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ABSTRACT
The effect of the tyrosine kinase inhibitor genistein on the accumulation of cisplatin (DDP) was investigated in DDP-sensitive and -resistant human 2008 ovarian carcinoma cell lines. DDP accumulation after a 1-h exposure was maximally increased by concurrent 40 μM genistein. The maximal stimulation of accumulation was observed after 2 h of total genistein exposure and was 83 ± 13% (n = 5) higher than controls. With resistant C13* cells, however, the stimulation of accumulation was delayed until 4 h and was increased only 46 ± 18% compared to controls. Revertant RH4 cells that retained the accumulation defect behaved like the C13* cells. Genistein stimulated [3H]mannitol accumulation (a marker of passive permeability) by 43 ± 9% (n = 3) in 2008 cells, and the effect was maximal after 2 h of total genistein exposure. Changes in [3H]mannitol accumulation in 2008 parent cells were highly correlated with DDP accumulation (r = 0.9010). These experiments also revealed that [3H]mannitol accumulation after 2 h in C13* cells was reduced 38% compared to 2008 cells, a decrease that reflected the DDP accumulation defect. Fluid-phase pinocytosis determined with lucifer yellow CH as a marker showed no difference between 2008 and C13* cells and no effect of genistein. Genistein was demonstrated to clearly inhibit protein-tyrosine phosphorylation initiated by the epidermal growth factor receptor kinase. Differences were noted in the phosphotyrosine pattern between the 2008 and C13* cells. Under the conditions that had the maximal effect on DDP accumulation in 2008 cells, genistein decreased the IC50 of DDP 8.2-fold in 2008 cells and 4.7-fold in C13* cells. We conclude that: (a) genistein stimulates DDP accumulation by modulating the passive permeability of the plasma membrane; (b) C13* cells are less permeable to passively diffusing small molecules, which offers a mechanism for the DDP accumulation defect without invoking carrier proteins; (c) the effect of tyrosine kinase inhibition on passive permeability is altered in C13* cells; and (d) pinocytosis contributes insignificantly to DDP accumulation. Genistein, a dietary isoflavone, thus seems to be a promising clinical candidate for combination with DDP.

INTRODUCTION
DDP4 is one of the most effective drugs for the treatment of human ovarian, testicular, bladder, lung, head and neck, and other solid tumors. Tumor cell resistance to this drug, however, is often encountered innately or acquired during chemotherapy. Among the known mechanisms of acquired resistance, impairment of cellular DDP accumulation seems to play an important role and has been shown to appear early in the development of resistance in vivo (1–6). Many in vitro studies have been conducted to determine the biochemical basis of this resistant phenotype by testing the ability of pharmacological agents to modulate DDP accumulation. Differential effects on DDP accumulation of treatments that affect the cellular physiology or architecture such as ouabain, low Na+, digitonin, amphotericin B, and taxol have been noted between accumulation-proficient and -deficient cells (7–11). Agents that modulate signal transduction pathways (forskolin, 3-isobutyl-1-methyl-xanthine, phorbol esters, and calmodulin inhibitors) have also been noted to evoke differential effects on DDP accumulation in cells with accumulation defects (12–14). The stimulation or inhibition of DDP accumulation by pharmacological agents and by the activation or down-regulation of some signal transduction pathways has suggested that DDP enters the cell not only by passive diffusion but also via a carrier-mediated system. Therefore, various laboratories have also attempted to identify changes in plasma membrane or structural proteins that could putatively account for the accumulation defect (11, 15–17).

Although numerous studies have reported the effect of activation or down-regulation of signal cascades on the cellular pharmacology of DDP, the effect of blockade of protein kinase activity has not been addressed carefully. Genistein is an isoflavone isolated from the fermentation broth of Pseudomonas sp. and is present as a phytoestrogen in soybeans and a variety of other leguminous foodstuffs (18, 19). Genistein has specific inhibitory activity against tyrosine kinases such as the EGF receptor, pp60c-src, and pp110γre turnover in vitro but not against serine and threonine-specific kinases such as the cyclic AMP-dependent protein kinase and protein kinase C (20). In addition to the ability of genistein to inhibit protein tyrosine kinases, genistein can affect cell proliferation by inhibition of phospholipase C and topoisomerase II (21, 22). These different

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4 The abbreviations used are: DDP, cis-diamminedichloroplatinum(II), cisplatin; LY, lucifer yellow CH; TPP⁺, tetraphenyl phosphonium cation; EGF, epidermal growth factor; CI, combination index.
activities are probably the expression of the same mechanism of action, noncompetitive alteration of the ATP binding site that is common to tyrosine kinases, serine/threonine kinases, and topoisomerase II (22). Genistein can thus inhibit a variety of changes in intracellular physiological, biochemical, and molecular events triggered by tyrosine phosphorylation. In the present study, the effect of genistein on DDP accumulation was investigated in the 2008 human ovarian carcinoma cell line and its DDP-resistant C13* subline.

MATERIALS AND METHODS

Drugs and Chemicals. DDP (clinical formulation) was obtained from Bristol-Myers (Syracuse, NY). Genistein and protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO). LY was obtained from Aldrich Chemical Co. (Milwaukee, WI). [3H]TPP* (37.4 Ci/mmol) and [3H]mannitol (30 Ci/mmol) were obtained from DuPont NEN Research Products (Boston, MA). RPMI 1640 powdered medium without glutamine, glucose, NaHCO₃, NaCl, and KCl was obtained from Flow Laboratories (McLean, VA). EGF was obtained from Life Technologies, Inc. (Gaithersburg, MD).

Cell Lines. The 2008 cell line (established from a patient with serous cystadenocarcinoma of the ovary) was used (23). The DDP-resistant C13* subline and revertant RH4 cells were generated as described previously (24, 25) and grown as monolayers in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Sigma) and 50 μg/ml gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). Cultures were equilibrated with humidified 5% CO₂ in air at 37°C. All studies were done with Mycoplasma-negative cells as determined with the Mycoplasma T.C. detection kit (Gen-Probe, San Diego, CA). Clonogenic assays on plastic were conducted as described previously (12); preplated cells were exposed to DDP ± genistein in RPMI 1640 in the absence of serum. Median effect analysis to assess whether the cytotoxic effects of DDP and genistein were antagonistic, additive, or synergistic was conducted using a computer program (Elsevier-Biosoft, Cambridge, United Kingdom) as described (26). Genistein and DDP were combined at a ratio of 116:1 for the 2008 cells and 18:1 for the C13* cells under conditions of continuous exposure. Based on the slope of the lines in the median effect plot [log (fraction killed)/log (fraction surviving) versus log (genistein concentration)], the analyses were conducted assuming that the drugs had similar modes of action (a mutually exclusive case).

Oxygen Consumption. Modulation of oxygen consumption by genistein in whole cells permeabilized by digitonin was assessed as described (27). Measurements were made in a thermostatically closed oxygenhgy vessel of 2.5 ml volume at 30°C with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) covered with an ultrathin Teflon membrane. Five 150-mm plates of subconfluent cells were harvested by trypsinization, washed once, suspended in wash buffer [150 mM NaCl, 5 mM KCl, and 10 mM Tris-HCl (pH 7.4)] and counted by hemocytometer. Cells were diluted immediately to a density of 7.5 × 10⁶ cells/ml in 2 ml of respiratory medium [125 mM sucrose, 50 mM KCl, 5 mM HEPES, 2 mM KH₂PO₄, and 1 mM MgCl₂ (pH 7.2)] and incubated under constant stirring. Oxygen disappearance was monitored after the addition to the cells in the chamber via Hamilton syringe of (final concentration) 50 μg/ml digitonin, 40 μM genistein, 10 mM succinate, 1 μg/ml antimycin A, or 0.05 mM N,N,N',N'-tetramethyl-p-phenylenediamine/ascorbate. Respiratory activity was expressed in nanogram atoms oxygen/min/10⁶ cells; the solubility of oxygen in the air-saturated medium was taken to be 435 ng atoms/ml at 30°C and 760 mm Hg (27).

Membrane Potential. The effect of genistein on mitochondrial membrane potential was determined by measuring the accumulation of [3H]TPP*, a membrane potential indicator (24). Cells were seeded into 6-well tissue culture plates. When the cells were subconfluent, the medium was aspirated and replaced with 1 ml RPMI 1640 at 37°C containing 5 μM [3H]TPP* (0.2 μCi/ml) in which the NaCl had been replaced by equimolar KCl (high K⁺ medium) to depolarize the plasma membrane. Genistein at the appropriate concentrations was added immediately. The final ethanol concentration in the medium did not exceed 0.1% by volume. The plates were returned to the incubator; at 1 h, the plates were removed, the medium was aspirated, and the wells were washed rapidly four times with PBS consisting of 8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, and 0.2 g KH₂PO₄ per liter (4°C). The cells were digested overnight in 1 ml of 1 N NaOH. A 0.7-ml aliquot was mixed with 7 ml acidified Ecolume, and radioactivity was measured on a 1209 RackBeta liquid scintillation counter (LKB Instruments, Gaithersburg, MD). Protein was determined on 20-μl aliquots by the Bradford method (28), using reagent obtained from Pierce Chemical Co. (Rockford, IL).

DDP Accumulation. One-h DDP accumulation experiments were performed with subconfluent monolayers as described (23). Experiments were performed in the cell culture medium without serum or antibiotics. To determine the effect of genistein preincubation time on DDP accumulation, the plates were incubated with 40 μM genistein for different times, and DDP was added at the last hour. Genistein was added from stock solutions in ethanol stored at 4°C, and the final ethanol concentration in the medium did not exceed 0.15% by volume. In studies with halenaquinone, the final acetone concentration did not exceed 0.05%. Incubations were terminated by washing four times with PBS at 4°C. Protein was determined by the Bradford method (28), and cell-associated platinum was measured by atomic absorption spectrophotometry as described previously (25). Each experiment was conducted with duplicate sets of plates.

Membrane Permeability. The permeability of the plasma membrane to a passively diffusing compound was assessed by the ability of cells to accumulate [3H]mannitol. The medium was aspirated from subconfluent cells grown in 6-well plates and replaced with 1 ml RPMI 1640 containing 1 mM [3H]mannitol (1 μCi/ml; Refs. 29 and 30). At the appropriate time points, the plates were washed and digested as described for membrane potential experiments.

Fluid-Phase Pinocytosis. Pinocytosis experiments were performed according to the method of Swanson et al. (31). LY was dissolved at 0.5 mg/ml (1.093 mm) in RPMI 1640 with 10% heat-inactivated fetal bovine serum, sterile filtered, and equilibrated with 5% CO₂. Subconfluent cells in 60-mm tissue culture dishes were incubated at 37°C with or without 40 μM genistein in 2.0 ml RPMI 1640 containing LY for various periods of time.
Dishes were drained and washed three times with PBS at 4°C containing 1 mg/ml BSA and three times with PBS at 4°C alone. Cells were scraped into 2.0 ml of 0.1% Triton X-100 and frozen. Thawed samples were sonicated with eight pulses at an output control setting of 80 (Vibracell VC40; Sonics and Materials, Danbury, CT). Protein was determined with 50-µl aliquots, and the remaining sample was centrifuged at 3000 × g for 15 min. A 1.5-ml aliquot of supernatant was mixed with 1.5 ml of 0.1% Triton X-100 containing 0.1 mg/ml BSA in a four-sided cuvette. Fluorescence at 540 nm was measured on an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, MD) with excitation at 430 nm. Standards curves of LY were prepared in Triton/BSA using plasticware. Accumulation was calculated for each sample as nanograms of LY per mg of protein, and each time point had duplicate plates. The apparent fluorescence of lysate from cells exposed for 30 s to LY was subtracted from other fluorescence values.

**Phosphotyrosine Immunoblotting.** Cells were seeded into 60-mm plates and grown until near confluence. The medium was removed, and the cells were rinsed with serum-free RPMI 1640 and replaced with serum-free medium ± 40 μM genistein. The cells were incubated for 2 h with or without 10 nM EGF added the last 10 min. At the end of the incubation, the medium was aspirated, and cultures were rinsed rapidly twice with PBS at 4°C containing 1 mM orthovanadate and scraped into the same buffer. Cell suspensions were centrifuged at 9000 × g, and pelleted cells were lysed by the addition of 250 μl lysis buffer [20 mm Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 86 μg/ml iodoacetamide]. Lysates were incubated on ice for 30 min, and then cell debris was pelleted for 10 min at 12,000 × g at 4°C. Equal protein (150 μg) from each cell lysate was mixed with 5 × Laemmli-reducing Tris-glycine SDS-PAGE buffer, boiled for 4 min, and loaded on an 8% SDS-polyacrylamide gel with a stacking gel. Proteins were separated by applying 90 V for 1 h and 150 V for 4 h. The separated proteins were then electroblotted to nitrocellulose paper (0.45 μm; Micron Separations, Westborough, MA) for 2 h (4°C, 24 V) or overnight (4°C, 8 V) with transfer buffer [39 mM glycine, 48 mM Tris, 0.037% SDS, and 20% methanol (v/v)]. After transfer, the blotted filters were immersed in blocking buffer consisting of 5% nonfat dried milk in TBS-T buffer [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.05% Tween-20] for 1 h on a rocking platform. After four washes in TBS-T buffer, phosphotyrosine-containing proteins were detected using PY20 mouse monoclonal antiphosphotyrosine antibody (Transduction Laboratories, Lexington, KY). Filters were incubated for 1 h with a 1:10000 dilution (0.1 μg/ml final) of PY20 diluted in blocking buffer. The filters were then washed four times in TBS-T buffer and incubated for 1 h with horseradish peroxidase-conjugated sheep antimouse immunoglobulin (Amersham, Arlington Heights, IL) diluted in blocking buffer (1:5000). All of the immunoblot steps were carried out at room temperature. Filters were washed six times with TBS-T buffer, and immunodetected bands were visualized using enhanced chemiluminescence (Amersham).

**Statistics.** All values report the mean ± SE unless otherwise indicated. Statistical significance (P < 0.05) was determined using Pharmacological Calculation System software (Microcomputer Specialists, Philadelphia, PA). SE bars that are absent on figures were smaller than the symbol size.

**RESULTS**

**Effect of Genistein on Cellular Accumulation of DDP.** The 2008 and C13* cells were treated concurrently with 100 μM DDP and various concentrations of genistein for 1 h (Fig. 1). DDP accumulation increased up to 50 μM genistein with no further increase up to 100 μM in 2008 cells, whereas at the same concentrations, there was no appreciable effect of genistein on DDP accumulation in C13* cells. In 2008 cells, the increased DDP accumulation at 50 and 100 μM genistein was statistically significant (t test) compared to cells treated with ethanol alone. Forty μM genistein were chosen for subsequent experiments as the lowest concentration with a near-maximal effect on DDP accumulation. The effect of genistein exposure time on the accumulation of DDP, which was added to the medium for the last hour of incubation, is shown in Fig. 2. In these experiments, revertant RH4 cells that have lost the majority of their resistance but retain their DDP accumulation defect were also used. The platinum accumulation increased in 2008 cells for the first 2 h of genistein exposure from 601 ± 46 (n = 5) to 1073 ± 119 pmol/mg protein (n = 4) and then returned slowly to the control value after 6 h of genistein exposure. In the other two cell lines, the maximum platinum accumulation was reached after 4 h of genistein exposure. After 4 h of total genistein exposure, accumulation had increased from 284 ± 17 to 361 ± 13 pmol/mg protein (n = 4) in C13* cells and from 411 ± 77 to 575 ± 65 pmol/mg protein (n = 3) in RH4 cells, although the apparent RH4 increase was not statistically significant (t test). The maximal percentage of increases in DDP accumulation in C13* and RH4 cells at 4 h, 46 ± 18% and 44 ± 11%, respectively, were not significantly lower (ANOVA) than the 83 ± 13% maximal increase in the parent line at 2 h.

![Effect of genistein concentration on 1-h DDP accumulation in 2008 (•) and C13* (○) cells. Cells were concurrently exposed to the indicated concentrations of genistein and to 100 μM DDP for 1 h. Points, mean values from three experiments; bars, SE.](https://clincancerres.aacrjournals.org)
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**Effect of Halenaquinone on Cellular Accumulation of DDP.** Because genistein is known to have other effects beside inhibition of tyrosine kinases, another tyrosine kinase inhibitor, halenaquinone, was tested as a modulator of DDP accumulation (32). Like genistein, halenaquinone increased 2008 cell net platinum accumulation in 1-h concurrent exposure experiments in a concentration-dependent manner (Fig. 3A). A significant increase (t test) was found at 50 μM halenaquinone (814 ± 53 pmol/mg protein) when compared to the control 2008 cells (623 ± 35 pmol/mg protein). Time course experiments in the presence of 50 μM halenaquinone showed that the maximum accumulation occurred after 2 h in both cell lines (Fig. 3B). Accumulation was increased 50 ± 1% by halenaquinone in 2008 cells and 62 ± 1% by halenaquinone in C13² cells. The data show that halenaquinone modulation of platinum accumulation in these cell lines was similar to the effect of genistein, with the exception that the delayed response was not seen in C13² cells.

**Membrane Permeability.** To explain the mechanism of the stimulation of DDP accumulation by the tyrosine kinase inhibitors genistein and halenaquinone, the effect of genistein on the general permeability of the plasma membrane was assessed using [³H]mannitol. Mannitol is a hydrophilic molecule that is not a substrate for any known carrier and penetrates biological membranes mainly by way of slow diffusion through the lipid bilayer (29, 30). Fig. 4A shows that the basal permeability of C13² cells to [³H]mannitol was significantly lower (t test) than DDP-sensitive 2008 cells, i.e., 928 ± 65 versus 1494 ± 110 nmol/mg protein, respectively. Concurrent exposure to 40 μM genistein for 2 h increased [³H]mannitol accumulation in the 2008 cells by 46 ± 6% (n = 4) but had no effect in either the C13² or the RH4 cells. The effect of genistein preincubation time on [³H]mannitol accumulation is shown in Fig. 4B. The time course of the modulation of [³H]mannitol accumulation by 40 μM genistein in 2008 cells was nearly identical to its effect on DDP accumulation (Fig. 2). Genistein did not increase the accumulation of [³H]mannitol in C13² cells at any time point. In this set of experiments, the mannitol content of 2008 cells after
phase pinocytosis was noted between the 2008 and C13* cell lines, as shown in Fig. 5A. The time course of LY accumulation in 2008 and C13* cells is shown in Fig. 5B. The effect of genistein on LY accumulation over 6 h (data not shown). When 2008: 0, C13* cells were treated with 40 μM genistein; three separate experiments; mean values from two to four experiments; bars, SE. The two substances; the line through the points (calculated by linear regression) had a correlation coefficient of 0.9010. The data were combined in Fig. 6A. A tight correlation existed between the accumulation of [3H]TPP* and the genistein concentration (μM). The effect of genistein on mitochondrial function. A, mitochondrial membrane potential as indicated by [3H]TPP* accumulation in high K+ medium. Cells (●, 2008; ○, C13*) were exposed to the indicated concentrations of genistein at the time of [3H]TPP* addition. B, oxygen consumption in digitonin-permeabilized cells. ●, basal consumption; □, with 40 μM genistein; fine hatch, succinate-dependent respiration; coarse hatch, succinate-dependent respiration with 40 μM genistein. Points, mean values of three separate experiments; bars, SE.

Fig. 6 Effect of genistein on mitochondrial function. A, mitochondrial membrane potential as indicated by [3H]TPP* accumulation in high K+ medium. Cells (●, 2008; ○, C13*) were exposed to the indicated concentrations of genistein at the time of [3H]TPP* addition. B, oxygen consumption in digitonin-permeabilized cells. ●, basal consumption; □, with 40 μM genistein; fine hatch, succinate-dependent respiration; coarse hatch, succinate-dependent respiration with 40 μM genistein. Points, mean values of three separate experiments; bars, SE.

2 h exposure to 40 μM genistein was 2525 ± 186 pmol/mg protein, an increase of 43 ± 9% from the untreated control. Fig. 4C shows that increasing the genistein concentration to 100 μM still had no effect on mannitol permeability in C13* cells, and that the RH4 cells were also refractory to modulation of passive permeability by genistein. To visualize the relationship between the time courses of genistein effects on DDP accumulation and mannitol accumulation in 2008 cells, the data were combined in Fig. 4D. A tight correlation existed between the accumulation of the two substances; the line through the points (calculated by linear regression) had a correlation coefficient of 0.9010.

Fig. 5 Fluid-phase pinocytosis as determined by LY accumulation. A, accumulation of LY versus time in 2008 (●) and C13* (○) cells. Points, mean values from two to four experiments; bars, SE. B, effect of genistein time on LY accumulation in 2008 (●) and C13* (○) cells. Cells were treated with 40 μM genistein for the indicated times and with 0.5 mg/ml LY for the last hour: Points, mean values from three experiments; bars, SE.

Effect of Genistein on Fluid-Phase Pinocytosis. To investigate whether the modulation of DDP accumulation by genistein could be explained by an effect on the ability of cells to engulf external medium, cells were incubated with LY, an impermeable fluorescent dye used as a marker of fluid-phase pinocytosis. The time course of LY accumulation in 2008 and C13* cells is shown in Fig. 5A. No difference in basal fluid-phase pinocytosis was noted between the 2008 and C13* cell lines. In addition, concurrent exposure to 40 μM genistein had no effect on LY accumulation over 6 h (data not shown). When cells were preincubated with genistein for varying amounts of time and LY added for the last hour of incubation, LY accumulation seemed to be increased with a maximum at 2 h after genistein exposure in both cell lines (Fig. 5B). However, the 2-h values were not significantly different from the values in cells that were not incubated with genistein. If all of the cell-associated LY arose through fluid-phase pinocytosis, then the approximate 40–50 ng/mg protein of LY at 1 h represented 80–100 pl of fluid internalized. A fluid-phase pinocytosis rate of 80–100 pl/mg protein/h could only account for 8–10 pmol platinum/mg protein of the platinum accumulated after a 1-h exposure to 100 μM DDP.

Effect of Genistein on Mitochondria. Changes in the mitochondria of C13* cells are central to their resistant phenotype. Although the DDP accumulation defect is not linked to the mitochondrial alterations (25), it was important to determine whether the differences in the response of 2008 and C13* cells to genistein as detected by DDP and mannitol accumulation were linked to the mitochondrial defects. Fig. 6A shows that genistein had the same effect on the mitochondrial membrane potential in both cell lines. The potential was depolarized...
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C13* cells was not reproducibly observed. Fig. 7B shows that a 2-h preincubation of the 2008 cells with 40 μM genistein was able to block the phosphorylation of proteins at Mr 130,000, 118,000, 73,000, and 71,000 (Lane 2) induced by a 10-min exposure to 10 nm EGF (Lane 1). Likewise, in C13* cells genistein blocked EGF-stimulated hyperphosphorylation of proteins at Mr 118,000, 73,000, and 71,000 (Fig. 7B, Lane 6). As noted in Fig. 7A, the Mr 130,000 immunoreactive band was missing in the C13* cells, and the Mr 183,000 and 170,000 bands were much fainter. Curiously, the bands at Mr 183,000 and 170,000, presumed to represent the EGF and erbB-2 receptors, were more phosphorylated in the genistein-treated cells.5 Exposure to 100 μM DDP did not change the EGF-stimulated hyperphosphorylation pattern or the inhibiting effect of genistein in either cell line (Fig. 7B, Lanes 3, 4, 7, and 8).

Genistein Modulation of DDP Sensitivity. The effect of genistein on DDP sensitivity in 2008 and C13* cells was investigated by colony-forming assay. Two days after seeding, cells were treated with 40 μM genistein for increasing lengths of time with DDP added for the last hour of incubation (Fig. 8A). With the 2008 cells, the survival significantly decreased (t test) from 71 ± 5% when treated with 1 μM DDP alone to 43 ± 4% when exposed to genistein for 1 h before and 1 h during the DDP exposure (n = 4). With C13* cells, the survival significantly decreased (t test) from 77 ± 4% when treated with 10 μM DDP alone to 52 ± 3% when exposed to genistein for 4 h (n = 4). For clinical applications of this work, i.e., combination chemotherapy of isoflavones with DDP, it would be encouraging to know whether the interaction of genistein and DDP on colony survival was synergistic. Median effect analysis of the interaction between these two drugs gave ambiguous results (Fig. 8B). In the 2008 cells, the interaction was additive or antagonistic (CI ≥ 1). In C13* cells, the interaction was antagonistic at low levels of colony inhibition but synergistic (CI < 1) when colony formation was inhibited by more than 50%. Cells were also incubated with graded concentrations of DDP alone for 1 h or incubated with 40 μM genistein for 1 h and then with both drugs during the second hour, the conditions that had the maximal effect on DDP accumulation in 2008 cells. Fig. 8, C and D, show that under these conditions, genistein decreased the IC50 of DDP in 2008 cells 9.6-fold from 4.1 ± 0.1 μM to 0.5 ± 0.1 μM (n = 3). Likewise, in the C13* cells, genistein decreased the IC50, 4.7-fold from 49.3 ± 8.3 μM to 10.6 ± 4.6 μM (n = 3). When the genistein concentration was varied during a 2-h concurrent exposure, the maximal enhancement of DDP cytotoxicity was found at 40 μM genistein for both 2008 and C13* cells (data not shown).

DISCUSSION

Much speculation surrounds the mechanism by which DDP enters cells (2, 33). Numerous studies have demonstrated that DDP accumulation can be stimulated or inhibited by pharmacological agents and the modulation of signal transduction pathways (2, 33). These data have been interpreted to mean that at

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5 Although the increased phosphorylation of the Mr 170,000 and 183,000 bands is not obvious in the figure, it was observable in the original film.
least a portion of DDP accumulation is mediated by a carrier or channel because it seems unlikely that so many diverse treatments could be modulating the passive diffusion of DDP through the plasma membrane. Nonetheless, one cannot dismiss the fact that definitive evidence of a carrier such as saturable accumulation, inhibition with structural analogues, or trans-stimulation has never been shown, despite regular attempts (23, 34–38). Conversely, little solid evidence exists that the accumulation of DDP is affected primarily by the passive permeability of the plasma membrane, or that accumulation defects in resistant cells can be explained by a decrease in plasma membrane passive permeability.

The experiments reported here show that DDP accumulation in a human ovarian carcinoma cell line can be modulated by a dietary isoflavone. Numerous important conclusions can be drawn from this data. Under exposure conditions that clearly caused inhibition of tyrosine kinase activity in these cells, genistein stimulated DDP accumulation by increasing the passive permeability of the plasma membrane. This was demonstrated by the effect of genistein on the accumulation of mannitol, which tightly paralleled the effect of genistein on DDP accumulation. Mannitol is a compound with no known way of entering cells other than simple diffusion (29, 30). Although genistein has other effects besides tyrosine kinase inhibition, we do not believe that these are contributors to the mechanism that modulates accumulation. For instance, genistein inhibits tyrosine phosphatase II, but it is difficult to imagine how this could affect plasma membrane permeability in the time frame of the experiment. In further support of this belief, the tyrosine kinase inhibitor helenalquione, which represents a different class of inhibitor than the isoflavones, evoked very similar effects on DDP accumulation. Unlike genistein, however, the maximal effect of helenalquione in C13* cells occurred at the same time as in 2008 cells. The explanation for this difference is not known.

The response of C13* cells to genistein as indicated by both DDP and mannitol accumulation was markedly different than that of 2008 cells. The ability of genistein to stimulate DDP accumulation was muted and was delayed 2 h compared to parental 2008 cells. Although genistein increased mannitol accumulation in the 2008 cells by 46%, it had no effect on mannitol accumulation in C13* or RH4 cells. Thus, a second conclusion is that C13* cells have an alteration in a tyrosine kinase-mediated signal transduction pathway that can modulate DDP accumulation. The possibility that genistein was not accumulated as efficiently by C13* cells as by 2008 cells was ruled out by the immunoblotting experiments, which showed that genistein very effectively blocked EGF receptor-mediated phosphorylation in C13* cells (Fig. 7).

Another important conclusion from these experiments is that C13* cells are less permeable to passively diffusing small molecules. The accumulation of mannitol in C13* cells was 38% less than 2008 cells after 2 h of exposure. It remains to be demonstrated whether other passively diffusing small molecules have diminished accumulation in C13* cells, but this offers an attractive mechanism for the DDP accumulation defect without invoking carrier proteins. Several investigators have speculated that changes in certain membrane proteins in DDP-resistant cells represent DDP transporters (15, 16, 39), but it is difficult to believe in DDP-carrier proteins without classic kinetic evidence of carrier-mediated transport. The underlying mechanism for the decrease in the passive permeability of C13* cells to mannitol is not known but could be due to changes in the lipid composition of the plasma membrane, the underlying structure of the cytoskeleton, or changes in the phosphorylation status of critical membrane proteins. Changes in the microtubules in C13* cells have been noted previously (11). An earlier report also documented subtle changes in the lipid composition of the plasma membrane of a precursor cell line to the C13* cells (40).

DDP accumulation exhibits many characteristics that are consistent with fluid-phase pinocytosis, i.e., it is energy dependent, temperature dependent, nonsaturable, and modulated by various signal transduction pathways (2, 31, 41–43). The accumulation of a compound that entered cells by fluid-phase pinocytosis would not be inhibited by structural analogues. Therefore, the hypothesis that inhibition of tyrosine kinase activity by genistein was stimulating pinocytosis and hence DDP accumulation was tested using LY as a marker of fluid-phase pinocytosis. No difference in LY accumulation was found between 2008 and C13* cells, and 40 µM genistein had no effect on pinocytosis in 2008 cells. Additionally, the 80–100 µM drug protein/h of fluid engulfed could only account for 1–2% of the total platinum accumulated during a DDP exposure. We, therefore, conclude that fluid-phase pinocytosis plays an insignificant role in the accumulation of DDP.

A major portion of the DDP resistance in C13* cells is mediated by the mitochondria (24, 25). Genistein has been reported to affect mitochondrial activity (44). Two approaches were used to determine whether the different effect of genistein on DDP accumulation in these cells was in any way mediated by the altered mitochondria. First, the effect of genistein on DDP accumulation in RH4 cells was demonstrated to clearly inhibit the effect of genistein on DDP accumulation in C13* cells. Second, the effect of genistein on respiratory activity was assessed. Genistein inhibited the basal oxygen consumption rate but stimulated succinate-dependent respiration. The effects were similar in 2008, RH4, and C13* cells. Together, these data indicate that the effect of genistein on DDP accumulation was not mediated through the mitochondria.

The conditions of genistein exposure that maximally affected DDP accumulation, passive permeability, and cytotoxicity in the 2008 cells were demonstrated to clearly inhibit the tyrosine kinase activity induced by EGF exposure. The inhibition, however, was only observed with secondary targets and not with the EGF receptor itself. Genistein actually intensified the autophosphorylation of the EGF receptor, suggesting that autophosphorylation was enhanced when downstream phosphorylation was blocked by genistein. This is consistent with reports that although genistein clearly obstructs EGF receptor autophosphorylation in cell-free incubations, in some intact cells it only inhibits downstream targets of the EGF receptor kinase (45, 46). Although the EGF receptor kinase was used to document an inhibitory effect of genistein on tyrosine kinases in 2008 and C13* cells, it is unlikely that the signal cascade initiated by the EGF receptor kinase is responsible for modulating accumulation because Christen et al. (47) reported that EGF stimulation does
not alter DDP accumulation in these same cells. Differences were noted in the phosphotyrosine pattern between 2008 and C13* cells. Pietras et al. (48) reported that the C13* cells have reduced levels of immunoreactive p135HER-2neu receptor. Thus, it is interesting to note that our study has also shown that C13* cells had diminished immunoreactive phosphotyrosine residues at M, 170,000 and 183,000. The identity of the other phosphotyrosine containing bands are unknown, although one could speculate that the M, 153,000 band might be phospholipase C (21).

Previous work has shown that the activation of protein kinase C, protein kinase A, and EGF signal transduction pathways and down-regulation of protein kinase C can modify the cellular pharmacology of DDP (12, 13, 47, 49, 50). We have now shown that the inhibition of tyrosine kinase systems can also affect both DDP accumulation and cytotoxicity. The biochemical origin of the DDP accumulation defect in resistant cells has remained undefined. The data presented here suggest that investigations of changes in the passive permeability of the plasma membrane should be pursued vigorously in accumulation-defective cells. Concurrently, the delayed and diminished response of DDP accumulation to genistein and the differences in the phosphotyrosine pattern in C13* cells suggest that the decreased accumulation in these cells might be attributable to alterations in cellular tyrosine kinase or tyrosine phosphatase activities that affect plasma membrane functions. It remains to be seen whether the other numerous modulators of DDP accumulation are also modulating the passive permeability of the plasma membrane.

Genistein is the aglycone of genistein, an isoflavone found in significant quantities in soy products (18, 19). Genistein is an antioxidant and antiestrogen and has been proposed to be anti-carcinogenic (45, 51). It has also been shown to inhibit tyrosine kinases, topoisomerase II, phospholipase C, and angiogenesis (20–22, 52). Despite these promising pharmacological properties, the pharmacokinetics and toxicology of genistein have not been well described. A recent study reported that plasma concentrations of genistein in women 6.5 h after an oral dose of 0.88 mg/kg genistein (as soy flour) were 2.15 ± 1.33 μM (53). Although this concentration is an order of magnitude lower than that needed to demonstrate effects on the cellular pharmacodynamics of DDP in our study, neither the lethal dose in animals nor the maximally tolerated dose of genistein in humans seems to be known. The present study, coupled with the significant therapeutic potential of genistein alone as an anticancer agent, make it particularly attractive to develop either this compound or an analogue in combination with DDP-based regimens.

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