Reversal of Multidrug Resistance in Vivo by Dietary Administration of the Phytochemical Indole-3-Carbinol

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ABSTRACT

A major obstacle to successful cancer chemotherapy is the development of multidrug resistance (MDR) by cancer cells. MDR is characterized by enhanced cellular efflux of many structurally and functionally diverse compounds, including many anticancer drugs, due to overexpression of the MDR-1 gene product, P-glycoprotein. We hypothesized that the phytochemical, indole-3-carbinol (I3C), and some of its acid-condensation derivatives may inhibit P-glycoprotein-mediated transport due to their aromatic and nitrogen components, thus increasing the accumulation and efficacy of anticancer drugs and acting as a dietary adjuvant to conventional chemotherapy. I3C was subjected to acid conditions similar to those occurring in the stomach following ingestion and three acid-condensation products; a dimer, a noncyclic trimer, and a cyclic trimer were isolated and purified by high-performance liquid chromatography. The ability of I3C and its acid-condensation derivatives to reverse MDR was investigated by using murine B16 melanoma cells that were transfected with the human MDR-1 gene (B16/hMDR-1 cells) and were cross-resistant to vinblastine and doxorubicin. The I3C acid-condensation product mixture, but not I3C, sensitized B16/hMDR-1 transfectants to the toxicity of vinblastine and doxorubicin. All three I3C acid-condensation products also increased the accumulation of the P-glycoprotein substrate, doxorubicin, in B16/hMDR-1 transfectants to levels comparable to parental B16 cells. The I3C acid-condensation product mixture competed with azidopine for binding to P-glycoprotein, suggesting that the observed MDR-reversing effect of the acid-condensation products was due to direct interaction with P-glycoprotein. The ability of p.o. administered I3C to reverse MDR was also tested in vivo. The resistance of B16/hMDR-1 transfectants to vinblastine and doxorubicin was preserved after i.p. injection and growth in nude mice. Tumor mass in mice that were provided with 333 or 500 mg/kg mouse/day I3C in their diet and injected s.c. with the anticancer drugs doxorubicin or vinblastine was significantly reduced as compared to tumor mass in mice provided with standard diet and injected with this anticancer drugs or mice provided with 500 mg/kg mouse/day I3C and not injected with anticancer compound. The concentrations of I3C used had no effect on survival or the general appearance and behavior of the mice. Collectively, these results indicate that ingestion of the common dietary constituent I3C results in its conversion to acid-condensation derivatives that sensitized MDR tumors to chemotherapeutic drugs without eliciting direct toxicity to the host.

INTRODUCTION

Resistence of tumor cells to multiple chemotherapeutic agents is a major obstacle to the treatment of several types of human cancer and has been closely associated with treatment failure (1, 2). This phenomenon has been termed MDR.1 One mechanism of MDR is associated with the overexpression of the MDR-1 gene product, P-glycoprotein (3–5). Other mechanisms such as overexpression of glutathione S-transferases (6, 7), mutation or loss of tumor suppressor p53 (8), or mutation of topoisomerase II may also play a role in MDR (9). Transfection of the MDR-1 gene into mammalian cells, however, confers the full MDR phenotype (10, 11). P-glycoprotein is a M, 170,000 membrane glycoprotein capable of the ATP-dependent cellular efflux of a variety of structurally and functionally diverse compounds across the plasma membrane (12–14). The mechanism of resistance in cells that overexpress P-glycoprotein is due to increased transport of various classes of anticancer compounds out of cells, resulting in decreased cellular accumulation and thus, decreased efficacy of the drugs. Anticancer compounds that are associated with P-glycoprotein-mediated drug resistance include Vinca alkaloids (i.e., vinblastine and vincristine), anthracyclines (i.e., doxorubicin), taxol, colchicine, actinomycin D, and mitomycin C (5, 11–13). Normal tissues such as liver, kidney, adrenal cortex, and secretory epithelia constitutively express P-glycoprotein. This suggests that P-glycoprotein may have a role in the cellular transport of endogenous compounds and may protect nonneoplastic tissues against cytotoxic compounds (15, 16).

Several classes of compounds that inhibit efflux by P-glycoprotein and enhance the accumulation and efficacy of anticancer compounds have been identified. MDR-reversing agents include calcium channel blockers (verapamil; Ref. 17), calmodulin inhibitors (phenothiazines; Ref. 17), indole alkaloids (reserpine; Ref. 18), and cyclosporins (19). Each of these compounds successfully reversed the MDR phenotype in vitro. The efficacy of these compounds in animal studies and clinical trials, however, has been disappointing due to dose-limiting toxicity. Accordingly, much effort is currently being expended toward identifying compounds that inhibit P-glycoprotein, reverse the MDR phenotype, and sensitize cancer cells to conventional chemotherapy without undesired toxicological effects.

Although P-glycoprotein mediates the transport of many structurally and functionally diverse compounds, many potent MDR-inhibiting compounds share some common physical characteristics such as cyclicity, lipophilicity, and a positive or neutral charge at physiological pH (12). Compounds that commonly share these characteristics readily interact with and are transported by P-glycoprotein. Based on its aromatic and nitrogen components, I3C and some of its acid-condensation derivatives were considered as candidate chemosensitizing agents. I3C is a derivative of glucobrassicin, a secondary plant metabolite that is abundant in cruciferous vegetables (e.g., cabbage, broccoli, Brussels sprouts, and others; Ref. 20). Glucobrassicin undergoes autoxidation during maceration to I3C, which is known to undergo acid-condensation in the stomach following ingestion. Incubation of I3C under conditions that mimic the acid conditions in the stomach results in the production of multimeric derivatives of I3C (21). The major acid-condensation products of I3C correspond to the dimeric, cyclic, and noncyclic trimeric derivatives DIM, CTI, and BII, respectively (22). These compounds have been extracted from the gastric contents, stomach tissue, small intestine, liver, lung, and blood following oral administration of I3C to rats (21, 23). I3C administration is known to induce cytochrome P450 and glutathione S-transferase activities, resulting in increased metabolic capacity toward chemical carcinogens (24–31). These properties of I3C are considered to...
contribute to the known anticarcinogenic properties of this compound, as well as to the reduced risk of cancer associated with diets rich in cruciferous vegetables (32, 33).

The purpose of the present study was to determine if oral administration of 13C reverses the MDR phenotype of tumors without significant toxicity. B16 murine melanoma cells, which were transfected with multiple copies of the human MDR-1 gene and shown to overexpress the human MDR-1 gene, exhibit resistance to multiple chemotherapeutic agents and accumulate less drug at steady state; these cells were grown in athymic nude mice to establish an in vivo model to assess chemical modulation of P-glycoprotein-mediated multidrug resistance. This model was used to assess the ability of p.o. administered 13C to sensitize drug-resistant tumors to conventional cancer chemotherapy. Results demonstrate a causal link between nutrition and improved cancer chemotherapy.

MATERIALS AND METHODS

Cell Culture. B16/hMDR-1 cells were derived from B16/F10 murine melanoma cells that were transfected with multiple copies of the human MDR-1 gene and subsequently selected for resistance to vinblastine (provided by A. C. King, Burroughs Wellcome, Research Triangle Park, NC). B16/F10 cells and B16/hMDR-1 transfectants were cultured in RPMI 1640 with l-glutamine supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) and housed in an incubator at 37°C and 5% CO2. Cells were passaged approximately every other day and cultured for no longer than 10 passages. The MDR phenotype was maintained by intermittently culturing cells in medium containing 150 ng/ml vinblastine.

Drugs. Doxorubicin (Adriamycin) was purchased from Adria Laboratories (a division of Erbamont, Inc., Columbus, OH). Vinblastine was purchased from Polysciences (Warrington, PA). Cisplatin, indole-3-carbinol, and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO).

Mice. Female, CD-1/CD-1, nu/nu inbred mice weighing between 22–24 g were purchased from Charles River Laboratories (Raleigh, NC). Animal care was provided by North Carolina State University Veterinary Center animal care staff. Mice were housed in a pathogen-free environment. All supplies, including food, water, and bedding, were autoclaved prior to use.

Synthesis and Purification of 13C Acid-Condensation Products. 13C acid-condensation products were synthesized as described previously (22, 34) with the following modifications. 13C (200 mg) was dissolved in 2 ml of DMSO, and the acid-condensation reaction was initiated by slowly adding the 13C solution to 200 ml of 0.05 M HCl (pH 1.3). The solution was stirred continuously for 80 min at room temperature and then was extracted twice with an equivalent volume of methylene chloride. Anhydrous magnesium sulfate was added to the methylene chloride solution to remove residual water. The solution was then filtered, condensed by rotary evaporation at 40°C, and stored in methylene chloride at −20°C.

The individual acid-condensation products were purified and separated by HPLC using a preparative C18 reverse phase column and a mobile phase of 50% acetonitrile/50% water at a flow rate of 2 ml/min. Products were detected at an absorbance of 280 nm (22). Three products were separated and purified by HPLC. The elution times of these products corresponded to the dimeric, cyclic trimeric, and noncyclic trimeric derivatives: DIM, CT!, and Bli, respectively (22, 34). Relative abundance of products in the mixture was approximated by HPLC. The elution times of these products corresponded to the dimeric, at an absorbance of 280 nm (22). Three products were separated and purified by HPLC using a preparative C18 reverse phase column and a mobile phase of 50% acetonitrile/50% water at a flow rate of 2 ml/min. Products were detected at an absorbance of 280 nm (22). Three products were separated and purified by HPLC.

Sensitization of Resistant Cells to Anticancer Drug Toxicity by 13C Acid-Condensation Products. To determine if 13C, its acid-condensation products, or verapamil sensitize MDR cells to the toxicity of vinblastine or doxorubicin, B16/F10 (5 × 105 cells) and B16/hMDR-1 (1 × 105 cells) were added to the wells of 96-well microtiter plates in 100 μl of culture medium. Different numbers of cells were plated to account for the more rapid growth of B16/F10 cells as compared to B16/hMDR-1 transfectants. After 4 h of incubation, B16/F10 cells and B16/hMDR-1 transfectants were incubated with vinblastine (0.1 μM) or doxorubicin (0.033 μM) and varying concentrations of 13C, the acid-condensation product mixture, or verapamil (in 100 μl of medium). Cells were incubated until cells in control wells were confluent (5 days), after which viability was assessed by the reduction of MTT (Sigma; Refs. 35 and 36). MTT (0.5 mg/ml PBS (0.01 M KPi (pH 7.4)) was added to each well of the microtiter plate, and plates were incubated for an additional 4 h in the dark at 37°C, after which the MTT solution was aspirated from the wells and 0.1 ml of acid isopropanol (0.04 N HCl in isopropanol alcohol) was added. The plates were allowed to stand for 10 min, after which they were read in an Anthos Microplate reader at 550 nm (35, 36). Each data point was replicated six times. Fold resistance was determined by comparing the IC50 of B16/hMDR-1 cells and B16/F10 cells. IC50 was defined as the concentration of the cytotoxic drug that caused 50% inhibition of cell growth as compared to untreated control and were calculated by the Spearman-Karber method (37).

Modulation of Doxorubicin Accumulation by 13C Acid-Condensation Products. The accumulation of doxorubicin, a drug transported by P-glycoprotein, in B16/F10 cells and B16/hMDR-1 transfectants was characterized. Cells (2 × 105) were incubated in the presence of 50 μM doxorubicin. At 1-h time intervals, cells were isolated by centrifugation and washed twice in cold PBS; then doxorubicin was extracted from cells using 50% ethanol/0.6 N HCl. The doxorubicin in the extract was measured by fluorometry at 470 nm excitation and 585 nm emission (38). Efflux of doxorubicin was measured first by incubating cells in 50 μM doxorubicin for 4 h. Following incubation, cells were removed by centrifugation, washed, and resuspended in medium containing no doxorubicin. Fluorescence was measured in the culture medium after removal of the cells at designated time intervals. The amount of doxorubicin transported into the medium was subtracted from the total amount of doxorubicin in the cells at the initiation of the efflux phase. The time required for cells to efflux 50% of the doxorubicin (T1/2) was calculated by linear regression analyses, following conversion of time to log scale.

The ability of 13C or individual 13C acid-condensation products to increase the accumulation of doxorubicin in B16/hMDR-1 cells (i.e., reverse the MDR phenotype) was assessed. B16/F10 and B16/hMDR-1 cells were incubated with 50 μM doxorubicin and increasing concentrations of 13C or individual 13C acid-condensation products for 4 h. The cells were washed, and doxorubicin was extracted and measured as described above.

Photoaffinity Labeling of P-glycoprotein by [3H]Azidopine and Competition with 13C Acid-Condensation Products. Membrane proteins were prepared from plasma membranes of B16/hMDR-1 transfectants and B16/F10 cells. Cells were suspended in cold hypotonic lysis buffer [10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM MgCl2] containing the protease inhibitors: leupeptin, 1 μg/ml; pepstatin A, 1 μg/ml; and phenylmethylsulfonyl fluoride, 50 μg/ml (Sigma Chemical Co., St. Louis, MO). After 15 min, unlysed cells were disrupted with a glass homogenizer and centrifuged at 400 × g for 10 min to remove nuclei. The supernatant was then centrifuged at 30,000 × g for 30 min, and the resulting pellet was resuspended in hypotonic buffer and stored at −80°C (39). Membrane protein concentration was measured according to Bradford (40) using BSA as a standard. B16/hMDR-1 and B16/F10 membrane preparations (50 μg) were incubated with 0.89 μM of the photoreactive P-glycoprotein substrate [3H]Azidopine (47 Ci/mmol) in buffer (10 mM HEPES and 1 mM EDTA, pH 7.4) in the dark, on ice for 2 h. B16/hMDR-1 membrane preparations were also subjected to simultaneous incubation with [3H]Azidopine, verapamil (100 μM), and 13C acid-condensation product mixture at concentrations of 75 and 150 μM to determine if these products competed with [3H]Azidopine for photoaffinity binding to P-glycoprotein. Samples were then irradiated with a 450 W mercury-vapor lamp for 10 min at 4–7°C (41). Proteins were separated on a 7.5% (v/v) SDS-polyacrylamide gel, transferred to nitrocellulose, and detected by autoradiography (42, 43).

Animal Model of P-glycoprotein-mediated Multidrug Resistance. The relative resistances of B16/hMDR-1 cells was determined in vivo by injecting B16/hMDR-1 transfectants (2.0 × 106/100 μl saline) or B16/F10 cells...
(1.0 × 10^6/100 μl saline) into the peritoneal cavity (i.p.) or s.c. in the flank region of CD-1 nu/nu mice on day 0. Different numbers of cells were injected to account for the more rapid growth of B16/F10 cells as compared to B16/hMDR-1 transfectants. The course of tumor growth was then followed by the excision of tumors from these sites, in separate groups of mice, every 3 days. Tumors grew more rapidly in the peritoneal cavity than they did s.c., and the mass of the tumors was also more consistent in the peritoneal cavity.

Pigmented, solid B16/hMDR-1 tumors grew in the peritoneal cavity to a size of about 1.0—1.5 g in about 2 weeks, whereas B16/F10 cells grew more rapidly to a size of 3—4 g in the same time period.

To determine whether B16/hMDR-1-derived tumors maintained the MDR phenotype in vivo, mice with i.p. tumors derived from both B16/hMDR-1 and B16/F10 cells (injected i.p. on day 0) were randomized and injected s.c. with anticancer drugs (vinblastine or doxorubicin) at varying concentrations or an equivalent volume of saline on days 3 and 5. Dermal irritation was observed at the site of doxorubicin administration. This irritation did not interfere with delivery of the compound and did not appear to have distressed the mice. On day 13, tumors derived from each cell type were removed and weighed, and their mass (relative to control) was compared at each dosage.

Sensitization of Drug-resistant Tumors in Vivo. The response of resistant tumors to anticancer drugs and I3C was determined by injection of B16/hMDR-1 cells (1–2 × 10^6/100 μl) into the peritoneal cavity (i.p.) of each mouse on day 0. Mice were randomized, and tumors were allowed to grow for 3 days prior to treatment with anticancer compounds. Each mouse was administered 333 or 500 mg I3C/kg mouse/day in their feed on days 0–8. I3C was provided by adding it to the feed (Rodent Chow 3500; Prolab, Agway, Inc., Syracuse, NY), homogenizing it in a food processor, and autoclaving it before use. Anticancer drugs vinblastine (6 mg/kg mouse) or doxorubicin (8 mg/kg mouse) were injected s.c. on days 3 and 5. Mouse weights and general observations were recorded every other day. Study groups included: (a) untreated controls; (b) animals injected only with anticancer drug on days 3 and 5; (c) animals provided only with 500 mg 13C/kg mouse/day; (d) animals provided with 500 mg 13C/kg mouse/day and injected with anticancer drug on days 3 and 5; and (e) animals provided with 333 mg 13C/kg mouse/day and injected with anticancer drug on days 3 and 5. All animals were euthanized on day 13, and tumors were removed and weighed. Groups included 8–15 animals, and each experiment was performed twice. Significant differences in tumor mass were assessed by the ANOVA and Dunnett's test (44, 45).

RESULTS

Differential Accumulation and Efflux of Doxorubicin in B16/hMDR-1 Transfectants and Parental B16/F10 Cells. The MDR phenotype is characterized by decreased accumulation and enhanced efflux of anticancer compounds, which is reversible upon treatment with P-glycoprotein inhibitors. Cells were first tested to determine the steady-state accumulation and efflux of doxorubicin in B16/hMDR-1 cells as compared to B16/F10 cells. The accumulation of doxorubicin at steady-state equilibrium was approximately 4-fold greater in B16/F10 cells as compared to B16/hMDR-1 cells (Fig. 1). B16/hMDR-1 cells also attained steady-state equilibrium after only 1–2 h, while B16/F10 cells attained steady-state after 3–4 h. The time steady-state equilibrium attained was confirmed by several replications of this experiment with time points carried out to 6 h. The rapid attainment of steady-state and reduced accumulation of doxorubicin in B16/hMDR-1 transfectants was due to the increased rate of efflux of doxorubicin. The T1/2 for the elimination of doxorubicin in B16/F10 cells was 41 min, while the T1/2 in B16/hMDR-1 cells was only 13 min.

Sensitization of B16/hMDR-1 Transfectants to the Toxicity of Anticancer Drugs by I3C Acid-Condensation Products but not I3C. The ability of I3C and its acid-condensation products to sensitize resistant B16/hMDR-1 transfectants to the toxicity of cytotoxic compounds was assessed. B16/hMDR-1 transfectants were shown previously to be cross-resistant to the toxicity of doxorubicin (30-fold) and vinblastine (>570-fold) but not cisplatin (a drug that is not transported by P-glycoprotein).4 Verapamil, I3C, and an acid-condensation product mixture were assessed at increasing concentrations for their ability to sensitize B16/hMDR-1 transfectants to the toxicity of doxorubicin (0.033 μM) and vinblastine (0.1 μM). These concentrations of vinblastine and doxorubicin represented the maximum concentration that had no effect on B16/hMDR-1 cell viability. IC_{50}s of doxorubicin were 0.45 μM for B16/hMDR-1 cells and 0.015 μM for B16/F10 cells, while IC_{50}s for vinblastine were 0.57 μM for B16/hMDR-1 cells and less than 0.001 μM for B16/F10 cells. I3C did not sensitize B16/hMDR-1 cells to the toxicity of vinblastine (Fig. 2A) or doxorubicin (Fig. 2B) at concentrations that were nontoxic to either cell line. The acid-condensation mixture sensitized B16/hMDR-1 cells to the toxicity of vinblastine with 50% sensitization at 12.5 μM, a concentration that had no effect on the viability of B16/hMDR-1 or B16/F10 cells (Fig. 2C). The acid-condensation product mixture also sensitized B16/hMDR-1 cells to the toxicity of doxorubicin with
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(100 μM), competed with [3H]azidopine and inhibited its binding to P-glycoprotein. Similarly, the acid-condensation product mixture competed with [3H]azidopine at 75 μM and, to a greater extent, at 150 μM. These results indicate that 13C acid-condensation products reverse the MDR phenotype by inhibiting the binding of ligand to human P-glycoprotein.

Protocol for Evaluation of B16/hMDR-1 Tumor Resistance in Vivo. A short-term model was developed to assess the in vivo resistance of tumors derived from B16/hMDR-1 transfectants and,

half-maximal sensitization at 4.4 μM (Fig. 2D). The known MDR-reversing agent, verapamil, sensitized B16/hMDR-1 cells to the toxicity of both vinblastine and doxorubicin, with half-maximal sensitization occurring at approximately 1 μM (results not shown).

Reversal of the MDR Phenotype with Respect to Doxorubicin Accumulation by 13C Acid-Condensation Products but not 13C. Experiments were next undertaken to establish: (a) whether the sensitization of the B16/hMDR-1 cells by the 13C acid-condensation products was due to increased accumulation of anti-cancer drugs; and (b) to determine which acid-condensation products elicited this effect. DIM, BII, and CTI, but not 13C, increased accumulation of doxorubicin in B16/hMDR transfectants in a concentration-dependent manner (Fig. 3). These acid-condensation products had no effect on accumulation in B16/F10 cells (Fig. 3). Concentrations of DIM, CTI, and BII required to increase the drug accumulation by 50% with respect to B16/F10 cells were 45, 35, and 70 μM, respectively.

Inhibition of [3H]Azidopine Binding to P-Glycoprotein by 13C Acid-Condensation Products. Azidopine has been demonstrated to act as a photoaffinity ligand for P-glycoprotein (19, 20). Photoaffinity labeling of plasma membrane preparations from B16/hMDR-1 cells revealed a major band at Mr, 170,000 (Fig. 4). Photoaffinity labeling of membrane preparations from B16/F10 cells did not reveal a band at Mr, 170,000, confirming that B16/hMDR-1 cells express P-glycoprotein on the plasma membrane while B16/F10 cells express little or no P-glycoprotein. As shown in Fig. 4, the positive control, verapamil
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A

0 25 50 75 100 125

Tumor mass (% control)

Vinblastine (mg/kg mouse)

B

0 5 10 15

Doxorubicin (mg/kg mouse)

Fig. 5. Relative resistance of B16/hMDR-1 transfectants to vinblastine (A) and doxorubicin (B) in vivo. B16/hMDR-1 (○) and B16/F10 cells (□) were injected i.p. into nude mice on day 0 and grown for 3 days. Animals were injected with increasing amounts of vinblastine or doxorubicin on days 3 and 5, and tumors were removed and weighed on day 13. Data points represent the mean weight of tumors removed from three animals. Bars, the SD associated with the mean.

ultimately, to assess the efficacy of oral 13C in sensitizing drug-resistant tumors to chemotherapeutic agents. The most appropriate site of tumor cell injection and the time course of tumor growth in C57Bl/6J nude mice was determined. Tumors derived from both cell types grew more rapidly when injected into the peritoneal cavity as opposed to s.c. injection, and the time course of tumor growth was more consistent in the peritoneal cavity than it was s.c. The peritoneal cavity was thus deemed to be the more suitable site for tumor growth in these experiments. Tumors in the peritoneal cavity grew as pigmented, solid masses that attached to the mesentery following injection. Tumors were easily extracted from the peritoneal cavity, and mass was determined by weighing.

Experiments were next conducted to determine if tumors derived from B16/hMDR-1 transfectants were resistant to the cytotoxicity of anticancer compounds at dosages that affected the growth of tumors derived from B16/F10 cells. Both types of cells were injected into nude mouse on day 0, followed by s.c. injection of various dosages of doxorubicin and vinblastine on days 3 and 5, and tumors were excised and weighed on day 13. Administration of 5.5 mg vinblastine/kg mouse reduced tumor mass of the B16/F10 cells by 70%, while mass of B16/hMDR-1-derived tumors was reduced by only 9% (Fig. 5A). Similarly, treatment with 9.75 mg doxorubicin/kg mouse reduced tumor mass of B16/F10 cells by 96%, while mass of B16/hMDR-1-derived tumors was reduced by only 48% (Fig. 5B). These results confirmed that B16/hMDR-1 tumors were more resistant to the toxicity of vinblastine and doxorubicin in vivo than were parental B16/F10-derived tumors.

Sensitization of Resistant B16/hMDR-1-derived Tumors in Vivo. Having established that B16/hMDR-1-derived tumors were drug resistant in vivo, the ability of 13C acid-condensation products to sensitize these tumors to the cytotoxicity of anticancer compounds vinblastine and doxorubicin was investigated. Treatment of mice with 6 mg/kg mouse vinblastine had no significant effect on the mass of the MDR tumors (Fig. 6). However, tumor mass was significantly reduced when mice were fed 13C (333 or 500 mg/kg mouse/day) during the vinblastine treatment period (Fig. 6). Feeding of 13C alone had no effect on tumor mass (Fig. 6). Similarly, doxorubicin (8 mg/kg) decreased slightly but had no significant impact on the drug-resistant tumors (Fig. 7). However, doxorubicin in combination with oral administration of 13C significantly reduced tumor mass (Fig. 7). 13C had no effect on the weight, behavior, and appearance of the mice. Collectively, these results demonstrate that 13C was capable of reversing the MDR phenotype in vivo while having no independent effects on tumor mass and no discernible effect on the tumor host.

DISCUSSION

Cross-resistance to multiple classes of chemotherapeutic agents is a major problem in the treatment of several types of human cancers. A major mechanism of this resistance is the enhanced efflux of a wide variety of structurally distinct classes of chemotherapeutic agents due to the overexpression of P-glycoprotein. Inhibition of P-glycoprotein-mediated transport results in an increase in cellular accumulation of cytotoxic chemotherapeutic agents, thus increasing the efficacy of treatments.

Fig. 6. Sensitization of B16/hMDR-1-derived tumors to vinblastine by dietary administration of 13C. Mice were injected with B16/hMDR-1 cells on day 0, provided with or without 13C in their diet on days 0–8, and treated with vinblastine on days 3 and 5; then tumors were removed and weighed on day 13. Data are presented as the mean tumor weight (16 animals/treatment); bars, SD. (* significant difference from vinblastine-treated controls (P < 0.05). Significant differences in tumor mass were assessed by the ANOVA and Dunnett's test.)

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cause they were produced in insignificant amounts as compared to the three major products, as indicated by HPLC retention data, they were not isolated. The major I3C acid-condensation derivatives were shown to increase the cellular accumulation of the P-glycoprotein substrate, doxorubicin, and sensitize resistant cells to cytotoxic chemotherapeutic agents. These derivatives also competed with azidopine for photoaffinity labeling of P-glycoprotein, indicating that they may function by competitively inhibiting P-glycoprotein-mediated transport.

*In vitro*, I3C acid-condensation products sensitize cells to doxorubicin more so than vinblastine. p.o.-administered I3C also sensitized tumor mass to doxorubicin more so than vinblastine. The difference in activity of the phytochemicals with the two anticancer drugs is probably due to the demonstrated greater resistance of the B16/hMDR-1 cells to vinblastine as compared to doxorubicin. This increased resistance may be due to higher affinity of vinblastine to P-glycoprotein. Accordingly, higher concentrations of the indolic phytochemicals would be required to successfully compete with vinblastine for binding for P-glycoprotein.

Inhibitors of P-glycoprotein must meet certain criteria to effectively reverse MDR *in vivo*. Concentrations of the MDR sensitizers that inhibit P-glycoprotein must be reached at the tumor site, and these compounds must not be cytotoxic to noncancerous cells at these concentrations. Mice treated by gavage with 50 mg [3H]I3C/kg mouse accumulated 100 μM of radiolabeled derivatives in the liver and displayed serum levels of 20 μM (46). Rats fed 300 mg [3H]I3C/kg rat/day for 6 days accumulated approximately 400 μM of I3C equivalents in the liver, 204 μM in the lung, and 274 μM in the blood, measured 48 h after first oral administration (23). Based on these and other studies (21, 23, 31, 46, 47), serum levels of I3C derivatives should exceed 200 μM in mice fed 500 mg I3C/kg mouse/day for 8 days. *In vitro* studies indicated that I3C derivatives reverse the MDR phenotype at concentrations below 200 μM. Individual acid-condensation products half-maximally increased doxorubicin accumulation at approximately 50 μM, an acid-condensation mixture that half-maximally sensitized resistant cells to doxorubicin at 4.4 μM and vinblastine at 12 μM, and the acid-condensation mixture inhibited azidopine binding to P-glycoprotein at 75 μM.

Clinical trials of MDR inhibitors have been largely disappointing because of dose-limiting cytotoxicity. Verapamil is among the best characterized modulators of P-glycoprotein-mediated MDR. Reversal of the MDR phenotype by verapamil in many cell lines overexpressing P-glycoprotein has been demonstrated *in vitro* (17, 48, 49). *In vivo*, however, results have been largely unsuccessful due to verapamil-associated severe cardiotoxicity at subtherapeutic doses (49–51). Cyclosporin A has also undergone clinical trials as an MDR-reversing agent. Dose-limiting toxicity such as immunosuppression, hyperbilirubinemia, and renal tubular dysfunction have precluded the attainment of therapeutic doses (52). In the present study, I3C effectively sensitized MDR tumors at dosages that elicited no discernible toxicity to the mice. We had shown previously that dietary administration of 750 mg/kg mouse/day I3C for 1 week had no effect on feeding behavior, weight, and general appearance of the mice. This dosage did alter several hepatic processes and hepatic cholesterol content (34, 53) Similarly, daily dietary dosages of 333 and 500 mg/kg mouse/day I3C used in the present study had no effect on mice treated with vinblastine or doxorubicin, although at necropsy, these mice did have enlarged livers.

Although I3C itself is not effective in reversing the MDR phenotype, this compound undergoes acid condensation in the stomach, yielding several oligomeric derivatives that are effective reversing agents. These oligomers likely exhibit distinct toxicological properties. For example, we have shown that the cyclic trimer CTI is a potent inhibitor of the enzyme acyl CoA:cholesterol acyltransferase, whereas...
the dimer DM is a rather ineffective inhibitor of this enzyme and the noncyclic trimer BI has intermediate potency (34). A similar order of potency was noted for the ability of these compounds to inhibit the oxidative metabolism of testosterone (54). However, results from the present study demonstrate that these compounds are similarly effective in inhibiting P-glycoprotein. Therefore, these derivatives would exhibit an additive effect on P-glycoprotein inhibition, while individually they may be present at nontoxic levels. The multiplicity of P-glycoprotein inhibitors generated during acid-condensation of II and III may thus contribute to its efficacy. These observations illustrate the principal that because P-glycoprotein is a relatively nonspecific transporter of lipophilic, cyclic compounds that carry a positive or neutral charge at physiological pH, combinations of compounds with these characteristics have the potential to reverse the MDR phenotype at individual concentrations that are nontoxic. The structural characteristics of these compounds add to the database of compounds that reverse the MDR phenotype and could also be used to aid in the design of more potent, nontoxic MDR-reversing drugs. In conclusion, these findings: (a) demonstrate an effective animal model to evaluate the efficacy of human P-glycoprotein inhibitors; (b) illustrate the additivity of P-glycoprotein inhibitors; and (c) indicate that III, via acid-condensation, may be effective as a dietary adjuvant in the treatment of MDR cancers.

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