost importance in the view that tumor cells often develop progressive resistance against chemotherapy in the clinic. Recent reports have suggested that the toxicity of cDDP toward tumor cells could be reduced by several ECM molecules such as collagens, fibronectin, and laminins, all of them being β1 integrin ligands (15, 18, 34, 35). These mechanisms were defined as CAM-DR (16, 17). These results are complementary to our previous work, where it was suggested that the nature of the matrices on which tumor cells attach plays a significant role in their ability to resist to necrosis or apoptosis (13) and that cDDP-resistant cells were equally sensitive to CEUs (4). Here, we present evidence that CEUs are also unaffected by CAM-DR, conversely, to the effect of cDDP on the same cells. CAM-DR not only failed to influence the cytotoxic action of CEUs, but fibronectin also appears to increase the level of cell death of CEU-treated tumor cells, an event that might be linked to the disturbance of focal contact integrity after cytoskeleton collapsing, which might be related to anoikis (49, 50) or to integrin-mediated death (51–53). Therefore, the ability of CEUs to circumvent many classical and recently described drug-resistance mechanisms is a significant advantage in the perspective of blocking tumor growth or

angiogenesis *in vivo*, because this may require extended drug exposure that might not be achieved if a rapid drug resistance is developed.

In summary, CEUs are small-molecule drugs having microtubule disrupting properties and biopharmaceutical characteristics that may present advantages in cancer chemotherapy. These molecules are easily prepared, and most of them meet all of the physicochemical requirement described by Lipinski *et al.* (54) to predict the oral bioavailability of a drug. Therefore, our results suggest that CEUs represent a promising new class of antiangiogenic and anticancer agents, targeting notably gastrointestinal cancers.

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