Gallic acid sensitizes paclitaxel-resistant human ovarian carcinoma cells through an increase in reactive oxygen species and subsequent downregulation of ERK activation

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Abstract. Paclitaxel (PTX) is currently used as a front-line chemotherapeutic agent for several types of cancer, including ovarian carcinoma; however, PTX-resistance frequently arises through multiple mechanisms. The development of new strategies using natural compounds and PTX in combination has been the aim of several prior studies, in order to enhance the efficacy of chemotherapy. In this study, we found the following: (i) gallic acid (GA), a phenolic compound, potentiated the capacity of PTX to decrease proliferation and to cause G2/M cycle arrest in the PTX-resistant A2780AD ovarian cancer cell line; (ii) GA exerted a pro-oxidant action by increasing the production of reactive oxygen species (ROS), and co-treatment with the antioxidant agent N-acetyl-L-cysteine (NAC) prevented GA+PTX-induced cell proliferation inhibition and G2/M phase arrest; (iii) PTX stimulated ERK phosphorylation/activation, and co-treatment with the MEK/ERK inhibitor PD98049 potentiated the proliferation inhibition and G2/M phase arrest; (iv) and finally, GA abrogated the PTX-induced stimulation of ERK phosphorylation, a response that was prevented by co-treatment with NAC. Taken together, these results indicate that GA sensitizes PTX-resistant ovarian carcinoma cells via the ROS-mediated inactivation of ERK, and suggest that GA could represent a useful co-adjuvant to PTX in ovarian carcinoma treatment.

Introduction

Ovarian carcinoma represents the sixth most common type of cancer and the fifth leading cause of cancer-associated deaths in developed countries. It affects ~204,000 women annually worldwide, and is responsible for ~125,000 deaths (1). Among the drugs currently used in ovarian cancer therapy are taxanes, a family of natural diterpenes that bind tubulin and promote microtubule stabilization in the polymerized state. One of these agents is paclitaxel (PTX), a natural product present in the Pacific yew, Taxus brevifolia. By causing microtubule stabilization, PTX blocks cell cycle progression at the G2/M phase, inhibiting proliferation and inducing cellular apoptosis (2-4). While chemotherapy is the most common approach to cancer treatment, its efficacy often includes drawbacks. One of these is the necessity of dose reduction in order to avoid unwanted toxicity, which limits the drug efficacy in monotherapy. Another is the generation of drug resistance upon prolonged treatment; prolonged PTX treatment may induce a multi-drug resistance phenotype, which represents a serious obstacle in ovarian cancer treatment (5-7). Hence, there is an urgent requirement to implement novel strategies to potentiate PTX efficacy, allowing a decrease in the effective dosage and the circumvention of resistance mechanisms.

Polyphenols and phenolic compounds represent a large collection of molecules present in the plant kingdom. At the low doses attainable through dietary intake, these compounds exert multiple protective functions, including against inflammation and tumorigenesis. Conversely, at high, albeit still pharmacologically attainable, concentrations, many phenolic compounds may promote the death of cancer cells, modulating key elements in signal transduction pathways linked to apoptosis (8). In this regard, gallic acid (GA), a phenol of natural origin with antioxidant activity, isolated from Caesalpinia mimosoides, exerts antitumor action in cholan-
giocarcinoma cell lines. GA was found to induce cell death in promyelocytic leukemia HL-60RG cells; morphological and biochemical studies indicated that the induced cell death occurs via apoptosis (9,10). Concerning biochemical mechanisms, phenols normally behave as antioxidant molecules, which may account for the aforementioned protective actions; but under some circumstances they may also behave as pro-oxidant agents. GA has been shown to prevent oxidative stress, but also to increase ROS production, depending on the experimental conditions (11,12). This is notable, since ROS are involved in the regulation of many physiological and pathological processes, including cell proliferation, invasion, cell death, tumor hypoxia, and drug resistance (13,14). A number of these effects can be explained by ROS-mediated upregulation or downregulation of critical protein kinase activities, such as PI3K/Akt, MEK/ERK and p38-MAPK (15-17). With these antecedents, we aimed to analyze the capacity of GA to improve the anti-proliferative action of PTX in ovarian carcinoma cells. Two cell models were used, namely the A2780 cell line and a doxorubicin-resistant variant A2780AD, which overexpresses P-glycoprotein (18,19). Our results indicated that A2780AD cells are less sensitive to PTX than A2780 cells, and that the cytostatic action of PTX is increased in both cell models via co-treatment with GA, which potentiates the PTX-induced G2/M phase arrest. Using the drug-resistant cell line, we also demonstrated that proliferation inhibition and G2/M phase arrest are mediated by GA-provoked ROS overproduction, and by ROS-mediated inhibition of PTX-provoked ERK activation.

Materials and methods

Reagents and antibodies. All components for cell culture were obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). 2’7’-Dichlorodihydrofluorescein diacetate (H$_2$DCFDA) and propidium iodide (PI) were obtained from Molecular Probes (Thermo Fisher Scientific, Inc., Eugene, OR, USA). The protein kinase inhibitors SB203580, PD98059 and SP600125, and the rabbit polyclonal antibodies for phospho-p38-MAPK (Thr180/Tyr182) (catalog #9211), phospho-Akt (Ser473) (catalog #4060), and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (catalog #4370), were obtained from Cell Signaling Technology (Danvers, MA, USA). All components for cell culture were purchased from Sigma-Aldrich Quimica SL (Madrid, Spain). GA and PTX were dissolved in deuterated dimethylsulfoxide (DMSO-D6) at 20 mM and the final concentration was obtained by successive dilutions with culture medium.

Cells. The human ovarian carcinoma A2780 cells (drug-sensitive) and A2780AD cells (multi-drug-resistant ovarian cancer) included in this study were a generous gift from Dr P. Giannakakou, Weill Cornell Medical College (New York, NY, USA). Cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 40 µg/ml gentamicin and penicillin-streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO$_2$.

Cell viability. To determine the cytotoxic effect of the compounds on cell lines A2780 and A2780AD, 10,000 cells/well were seeded in a 96-well cell culture plate, and treated for 48 h with the desired concentration of GA and PTX, alone or in combination. At the end of treatment, 20 µl MTT (2.5 mg/ml) was added to each well and the plate was incubated for 4 h at 37°C. The reaction was then stopped by adding 100 µl of MTT solubilizer 10% SDS (sodium dodecyl sulfate) and 45% DMF (N,N-dimethylformamide) pH 5.5 (20). The plate was incubated at 37°C overnight to dissolve the formazan precipitates, and the absorbance of each well was measured at a wavelength of 595/690 nm in an Appliskan (Thermo Electron Corporation, Vantaa, Finland) plate reader. The targets used were wells without cells and the growth controls were wells with cells containing the same proportion of DMSO present in the wells with the treatments. The results were analyzed in the GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) and were expressed as the mean ± standard error of several independent experiments.

ROS production. To measure the drug effects on the relative intracellular ROS accumulation, at the end of the treatments (24 h), the cells were washed twice with PBS and incubated for 30 min at 37°C with RPMI-1640 medium containing 5 µM H$_2$DCFDA, a non-specific ROS-sensitive probe. H$_2$DCFDA is the cell is cleaved by intracellular esterases, producing the membrane-impermeable product H$_2$DCF, which accumulates into the cell. H$_2$DCF is not a fluorescent molecule, but is oxidized by intracellular ROS to give the fluorescent product DCF (21). The resulting fluorescence was then measured in a fluorimeter FluoroMax-2 (Horiba Scientific, Minami-ku Kyoto, Japan).

Flow cytometry. The effects of the drug on cell cycle progression were determined via flow cytometry. Following treatments (24 h), the cells were washed with PBS and fixed in 70% ethanol at 4°C for at least 1 h. The cells were then washed twice with PBS, resuspended in 500 µl PBS containing 60 µl/ml DNase-free RNase A and 50 µl/ml PI, and the fluorescence was measured using a Coulter Epics XL flow cytometer (Beckman Coulter Inc., Brea, CA, USA), as previously described (22). The resulting flow cytometry histograms were analyzed with the FlowLogic 7.0 program (Innui, Victoria, Canada), to obtain the relative percentages of cells at the G1, S and G2/M phases of the cell cycle. In addition to the typical cell cycle phases, the flow cytometry histograms showed a sub-G$_1$ population. This suggests reduction and damage of DNA related to cell death (23).

Western blotting. Following treatments (24 h), the cells were collected and lysed in lysis buffer (Tris-HCl 20 mM, pH 7.5, glycerol 10%, NaCl 137 mM, NP40 1%) containing a protease inhibitor cocktail (Thermo Fisher Scientific, Inc.), and the proteins were quantified. Equal amounts of protein were dissolved in 2X Laemml buffer with β-mercaptoethanol, heated to 95°C for 3 min and resolved via 10% SDS-PAGE. After electrophoretic separation, the proteins were transferred to a PVDF membrane using the Trans-Blot™ Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA), following which the membranes were blocked with 5% milk in TBS-Tween 20 for
1 h, and then incubated overnight at 4°C with the following primary antibodies: phospho-p38-MAPK (Thr180/Tyr182), 1:100 dilution; phospho-Akt (Ser473), 1:200 dilution; phospho-p44/42 MAPK (Erk1/2) Thr202/Tyr204, dilution 1:1,000. The membrane was then washed twice in TBS-Tween, and incubated for 1 h at room temperature with the corresponding secondary antibody (anti-rabbit IgG, HRP-linked, dilution 1:2,000). The proteins were visualized using the ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Inc.), using NZY Supreme ECL HRP Substrate (NZYTech, Lda., Lisbon, Portugal) as a developer. β-actin was used as the loading control.

**Statistical analysis.** Data were analyzed using the GraphPad Prism 5.0 statistical program (GraphPad Software, Inc.), and statistical differences between groups were evaluated using one-way analysis of variance followed by the Dunnett's test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Cell proliferation.** As an initial approach, we aimed to comparatively examine the effect of GA, in a concentration range of 10-200 µM, on total cell proliferation activity, as measured by the number of viable cells in sensitive (A2780) and multi-drug-resistant (A2780AD) ovarian carcinoma cell cultures. As indicated in Fig. 1A, GA caused a concentration-dependent reduction in the percentage of viable cells, an effect that was of lower intensity in the resistant cell line. The approximate reduction rates were 25% with 50 µM GA in A2780 cells, and 20% with 100 µM GA in A2780AD cells. These concentrations were selected for the combination treatment assays with the respective cell lines in the following determinations of ROS generation, cell cycle and protein kinases.

Subsequently, we comparatively analyzed the effect of PTX, at concentrations ranging from 10-400 nM, alone and in combination with the previously indicated concentrations of GA. As depicted in Fig. 1B and C, PTX caused a drug-dependent decrease in the number of viable cells, which was less pronounced in the resistant cell line. Thus, treatment with 50 nM PTX caused an ~30% decrease in the number of A2780 cells, whereas 200 nM caused only an ~20% decrease in the number of A2780AD cells. In addition, we observed that PTX and GA cooperated in more than an additive manner to inhibit cell viability, even in the resistant A2780AD cell line. For example, 50 nM PTX plus 50 µM GA caused an almost 60% decrease in the number of viable cells in the A2780 cell cultures; and the same result was obtained using 200 nM PTX plus 100 µM GA in the A2780AD cell cultures. Therefore, the concentrations of 50 and 200 nM PTX were adopted for further experiments with the A2780 and A2780AD cell lines, respectively.

**ROS generation.** As aforementioned, phenolic agents often provoke oxidative stress in cultured tumor cells, which may be a determinant of proliferation inhibition and cell death. The generation of oxidative stress by GA and PTX, evaluated alone and in combination, was determined by measuring ROS production, using an H$_2$DCFDA ROS-sensitive probe. The results are indicated in Fig. 2A and B. Treatment with PTX alone did not significantly affect ROS production, while treatment with GA, either alone or in combination with PTX, caused an approximate 2-fold increase. At the drug concentrations used, the results were approximately the same in the two cell lines.

In order to investigate the role of ROS overproduction in the inhibition of cell proliferation, we used the antioxidant/ROS-scavenging agent NAC. The results presented in Fig. 3 indicate that NAC markedly attenuated the decrease in viability caused by the combination of PTX plus GA in the A2780AD cell cultures, in such a manner that the decrease in viability caused by the NAC + PTX + GA triple combination was similar to that obtained with PTX alone. This seems
congruent with the observation that GA causes ROS overproduction, while PTX does not. Taken together, the results in Figs. 2 and 3 indicate that ROS overproduction is an essential determinant of the capacity of GA to potentiate PTX toxicity in drug-resistant ovarian cancer cells.

Cell cycle distribution. PTX is a potent microtubule-targeting agent known to cause mitotic cell cycle arrest (3). After establishing the inhibitory action of PTX and GA on total cell proliferation activity, as well as the protective action of NAC, we aimed to comparatively analyze the effects of these agents on cell-cycle progression in drug-resistant A2780AD cells. The flow cytometry assay results are indicated in Fig. 4. As hypothesized, treatment with PTX increased the proportion of cells in the G2/M phase, a response that was potentiated by co-treatment with GA. Of note, G2/M phase accumulation was suppressed by the presence of NAC.

In addition to discerning the distribution of the typical cell cycle phases (G1, S and G2/M), a flow cytometry assay can also reveal a subpopulation (sub-G1) of cells with reduced DNA content, normally interpreted as dead cells (23). In the present study, this subpopulation was negligible in cells treated with PTX and GA alone; but it reached a modest, albeit significant, value upon treatment with GA plus PTX. As in the case of G2/M, the sub-G1 fraction was suppressed by co-treatment with NAC. Taken together, these results suggest that the decrease in total cell proliferation in the PTX plus GA-treated cultures (Fig. 1B and C) is a consequence of both cell cycle blockade and induced cell death.

Protein kinases. It is known that Akt and ERK normally function as defensive protein kinases, which prevent apoptotic cell death while favoring cell proliferation/cell cycle progression (24-26). In addition, MAPK p38 is also activated in response to oxidative stress (27,28), and may behave as either a positive or negative regulator of apoptosis and the cell cycle, depending on the cell line and the experimental conditions (29,30). Thus, western blot assays were carried out in A2780AD cells in order to investigate the phosphorylation/activation of these kinases in response to GA and PTX, alone and in combination, and in the absence or the presence of NAC. The results depicted in Fig. 5 indicate that these treatments did not significantly affect Akt and p38 phosphorylation. In contrast, we observed that PTX stimulated ERK phosphorylation and that GA (which increased ROS production; Fig. 2) decreased the basal phosphorylation level of ERK and prevented the aforementioned PTX-induced increase. We also observed that the inhibitory action of GA in combination therapy was suppressed by the presence of NAC. Taken together, these results suggest that ROS negatively regulated ERK activation under experimental conditions used in the present study.

Finally, the functional importance of ERK activation as a regulator of cell proliferation and cell cycle progression was examined using the MEK/ERK inhibitor PD98059 (20 µM).
It was observed that PD98059, which was innocuous per se, potentiated the PTX-induced decrease in cell viability, alone and in combination with GA (Fig. 6A). Accordingly, PD98059 potentiated the G2/M arrest caused by GA plus PTX, although it did not potentiate, and even slightly reduced, the size of the sub-G1 cell fraction (Fig. 6B). Taken together, these results indicate that ERK activation exerts a defensive role, attenuating the excessive cytostatic action induced by PTX in A2780AD cells.

**Discussion**

The efficacy of chemotherapy is frequently hampered by the limited potency of antitumor drugs when used alone, and by the acquisition of a multi-drug-resistant phenotype after prolonged exposure. The use of adjuvants, such as selected phenolic compounds, may help to partially overcome these issues. To date, there has been great interest in the study of these compounds, particularly regarding their application in cancer therapies, alone or as adjuvants, and for their chemosensitizing effects (31-33).

In the present study, we investigated the ability of gallic acid (GA), a phenolic compound, to potentiate the anti-proliferative action of PTX using two ovarian carcinoma cell models: A2780 drug-sensitive cells and A2780AD cells, a drug-resistant variant due to P-glycoprotein overexpression. Our results showed that the anti-proliferative action of PTX was reduced in A2780AD cells, concordant with the multi-drug-resistance phenotype. In addition, the anti-proliferative action corresponded to cell cycle arrest in the G2/M phase, which reflects the action of PTX as a microtubule stabilizer. Notably, co-treatment with GA potentiated the anti-proliferative action and G2/M phase arrest induced by PTX in the A2780AD cells, in which it was demonstrated that the efficacy of PTX...
monotherapy was low. Furthermore, in the combined treatment, we observed an accumulation of cells in the sub-\(G_1\) phase, suggesting the induction of cell death. Prior reports demonstrated the antitumor effect of GA in certain cancer cell lines, and indicate that it could be a chemo-sensitizer and potentiate the cytotoxicity of PTX in ovarian cancer cells with a resistance phenotype (34,35).

ROS are a by-product of the respiratory chain, and at low levels have positive effects on cells (mitogenic, signaling, etc.). However, many agents, including phenolic compounds under certain conditions, can overinduce ROS, which can have deleterious effects on cells (36,37). It is known that GA has both antioxidant and pro-oxidant activities, which are responsible for inducing death in certain cancer cells (11). Our results showed that GA in A2780 and A2780AD cells induced the generation of ROS, presenting a pro-oxidant effect, and that co-treatment with NAC greatly attenuated the reduction in the number of viable A2780AD cells caused by PTX plus GA; in fact A2780AD cells exhibited similar response to treatment with PTX alone. This pro-oxidant effect of GA has also been shown in A549 lung cancer cells, wherein GA inhibited cell growth and increased the production of intracellular ROS (38-40).

Prior treatment of A2780AD cells with the antioxidant NAC prevented the combined effect of GA plus PTX on the \(G_2/M\) phase, showing a similar effect in those cells treated with only PTX.

The protective action of NAC confirms that ROS effectively mediate the anti-proliferative action and \(G_2/M\) phase arrest observed with the combined treatment.

It is known that ROS can regulate signaling pathways that promote cell survival, including the mitogen-activated protein kinase (MAPK) signaling pathway, which includes ERK and p38. Reports indicate that the downregulation of these pathways is important for inducing cell-cycle arrest and cell death (17,24). We examined the actions of p38, Akt and ERK inhibitors, and the results of western blot analysis revealed modulation only of the ERKs. We observed pERK activation by PTX, but also pERK inhibition by GA and the ability of GA to attenuate PTX-induced pERK activation. Reports have indicated that propyl gallate induces death in HeLa cells with concomitant inhibition of MAPK and promotion of ROS levels (41). In a study of osteosarcoma cells, it was observed

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Figure 6. Effect of MEK/ERK inhibitor on cell proliferation and cell cycle disruption. (A) Changes in the frequency of viable cells in A2780AD cell cultures treated for 24 h with PD 98049 (PD), PTX, and GA, alone or in combination. Values are represented in relation to untreated cells (arbitrary value of 100). (B) Cell cycle distribution in untreated A2780AD cell cultures (Cont) and cultures treated for 24 h with PD 20 \(\mu\)M and with GA plus PTX, alone and in combination. PD98094 was applied 30 min in advance to the other drugs. GA, gallic acid; PTX, paclitaxel.
that GA inhibited the activation of pERK and pAKT, regulators of the MAPK pathways, modulating cell proliferation, apoptosis, and angiogenesis (42).

The use of a commercial inhibitor of ERK (PD98059) on PTX-resistant A2780AD cells showed that, with the inhibition of ERKs, the effects induced by GA plus PTX, namely inhibition of cell proliferation and G2/M cell cycle arrest, were potentiated. Taken together, the results appear to suggest that the activation of ERKs by PTX acts as a defensive factor, attenuating excessive cytostatic action. Other reports have associated PTX with the activation of cell survival pathways, such as the MAPK p38/AKT and ERK signaling pathways, and, therefore, the inhibition of apoptosis. Furthermore, PTX promotes the expression of various survival factors that induce the phosphorylation and stabilization of surviving proteins (17,43-45).

Earlier studies indicated that ERK activation negatively regulates apoptosis in various cancer cell lines (39,40,46). In particular, co-treatment with MEK/ERK inhibitors enhanced PTX-induced apoptosis in breast, ovarian, lung and prostate cancer cells; and in the case of prostate cancer, apoptosis potentiation was accompanied by Bcl-2 inactivation and increased Bax expression (44,47,48). Thus, co-treatment with MEK/ERK inhibitors could be a clinically useful strategy against tumours with constitutively high or therapeutically induced ERK activation (42,49).

In conclusion, ROS overproduction negatively regulates ERK activation and it is also an essential determinant of the capacity of GA to potentiate PTX toxicity. Thus, the combined therapy of PTX plus GA may represent a novel strategy for combating cancer resistance, requiring lower doses as compared with PTX alone. The use of GA could be effective as an adjuvant in combination with PTX in ovarian cancer treatment.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LGM, PA and JNSC formulated the original ideas and working hypothesis, together with JFD designed the research study. JNSC participated in the entire experimental process of the study; MRH and IB supervised ROS production and antiproliferative studies; LA and ARE supervised the western blot assays; PL, MRH and PA participated in the flow cytometry analysis. All authors analyzed and interpreted the data. PA, LGM and JNSC wrote the manuscript and IFD and LA provided important reviews and considerations. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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