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Evaluation of a Gemcitabine-Doxorubicin-Paclitaxel Combination Schedule through Flow Cytometry Assessment of Apoptosis Extent Induced in Human Breast Cancer Cell Lines

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Combination chemotherapy with gemcitabine (Gem), doxorubicin (Dox), and paclitaxel (Pac) (GAT) has been considered attractive as first-line treatment in metastatic breast cancer. We compared the potential of various schedules of GAT to induce apoptosis on MDA-MB-231, MCF7, and T47D human breast cancer cell lines. The extent of apoptotic induction was analyzed by flow cytometry with 7-aminoactinomycin D (7AAD) staining. Differences between various schedules in terms of apoptotic induction were statistically significant (P<0.05). The most effective apoptotic induction regimen was achieved by the sequence: Dox for 16 h followed by Pac+Gem. Schedules employing a 16-h interval between drug administrations induced higher levels of apoptosis in human breast cancer cell lines compared with schedules using a 4-h interval. The therapeutic efficacy of the experimental results shown in this paper has been clinically corroborated in a phase II trial in metastatic breast cancer patients.

Key words: Breast cancer — Gemcitabine — Doxorubicin — Paclitaxel — Apoptosis

Clinical trials designed to evaluate the effectiveness and safety of new treatments for patients with all stages of breast cancer are under way. Combination therapy offers exciting possibilities of enhanced antitumor efficacy. In fact, the most effective proven chemotherapeutic regimens are combinations of active antineoplastic agents. Criteria for an effective combination include use of drugs with different mechanisms of action, relative non-cross-resistance, and partially non-overlapping toxicities. Tumor heterogeneity and the presence of subsets of cells resistant to certain drugs provide a rationale for treatment with multiple non-cross-resistant drugs. Several groups have reported that the combination of doxorubicin (Dox) plus paclitaxel (Pac) produces a high response rate, including complete responses, in metastatic breast cancer, but that this effect is offset by an 18% to 20% incidence of congestive heart failure.[1)] Furthermore, the schedule of Pac before Dox appears more toxic than that of Dox before Pac, in that Pac has been shown to increase the myocardial concentration of Dox if there is a relatively short interval between administration of the drugs.[2)] Gemcitabine (Gem) has also shown a wide range of antitumor activity and moderate toxicity in metastatic breast cancer, without cross-resistance with Pac and Dox.[3,4)] Thus, the combination of Gem-Dox-Pac is considered an attractive first-line treatment for these patients.

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It is now well documented that cytotoxic chemotherapy induces an increase in apoptosis within 24 h after the start of treatment.[5)] Malignant transformation of breast epithelial cells is associated with a dysregulation of proliferation/apoptosis control mechanisms. It seems that alterations in the genes involved in the apoptosis pathway play a crucial role in the process of progression and invasion in breast carcinogenesis.[6)] Therefore, the study of apoptotic induction in breast tumor cells, an important underlying mechanism of the antitumor activity of chemotherapeutic combinations, is a subject of growing research. In this work, we investigated experimentally the effect of the chemotherapeutic combination of Gem-Dox-Pac on apoptotic induction in human breast cancer cells.

The main objective of this study was to evaluate the ability of this chemotherapeutic combination (Dox+Pac+Gem) to promote apoptosis in 3 human breast cancer cell lines: 2 estrogen and progesterone receptor positive (MCF-7, T47D), and 1 estrogen and progesterone receptor negative (MDA-MB-231). Because the interaction of Dox, Pac, and Gem is highly schedule- and time-dependent, we attempted to find the combination schedule with the highest antitumor activity and the lowest cardiotoxicity by adjusting the interval between Dox and Pac administration.

These results could be of great interest to the design of optimal clinical treatment scheduling in breast cancer patients, which could improve the therapeutic efficacy of the combination. Thus, this *in vitro* study on human cell lines represents a rational step in the formulation of the most effective treatment schedule.

MATERIALS AND METHODS

Cell lines Breast carcinoma MDA-MB-231 (estrogen and progesterone receptor-negative), T47D, and MCF-7 cells (both estrogen and progesterone receptor-positive) were obtained from the American Type Culture Collection. All cells were grown as monolayers in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 2 m*M* L-glutamine, 10% heat-inactivated fetal bovine serum, and a 1% penicillin-streptomycin mixture, at 37°C in a 5% carbon dioxide atmosphere. Cells were grown to confluence and passaged with the use of trypsin ethylenediaminetetraacetic acid (EDTA). Cells in the exponential growth phase were used for all experiments.

Drugs Paclitaxel ("Taxol"), supplied in a "Cremophor" EL (polyoxyethylated castor oil, BASF Aktiengesellshaft, Ludwigshafen, Germany, and ethanol solution), was purchased from Bristol-Myers Squibb Oncology, Princeton, New Jersey. Doxorubicin ("Farmiblastina") was purchased from Pharmacia & Upjohn, Peapack, New Jersey. Gemcitabine ("Gemzar", Eli Lilly and Company, Indianapolis, Indiana) was supplied as a lyophilized powder and diluted with sterile sodium chloride 0.9% at a concentration of 20 mg/ml, divided into aliquots, and stored at −70°C until used. Drug stocks were freshly diluted in culture medium before each experiment.

Chemosensitivity assays Cell survival in untreated or treated cells was assessed by a 7-day MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma Chemical Company, St. Louis, Missouri) assay. MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml and the solution was filtered. Cells were collected during the exponential growth phase of culture by trypsinization and seeded at 2×10^3 cells per well in 96-well plates (100 μl of cell suspension per well). At 24 h after plating, 100 μl of fresh culture medium, with or without drugs, was added to the wells. Cells were exposed to the drugs for 72 h. Thus, this assay is a measure of longer-term cytotoxicity rather than apoptotic endpoints. At the end of drug exposure, the medium was removed from the plates and 200 μl of fresh culture medium, without drugs, was added to the cells and incubated for an additional 96 h. After incubation, 40 μl of stock MTT solution was added and the plates were incubated at 37°C in 5% carbon dioxide for 4 h. In cell culture, MTT is converted from a yellow-colored salt to a purple-colored formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenases, the activity of which is linearly related to the cell number. Acid-isopropanol (100 μl of 0.04 *N* HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the formazan crystals. Absorbance at 550 nm (reference wavelength of 620 nm) was determined in an enzyme-linked immunosorbent assay (ELISA) reader (Whittaker Microplate Reader 2001; Anthos Labtec Instruments, Salzburg, Austria). Survival was expressed as the percentage of viable cells in treated samples relative to nontreated control cells.

Analysis of apoptosis by flow cytometry with 7-amino-actinomycin D (7AAD) staining This assay was developed according to the method previously described by Schmid *et al.*[7] and validated by Philpott *et al.*[8] Briefly, 7AAD (Sigma Chemical Company) was dissolved in acetone and diluted in PBS to a concentration of 200 μg/ml. This solution was kept at −20°C and protected from light until use. A total of 100 μl of 7AAD solution was added to 10^6 cells suspended in 1 ml of PBS and mixed well. Cells were incubated in the dark for 20 min at 4°C and harvested by low-speed centrifugation. Finally, cells were resuspended in 500 μl of 2% paraformaldehyde solution and analyzed by flow cytometry using an EPICS Elite ESP flow cytometer (Coulter, Hialeah, Florida) within 30 min of fixation. Scattergrams were generated by combining

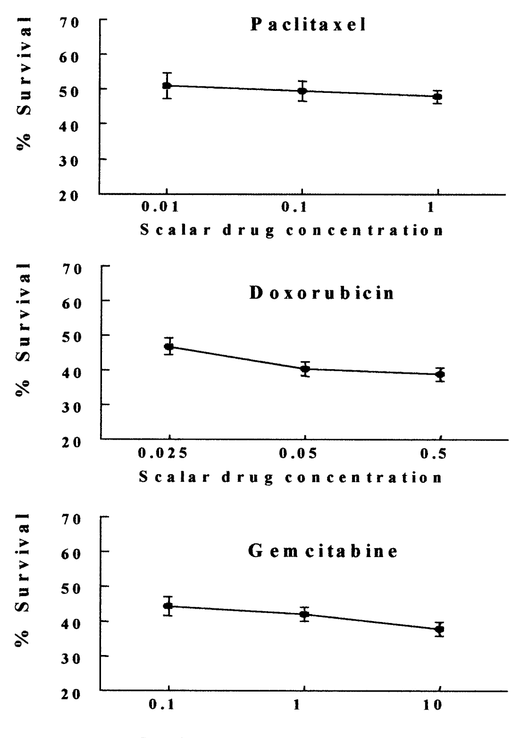

Fig. 1. Cell viability assessed by MTT assay. Dose-response curves of MDA-MB-231 cell lines exposed to single drugs (paclitaxel, doxorubicin, and gemcitabine) for 72 h. Results are mean values±standard deviation of three independent experiments in triplicate. Scalar drug concentrations (*X*-axis) are expressed in μg/ml.

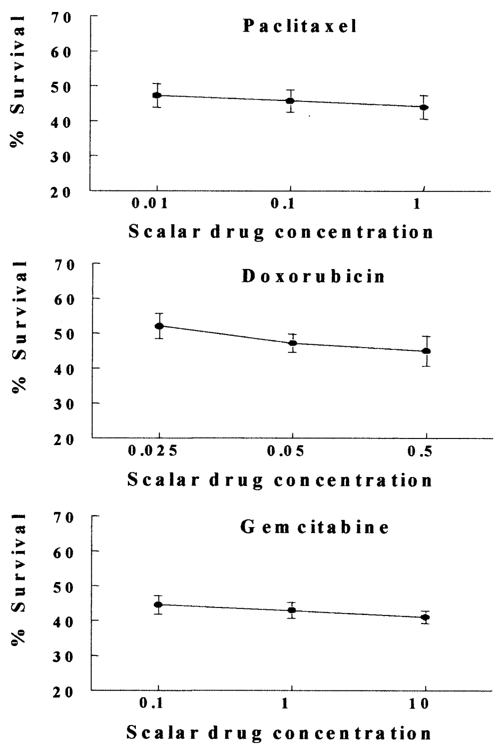

Fig. 2. Cell viability assessed by MTT assay. Dose-response curves of MCF7 cell lines exposed to single drugs (paclitaxel, doxorubicin, and gemcitabine) for 72 h. Results are mean values±standard deviation of three independent experiments in triplicate. Scalar drug concentrations (*X*-axis) are expressed in μg/ml.

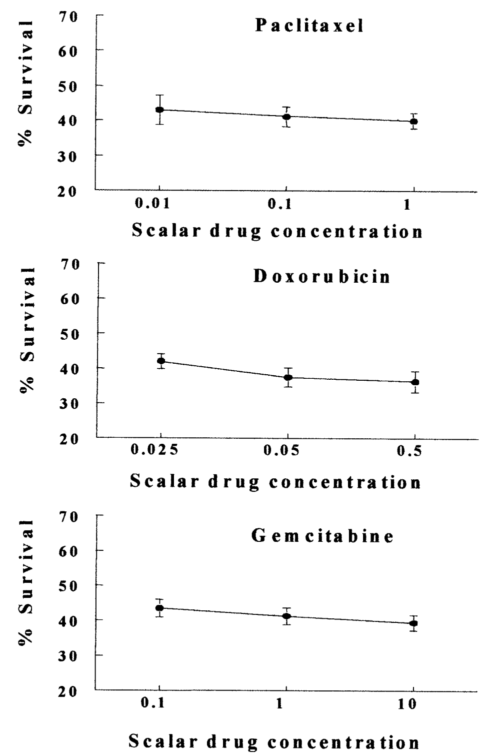

Fig. 3. Cell viability assessed by MTT assay. Dose-response curves of T47D cell lines exposed to single drugs (paclitaxel, doxorubicin, and gemcitabine) for 72 h. Results are mean values±standard deviation of three independent experiments in triplicate. Scalar drug concentrations (*X*-axis) are expressed in μg/ml.

forward light scatter with 7AAD fluorescence (FL3) and regions were drawn around clear-cut populations having negative (live cells), dim (early-apoptotic cells), and bright fluorescence (late-apoptotic/dead cells).

Following cell treatment, all cells were stained with 7AAD, which together with flow cytometry, allowed the easy quantitation of live, apoptotic, and late-apoptotic/dead cells. The whole nucleated cell population was analyzed. Unstained tumor cell lines were used as negative controls.

The 7AAD method and flow cytometry were used to assess the induction of apoptosis of breast cancer cells by each drug for an exposure period of 24 h. We also used this method to determine whether the induction of apoptosis by this drug combination could be improved by varying the sequence and schedules of Dox, Pac, and Gem. We used 4 schedules of drug administration: 1) cell exposure to Dox for 4 h, followed by Pac for 24 h, a 24-h washout period, and 24-h treatment with Gem; 2) exposure to Dox for 4 h, followed by Pac for 24 h, a 48-h washout period, and 24-h treatment with Gem; 3) exposure to Dox for 16 h, followed by Pac+Gem for 48 h, and a 48-h washout period; and 4) exposure to Dox for 16 h, followed by Pac+Gem for 96 h. Differences between treated cell lines were statistically analyzed by using the two-tailed Student's *t* test. A *P* value <0.05 was considered statistically significant.

RESULTS

Effect of individual drugs on cell growth The colorimetric MTT assay was used to determine the effect of individual drugs on cell survival of the three cell lines evaluated (MDA-MB-231, MCF7, and T47D) as shown in Figs. 1, 2 and 3. The scalar drug concentrations used were 0.01, 0.1, and 1 μg/ml for Pac, 0.025, 0.05, and 0.5 μg/ml for Dox; and 0.1, 1, and 10 μg/ml for Gem. The drug concentrations selected were close to the IC_{50} detected for each of the three cell lines employed in these experiments. This assay was previously performed in our laboratory to establish the optimal drug concentrations to be further used in the analysis of apoptotic induction (Figs. 1, 2 and

3). The extent of growth inhibition rates (% survival) for each drug was as follows: T47D > MCF7 > MDA-MB-231 for Pac; T47D > MDA-MB-231 > MCF7 for Dox; and a similar effect across the 3 cell lines for Gem.

Analysis of apoptosis induced by individual drugs The objectives were i) to analyze if Pac, Dox and Gem induce apoptosis in the three breast cancer cell lines tested, and ii) to determine if the apoptosis induced by these chemotherapeutic drugs is accurately assessed by the 7AAD method. Flow cytometric analysis using 7AAD was then used to assess the induction of apoptosis in breast cancer cells to a 24-h exposure of the individual drugs. The scalar drug concentrations used for MDA-MB-231, MCF7, and T47D cell lines and the corresponding drug-induced apoptotic effect of each drug are presented in Tables I, II and III, respectively. A wide range of concentrations of each of the chemotherapeutic drugs was used. Our results show that significant alteration of the apoptotic cell population was detected by this assay, and the percentage of early-apoptotic and late-apoptotic/dead cells increased in a dose-dependent manner for all drugs. We found that at low drug concentrations an increase of early apoptotic cells was observed while at high concentrations an increase of late-apoptotic/dead cells was found. It is important to note that differences in the sensitivity to chemotherapeutic drugs among the three breast cancer cell lines were observed. Thus, MDA-MB-231 cells were found to be highly sensitive to the apoptotic drugs compared to MCF-7 and T47D cells. The extent of apoptosis induced in tumor cell lines for all drugs tested was as follows: MDA-MB-231 > MCF7 > T47D.

Analysis of apoptosis induced by varying drug sequence and schedule Flow cytometric analysis using 7AAD was also used to assess the drug-induced apoptotic effect of different drug sequences and schedules on the three cell lines (MDA-MB-231, MCF-7 and T47D) (Tables IV, V and VI, respectively). The sequence/schedules that we compared in this study are: 1) Dox for 4 h, followed by Pac for 24 h, a 24 h washout period, and Gem for 24 h; 2) Dox for 4 h, followed by Pac for 24 h, a 48 h washout period, and Gem for 24 h; 3) Dox for 16 h, followed by (Pac+Gem) for 48 h, and a 48 h washout period; 4) Dox for 16 h, followed by (Pac+Gem) for 96 h. The scalar drug concentrations used for this evaluation were 0.01 μg/ml for Pac, 0.025 μg/ml for Dox, and 0.1 μg/ml for Gem. These doses were selected on the basis of results shown in Figs. 1–3 and Tables I–III. At these doses, we found that the drugs are cytotoxic by inducing

able I. Drug-induced Apoptosis in MDA-MB-231 Cell Lines retreated for 24 Hours

Treatment[a]	% Live cells	% Early-apoptotic cells	% Late-apoptotic and dead cells
Untreated control[b]	89.4±4.4	6.7±2.9	3.9±1.5
Paclitaxel (μg/ml)			
0.001	73	23.9	3.1
0.005	36.9	50.2	12.9
0.01	31.6	54	14.4
Doxorubicin (μg/ml)			
0.01	67.2	30	2.8
0.025	60	36.5	3.5
0.05	32.2	63.1	4.7
Gemcitabine (μg/ml)			
0.01	70.5	27	2.5
0.05	61	34.6	4.4
0.1	57.1	38.7	4.2

a) Cells were incubated with different concentrations of doxorubicin, paclitaxel, or gemcitabine; apoptosis was determined after 24 h by flow cytometry using 7-aminoactinomycin D (7AAD) staining. Results are expressed as the mean percentage of live, early-apoptotic, or late-apoptotic/dead cells of at least 3 experiments.
b) Mean percentage±standard deviation of experimental data detected in control samples.

able II. Drug-induced Apoptosis in MCF7 Cell Lines Prereated for 24 Hours

Treatment[a]	% Live cells	% Early-apoptotic cells	% Late-apoptotic and dead cells
Untreated control[b]	78.3±4.1	11.7±2.8	10±2.5
Paclitaxel (μg/ml)			
0.001	71.3	13.7	15
0.005	70.4	14.1	15.5
0.01	66	14.5	19.5
0.1	65.1	9.6	25.3
1	64.7	5.5	29.8
Doxorubicin (μg/ml)			
0.01	68	20.1	11.9
0.025	65	20.8	14.2
0.05	62.5	23.4	14
0.5	10.7	68.3	21
5	0	28.7	71.3
Gemcitabine (μg/ml)			
0.01	72	7.6	20.2
0.05	68.2	14	17.8
0.1	62.7	11.7	25.6
1	60.1	12.2	27.7
10	50.7	31	18.3

a) Cells were incubated with different concentrations of doxorubicin, paclitaxel, or gemcitabine; apoptosis was determined after 24 h by flow cytometry using 7-aminoactinomycin D (7AAD) staining. Results are expressed as the mean percentage of live, early-apoptotic, or late-apoptotic/dead cells of at least 3 experiments.
b) Mean percentage±standard deviation of experimental data detected in control samples.

Table III. Drug-induced Apoptosis in T47D Cell Lines Pretreated for 24 Hours

Treatment[a)]	% Live cells	% Early-apoptotic cells	% Late-apoptotic and dead cells
Untreated control[b)]	79.4±4.1	14.8±3.2	5.8±2.9
Paclitaxel (μg/ml)			
0.01	74.4	19.5	6.1
0.1	72.8	19	8.2
1	2	47	51
Doxorubicin (μg/ml)			
0.025	74.9	18.9	6.2
0.05	69.8	24.1	6.1
0.5	25.3	66	8.7
5	2	45.4	52.5
Gemcitabine (μg/ml)			
0.1	75.1	19.7	5.2
1	70.8	23.3	5.9
10	66.7	28.2	5.1

a) Cells were incubated with different concentrations of doxorubicin, paclitaxel, or gemcitabine; apoptosis was determined after 24 h by flow cytometry using 7-aminoactinomycin D (7AAD) staining. Results are expressed as the mean percentage of live, early-apoptotic, or late-apoptotic/dead cells of at least 3 experiments.
b) Mean percentage±standard deviation of experimental data detected in control samples.

Table IV. Apoptosis Induced by Varying Drug Sequence and Schedule in MDA-MB-231 Cell Lines

Treatment	% Live cells	% Early-apoptotic cells	% Late-apoptotic and dead cells
Untreated control[a)]	89.4±4.4	6.7±2.9	3.9±1.5
Dox (4 h) → Pac (24 h) → 24-h washout → Gem (24 h)	39.2	52.7	8.1
Dox (4 h) → Pac (24 h) → 48-h washout → Gem (24 h)	32.8	53.7	13.5
Dox (16 h) → Pac+ Gem (48 h) → 48-h washout	23.1	58.3	18.6
Dox (16 h) → Pac+ Gem (96 h)	24.5	56.6	18.9

Abbreviations: Dox, doxorubicin; Pac, paclitaxel; Gem, gemcitabine.
a) Mean percentage±standard deviation of experimental data detected in control samples.

Table V. Apoptosis Induced by Varying Drug Sequence and Schedule in MCF-7 Cell Lines

Treatment	% Live cells	% Early-apoptotic cells	% Late-apoptotic and dead cells
Untreated control[a)]	79.3±4.1	10.7±2.8	10±2.5
Dox (4 h) → Pac (24 h) → 24-h washout → Gem (24 h)	65.1	19.4	15.5
Dox (4 h) → Pac (24 h) → 48-h washout → Gem (24 h)	60.9	22.3	16.8
Dox (16 h) → Pac+ Gem (48 h) → 48-h washout	50.1	33.6	16.3
Dox (16 h) → Pac+ Gem (96 h)	44.3	34.2	25.5

Abbreviations: Dox, doxorubicin; Pac, paclitaxel; Gem, gemcitabine.
a) Mean percentage±standard deviation of experimental data detected in control samples.

Table VI. Apoptosis Induced by Varying Drug Sequence and Schedule in T47D Cell Lines

Treatment	% Live cells	% Early-apoptotic cells	% Late-apoptotic and dead cells
Untreated control[a)]	79.4±4.1	14.8±3.2	5.8±2.9
Dox (4 h) → Pac (24 h) → 24-h washout → Gem (24 h)	67.7	22.9	9.4
Dox (4 h) → Pac (24 h) → 48-h washout → Gem (24 h)	45.6	41.2	13.2
Dox (16 h) → Pac+ Gem (48 h) → 48-h washout	28.3	55.2	16.5
Dox (16 h) → Pac+ Gem (96 h)	31.5	54.7	13.8

Abbreviations: Dox, doxorubicin; Pac, paclitaxel; Gem, gemcitabine.
a) Mean percentage±standard deviation of experimental data detected in control samples.

apoptosis (assessed chiefly as early-apoptotic cells). In addition, similar concentrations of Pac, Dox and Gem were also used by Zoli *et al.*[9)] in their drug combination studies. The results show a similar effect of the sequence/schedules 3 and 4 with regard to apoptosis induction on the three cancer cell lines tested. If we compare sequence/schedules 1 and 2, the maximun apoptotic induction on the three cancer cell lines tested is achieved by the sequence/schedules 2. This is in agreement with the results obtained by Zoli *et al.*,[9)] as they observed that the maximum cytocidal effect was achieved by the sequence 2. When the sums of the early-apoptotic and late-apoptotic/dead cells among schedules were compared, the schedules using a 16-h interval between drug administrations (sequence/schedules 3 and 4) induced higher levels of apoptosis compared with those using a 4-h interval (sequence/schedules 1 and 2) ($P<0.05$). These results were again similar for the three cancer cell lines tested (MDA-MB-231, MCF-7 and T47D).

DISCUSSION

To date, the effectiveness of new combination chemotherapy protocols for breast cancer patients has been based on the results obtained from clinical trials. One of the most important endpoints of clinical trials is identification of the optimal schedule of sequential drug administration. Our current study suggests that *in vitro* assessment of the extent of apoptosis induced by the combination regimen could be a good tool for identifying the most effective treatment schedule.

Apoptosis and cell proliferation determine the growth dynamics of breast carcinomas, including their response to drugs. Ellis and coworkers[10, 11] showed that increased apoptosis is a common factor in breast cancer's response to chemotherapy and that this change in apoptosis may predict the clinical response. We focused our study on the interaction between Dox, Pac, and Gem in terms of their antitumor activity to treat breast cancer. We demonstrated that each drug inhibits the *in vitro* growth of MDA-MB-231, T47D, and MCF7 breast cancer cell lines (Figs. 1–3).

The capacity of Dox, Pac, and Gem to induce apoptosis in tumor cells is well established.[12–15] There are many ways to detect apoptosis: detection of typical morphologic features of the cell population by light or electron microscopy, time-lapse photography, detection of DNA fragmentation by gel electrophoresis, the TUNEL assay, and flow cytometry-based methods (for example, PI staining). In this study, flow cytometric analysis with 7AAD was used[7, 8] to determine the extent of drug-induced apoptosis on tumor cell lines. 7AAD stains live, apoptotic, and dead cells differentially because of the altered accessibility of DNA in each subpopulation. This method has several advantages over the methods outlined above: it gives information about cell numbers, it does not require dye permeation through the cell membrane, and moreover, large numbers of cells can be rapidly and accurately examined by flow cytometry. The main advantage is that it is able to identify early-apoptotic cells (cells retaining membrane integrity) separately from late-apoptotic/dead cells (membrane integrity has been lost). This is of particular interest in our study, where apoptosis is not induced simultaneously in all cells.

The utility of the 7AAD method is well established in the assessment of apoptosis in human tumors. Pallis and coworkers[16] used flow cytometric analysis with 7AAD to analyze the chemosensitivity of blasts from patients with acute myeloblastic leukemia and myelodysplastic syndromes. The 7AAD method was also performed to detect apoptosis induced in human malignant melanoma cell lines[17] and in breast cancer cell line MDA-MB-231.[18] The results we obtained using the 7AAD method suggest that it could be a good tool to detect and check the extent of apoptosis induced by chemotherapeutic drugs in human breast tumor cell lines. In all tumor cells lines, the extent of apoptosis induced by the individual drugs (Pac, Dox, or Gem) was MDA-MB-231 > MCF7 > T47D, and the increase in the percentage of early-apoptotic and late-apoptotic/dead cells was dose-dependent (Tables I, II and III). A wide range of drug concentrations was used to assess cell apoptosis and we found that, in general, at low drug concentrations an increase of early-apoptotic cells was detected while at high drug concentrations an increase of late-apoptotic/dead cells was observed. These results are in agreement with the statement that at high concentrations most chemotherapeutic drugs lead to the inhibition of tumor cell proliferation by the induction of necrosis.

We found only one study that presented *in vitro* results with the Dox-Pac-Gem combination.[9] They evaluated the cytotoxic effects of a combination of Dox, Pac, and Gem in BRC-230 and MCF7 human breast cancer cells. The maximum cytocidal effect was achieved by the sequence of Dox for 4 h, followed by Pac for 24 h, a 48-h washout period, and Gem for 24 h.

In this study, we have also presented data on the extent of apoptotic induction in MDA-MB-231, MCF7, and T47D human breast cancer cell lines by our proposed sequential combination of Dox, Pac, and Gem (Dox for 16 h, followed by Pac+Gem) and those proposed by Zoli and coworkers[9] (Dox for 4 h, followed by Pac for 24 h, a 24 h washout period, and Gem for 24 h; and Dox for 4 h, followed by Pac for 24 h, a 48 h washout period, and Gem for 24 h) (Tables IV–VI). Cells were stained with 7AAD and then analyzed by flow cytometry. The best schedule of drug administration for the promotion of apoptosis was achieved by the sequence/schedule: exposure to Dox for 16 h, followed by Pac+Gem. This drug combination scheme was shown to be active in both estrogen receptor-negative and -positive breast cancer cell lines, although there were significantly more apoptotic cells observed in estrogen- and progesterone-negative cells (MDA-MB-231) than in estrogen- and progesterone-positive cells (MCF7 and T47D). These data are in agreement with the results obtained when we used the individual chemotherapeutic drugs (Tables I–III).

In experimental estrogen deprivation conditions, as in this study, the apoptotic effects of the individual drugs and the chemotherapeutic combination schedules tested were higher in estrogen receptor-negative breast cancer cell lines (MDA-MB-231) than in estrogen receptor-positive cell lines (MCF7 and T47D). Zoli *et al.*[9] also reported a higher cytocidal effect in estrogen receptor-negative breast cancer cell lines than in estrogen receptor-positive cell lines. It is important to note that estrogen-negative breast cancers are highly aggressive and they must be treated with polychemotherapy. In this context, Rochefort *et al.*[19] found that estrogens in estrogen-positive breast cancers have a dual effect, since they stimulate tumor growth, but inhibit invasion and motility. On the other hand, no direct involvement of estrogen and/or progesterone receptors in apoptotic cell death susceptibility induced by chemotherapeutic drugs has been described so far.

Theoretically, Dox separated from Pac by a 16-h washout interval could minimize the inhibitory effects exerted by Pac on P-glycoprotein-mediated biliary clearance of Dox, which could reduce the risk of severe cardiotoxicity. The following phase II trial conducted by our group,[20, 21] supports our belief that the 16-h interval between Dox and Pac could reduce the risk of cardiotoxicity. The toxicity and activity of Dox at 30 mg/m^2 (day 1), Pac at 135 mg/m^2 (day 2), and Gem at 2500 mg/m^2 (day 2) given biweekly in a 28-day cycle for 6 cycles was evaluated as first-line treatment in 41 patients with metastatic breast cancer. Dox was administrated 16 h before Pac. The activity observed was extremely encouraging, with an overall response rate of 82.9% and a complete response rate of 43.9%. Median duration of response was 14.1 months and median time to progression was 13.9 months. Median survival was 26.2 months. Decreased left ventricular ejection fraction was observed in 3 patients, none of whom developed symptomatic heart failure. In addition, Frassineti and coworkers[22] did not observe any relevant clinical cardiotoxicity in patients who received Dox then Pac with a 16-h intervening interval.

In summary, we have shown that apoptosis induced by Pac, Dox, and Gem in breast tumor cell lines is accurately measured by 7AAD staining and flow cytometric analysis. We propose that the 7AAD method is a good tool to evaluate the impact of different schedules of drug administration on the apoptotic effect of drugs used in breast cancer treatment. We have found that a schedule of Dox followed 16 h later by Pac+Gem induces higher levels of apoptosis than drug schedules with a 4-h intervening period (statistically significant, $P<0.05$). The potential benefit of reducing the risk of cardiotoxicity with this schedule was corroborated in a phase II trial in metastatic breast cancer patients, in whom there was no clinical evidence of heart failure when Dox was administered 16 h before Pac.

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