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ORIGINAL ARTICLE

Probenecid is a chemosensitizer in cancer cell lines

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Abstract Resistance and toxicity are the major barriers to successful cancer chemotherapies. Developing molecules that reduce drug resistance and improve antineoplastic effects is of great interest for cancer research; ideally, these substances should not affect the pharmacodynamics of the chemotherapeutic agent while providing a synergistic antineoplastic effect. In this study, we tested in vitro co-administration of the antineoplastic agents cisplatin or paclitaxel with probenecid, an anion channel inhibitor, in a panel of cancer cell lines to determine the cytotoxicity and synergistic effects of these drug combinations. In addition, we measured the clonogenicity and apoptotic index in these cells. We observed a synergistic interaction between probenecid and the chemotherapeutic agents, and increasing doses of probenecid resulted in a significant decrease in the effective doses of the chemotherapeutic agents. For the antineoplastic agent and probenecid combinations, we found increased cell death, reduced colony formation, and a higher number of apoptotic cells, compared with treatment of cisplatin or paclitaxel alone. Further research is necessary to elucidate the molecular mechanisms by which the synergistic effect occurs. If these synergistic effects can be reproduced in vivo, the co-administration of probenecid with different chemotherapeutic agents may provide a valid treatment in patients with chemotherapy resistance.

Keywords Cisplatin · Paclitaxel · Probenecid · Synergism · Resistance

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Introduction

Chemotherapy remains the main therapeutic option for most types of cancers. However, this treatment modality is associated with important toxicity [1–7] and is prone to the development of resistance to anticancer drugs [8–10]. The major phenotype of multidrug resistance (MDR) is composed of a group of ATP-binding cassette (ABC) drug transporters that include P-glycoprotein (MDR1), several multidrug resistance proteins (MRP), and breast cancer resistance protein (BCRP) [11–14]. There has been an intense search for compounds that can act as MDR modulators to reduce the resistant phenotype of cancer cells and to increase the effectiveness of chemotherapeutic drugs. Compounds that act directly on the ABC transporter proteins to block their activity have been shown to reverse the MDR phenotype in cultured cells, animal models, and, more recently, patients. This research has allowed the identification of several potent molecules with long-lasting MDR reversal properties and minimal pharmacokinetic interaction with the co-administered cytotoxic agent [15–17]. Many trials of combination therapies have proved that this approach is a valid strategy for overcoming drug resistance and improving responses and cure rates [18–24].

Probenecid, a uricosuric agent used in the treatment of gout, acts on organic anion transporters, such as MDR1,

methotrexate (MTX) efflux, canalicular multispecific organic anion transporter (cMOAT), solute carrier family 22 member 6 (SLC22A6), solute carrier family 22 member 8 (SLC22A8), and MPR4 [25–32]. Probenecid is a good modulator of MRP activity and a potential candidate for clinical use to reverse MDR-associated MRP and to increase the effective serum concentrations of antibiotics, chemotherapeutics, and other medications [33–39].

This paper explores the possible use of probenecid as a sensitizer to commonly used chemotherapeutic drugs.

Materials and methods

Cell culture

The following human cancer cell lines were used: SH-SY5Y, T47D, and HeLa, which correspond to neuroblastoma, breast cancer, and cervical cancer, respectively. Cells were obtained from the American Type Culture Collection (ATCC), maintained as a monolayer at 37°C, and cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum in a humidified atmosphere of 5% (v/v) carbon dioxide in air.

Drugs and chemicals

Cisplatin, paclitaxel, and probenecid were obtained from Sigma Chemical Company (St. Louis, MO) and were dissolved in dimethyl sulfoxide (DMSO). Cell culture reagents and consumables were obtained from GIBCO (Gaithersburg, MD) or Corning (USA). All chemicals not otherwise specified were of the highest grade and were purchased from local suppliers.

Cytotoxicity assay

Cells were seeded in 24- or 96-well chamber dishes and exposed to several concentrations of cisplatin (5–100 μM), paclitaxel (5–100 nM), or probenecid (50–1,000 μM) for 24 h. Drugs were investigated for their activity as single agents and in combinations. The cisplatin and probenecid combination was used at a ratio of 1:10, and the paclitaxel and probenecid combination was used at a ratio of 1:10,000. After incubation, the medium was changed, and viable cells, which remained attached to the dish, were fixed in 70% ethanol at −20°C, washed in phosphate buffer saline (PBS) and stained with crystal violet (1% in water). After washing, the stain was solubilized in 33% acetic acid, and the absorbance was determined in a microplate reader at 570 nm. The analysis was performed at least in triplicate in three independent experiments.

Synergistic analysis

To evaluate the effects of combined drug treatments, the results from the cytotoxicity assays were analyzed with the CalcuSyn program (Biosoft, Cambridge, United Kingdom) to determine synergism between probenecid and either cisplatin or paclitaxel. The software uses a median-effect method, which is a well-established procedure to quantify the effects of drug combinations and to determine whether they produce greater effects together than expected from simple summation of their individual effects [40]. The combination index (CI) values are obtained from the data and reflect the nature of the interaction between either cisplatin and probenecid or paclitaxel and probenecid, that is, values <1 reflect synergistic activity, values equal to 1 reflect additive activity, and values >1 reflect antagonism.

However, within the categories of synergistic or antagonistic effects, there are different levels of drug interactions, which are dependent on CI values [41] (Table 1).

The dose reduction index (DRI) defines the possible extent of drug dose reduction in a combination for a given degree of effect, compared with the dose of each drug alone [42–44].

Clonogenicity assays

Cancer cell lines were exposed to cisplatin (10 μM), probenecid (100 μM), or a combination of both in a 1:10 ratio for 24 h. Similarly, cells were exposed to paclitaxel (0.01 μM), probenecid (100 μM), or both in a 1:10,000 ratio for 24 h. The cells were trypsinized and seeded in

Table 1 Recommended symbol for describing synergism or antagonism in drug combination studies analyzed with the combination index (CI) method

Range of CI	Symbol	Description
<0.1	+++++	Very strong synergism
0.1–0.3	++++	Strong synergism
0.3–0.7	+++	Synergism
0.7–0.85	++	Moderate synergism
0.85–0.90	+	Slight synergism
0 90–1 10	±	Nearly additive
1.10–1.20	−	Slight antagonism
1 20–1 45	−−	Moderate antagonism
1.45–3.3	−−−	Antagonism
3.3–10	−−−−	Strong antagonism
>10	−−−−−	Very strong antagonism

The combination index method is based on that described by Chou and Talalay [40, 43], and the computer software of Chou and Chou [41, 42], and CalcuSyn. The ranges of CI and the symbols are refined from those described earlier by Chou [43]. CI < l, =1, and >1 indicate synergism, additive effect, and antagonism, respectively [40, 42]

24-well chamber dishes at low density (100–200 cells/dish) for 14 days in an atmosphere containing 5% CO_2 in an incubator at 37°C. The cells were then fixed and stained with crystal violet. Total colony numbers and the percentage of colonies >4 mm in size were counted and analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland).

Detection of apoptotic cells

The percentage of apoptotic cells was determined by staining with ethidium bromide (EB) and performing a nuclear morphology analysis. Cells were exposed to different drugs alone or in fixed ratio combinations (cisplatin/probenecid; 1:10 or paclitaxel/probenecid; 1:10,000) for 24 h. Cells were trypsinized, centrifuged, and washed with 1 ml of cold PBS once, and the cell pellets were then resuspended in 25 µl of cold PBS and 2 µl of EB (100 µg/ml). Stained cell suspensions (10 µl) were placed on a clean microscope slide and covered with a coverslip. Cells were viewed and counted using a fluorescence microscope (Carl Zeiss, Germany) at 40× magnification. Tests were done in triplicate, counting a minimum of 100 total cells for each test.

Statistical analysis

All statistical analyses were performed using the statistical software program Prism 4 (GraphPad Software, Inc.). In clonogenicity assays, data analyses for multiple comparisons of drugs alone and in combination were performed using one-way ANOVA followed by Tukey's test. Statistical analysis was performed at the $P < 0.001$ (denoted as **) and $P < 0.01$ (denoted as *) levels of significance. In apoptosis assessment, the experimental and control groups were compared statistically using an unpaired two-tailed Student's t test. Statistical analysis was performed at the $P < 0.001$ (denoted as *).

Results

Cytotoxic effects and synergism analyses

Median-effect plot analyses (logarithm of fraction affected/fraction unaffected versus logarithm of dose) and calculation of the combination index (CI) versus fraction-affected (fa) plots were performed using the method of Chou and Talalay [40] with Calcusyn (Biosoft). In these analyses, we observed a dose–effect-dependent decrease in cell number, as measured by crystal violet assay (Fig. 1). The data were also examined using a median-effect analysis to determine the type of interactions produced, i.e., antagonism (CI > 1), additivity (CI = 1), or synergism (CI < 1). CI values were calculated for different dose–effect levels based on parameters derived from median-effect plots of cisplatin or paclitaxel alone or in combination with probenecid at fixed ratios (Fig. 2).

In SH-SY5Y cells, decreasing doses of cisplatin in combination with probenecid at fixed ratio of 1:10 showed synergism, with CIs of 0.63–0.25 for effect fraction affected ranging from 0.29 to 0.75. In T47D, we found nearly additive to moderate synergism, with CIs of 1.10–0.73 for fa ranging from 0.19 to 0.61. Less synergy was found in HeLa cells, with CIs of 0.96–0.61 for fa ranging from 0.20 to 0.65 (Table 2). Similar effects were found for paclitaxel in combination with probenecid at a fixed ratio of 1:10,000. In SH-SY5Y, a synergistic effect was observed, with CIs of 0.45–0.088 for fa ranging from 0.43 to 0.90, whereas, in T47D cells, a moderate synergistic effect was observed, with CIs of 0.44–0.85 for fa ranging from 0.28 to 0.70. Finally, a synergistic effect was observed in HeLa cells, with CIs of 0.35–0.20 for fa ranging from 0.35 to 0.86 (Table 3).

The combined effects of either cisplatin or paclitaxel with probenecid in the SH-SY5Y, T47D, and HeLa cell lines, as represented by the dose reduction index (DRI), the CI, and the dose–effect levels of cell growth inhibition (ED_{30}, ED_{50}, ED_{70}), are summarized in Table 4. The DRI showed a considerable dose reduction for the combinations of cisplatin or paclitaxel with probenecid as a result of the synergistic effects (Table 4). When using synergistic drug combinations at corresponding dose levels, the DRI indicated that the concentration of cisplatin or paclitaxel necessary to inhibit the growth of 50% of cancer cells (ED50) could be decreased 7.03-fold (SH-SY5Y, cisplatin/probenecid), 3.06-fold (T47D, cisplatin/probenecid), 1.88-fold (HeLa, cisplatin/probenecid), 4.60-fold (SH-SY5Y, paclitaxel/probenecid), 2.91-fold (T47D, paclitaxel/probenecid), and 4.40-fold (HeLa, paclitaxel/probenecid). The dose reduction level was different and specific to each combination and cell line (Table 4).

Colony-forming ability

To further support our findings, we performed clonogenic analyses. As shown in Fig. 3, probenecid alone induced a discreet loss of colonies in the three cell lines. Interestingly, addition of the uricosuric drug to either paclitaxel or cisplatin significantly increased the effects of the chemotherapeutic drugs (Fig. 3). This result was reproducible in the three cell lines. Our data are consistent with the dose reduction effects shown in the combined assays.

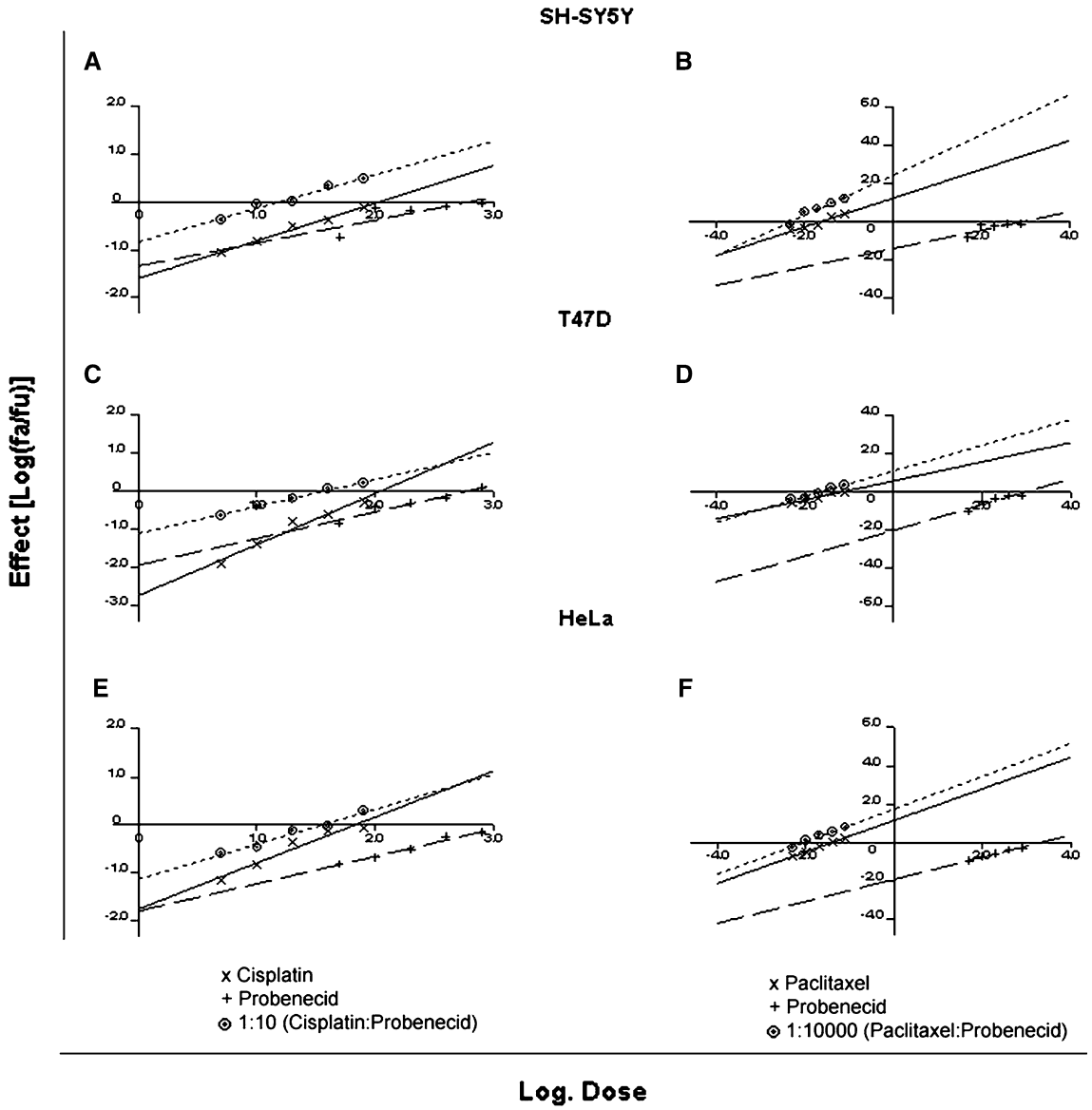

Fig. 1 Median effect plots of cisplatin, paclitaxel, or probenecid alone, or in combination at fixed ratios. All assays were done at least in triplicate in three independent experiments. Cellular death was determined by the crystal violet method. Effective concentration was analyzed using CalcuSyn software Cambridge, UK. **a**, **b** SH-SY5Y cells; **c**, **d** T47D cells; **e**, **f** HeLa cells

Apoptosis induction

Finally, to address the mechanism of the observed synergistic effects, we evaluated apoptosis induction by nuclear staining with ethidium bromide. We measured apoptosis induction for cells treated with cisplatin or paclitaxel in combination with probenecid at synergistic fixed ratio doses, compared with cells treated with single agents. Figure 4 shows a significant increase in the number of cells with nuclear condensation and segmentation for the cells exposed to either the combination of cisplatin-probenecid or paclitaxel-probenecid compared with those cells exposed to either cisplatin or paclitaxel alone.

Discussion

In this paper, we have demonstrated a synergistic effect between probenecid and two antineoplastic agents, cisplatin and paclitaxel. The drug combinations increased cell death by apoptosis and reduced the formation of colonies in human cancer cell lines.

For this study, drug synergy was quantified using the combination index equation of Chou and Talalay, which allows the evaluation of 2 or more chemotherapeutic agents at different concentrations and effect levels [41]. Through this methodology, combinations of drugs can be analyzed for synergy versus antagonism, as well as their maximal

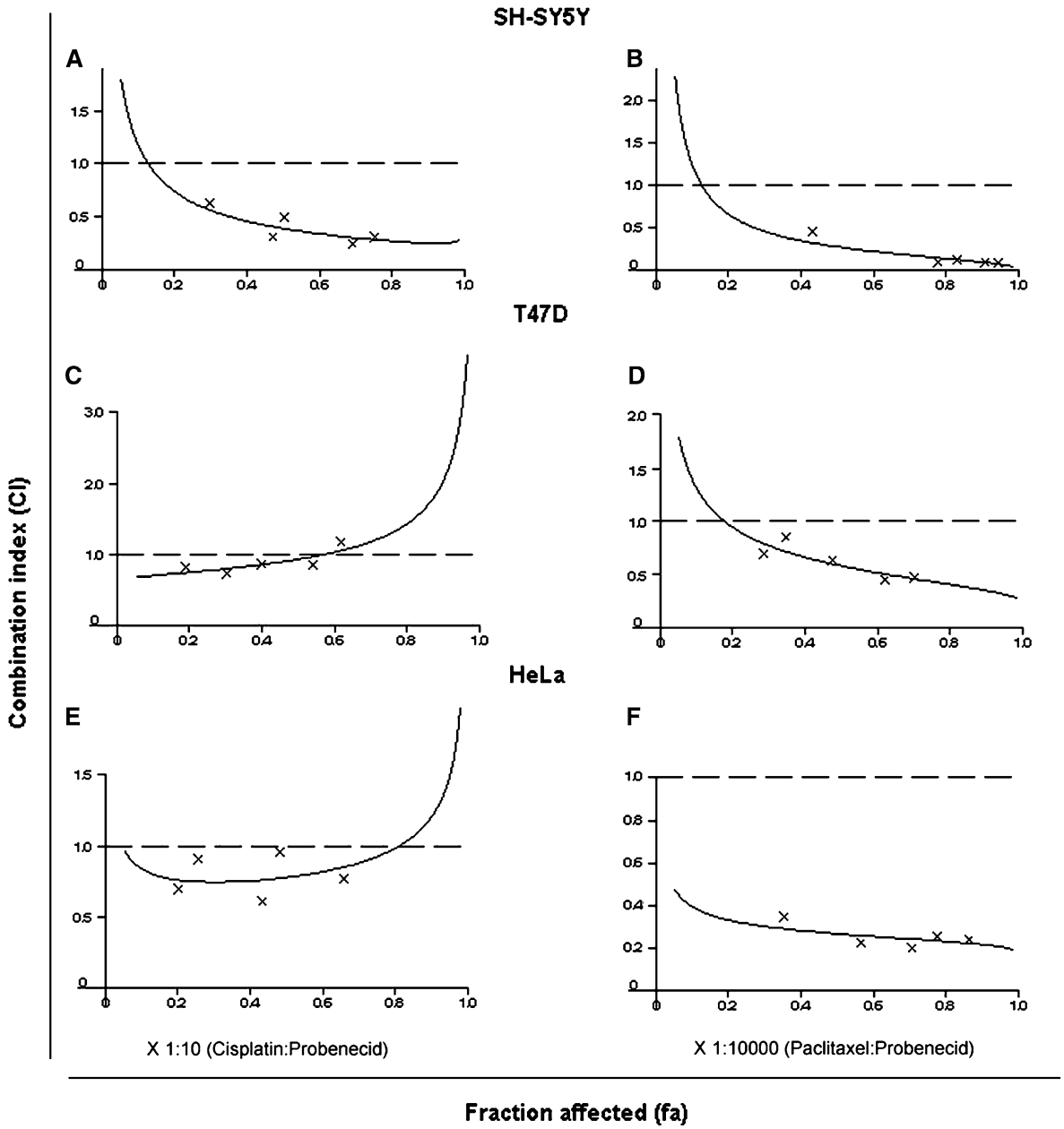

Fig. 2 Analysis of combined drug effects. To quantitative the interaction between the treatments, CI values versus cytotoxicity plots were generated for the cancer cell lines. **a**, **b** SH-SY5Y cells; **c**, **d** T47D cells; **e**, **f** HeLa cells. *Left-side* combination of cisplatin and probenecid at fixed ratio 1:10, *right-side* combination of paclitaxel and probenecid at fixed ratio of 1:10000. Xs represent combination data points. CI < 1, =1, and >1 indicates synergism, additive effect, and antagonism, respectively

antitumor efficacy. Such analyses have been the scientific basis for clinical protocol designs [45– 47]. New combinations of therapeutic treatments for cancer have been developed through the addition of a new drug to a preexisting, established regimen. Given the large number of new and old drugs available, this strategy is a useful and efficient way to identify combinations with significant synergistic interactions in vitro, thus narrowing the field of potential combinations that may have the greatest clinical benefit and therefore deserve to be tested in human trials.

In this study, the growth-inhibitory activities of cisplatin, paclitaxel, and probenecid, alone and in combination, were investigated in SH-5YSY, T47D, and HeLa cells. Our results indicate that cisplatin or paclitaxel alone mediated significant growth-inhibitory effects on the three cell lines tested in a dose-dependent manner; however,

Table 2 Combination index values and fraction affected for SH-SY5Y, T47D, and HeLa cell lines treated with cisplatin and probenecid

Cisplatin (μm)	Probenecid (μm)	CI SH-SY5Y	Fa SH-SY5Y	CI T47D	Fa T47D	CI Hela	Fa Hela
5	50	0.634	0.2977	0.824	0.1903	0.697	0.2037
10	100	0.315	0.4726	0.737	0.3033	0.909	0.2567
20	200	0.502	0.5039	0.86	0.4001	0.613	0.4350
40	400	0.256	0.6915	0.865	0.5402	0.962	0.4816
80	800	0.3l2	0.7521	1.106	0.6190	0.772	0.6592

CI indicates combination index, *Fa* indicates fraction affected

Table 3 Combination index values and fraction affected for SH-SY5Y, T47D, and HeLa cell lines treated with paclitaxel and probenecid

Paclitaxel (μM)	Probenecid (μM)	Cl SH-SY5Y	Fa SH-SY5Y	Cl T47D	Fa T47D	Cl Hela	Fa Hela
0.005	50	0.456	0.4308	0.697	0.2876	0.351	0.3525
0 01	100	0.090	0.7782	0.854	0.34500	0.224	0.5660
0.02	200	0.123	0.8299	0.635	0.47592	0.203	0.7078
0.04	400	0.093	0.9085	0.447	0.62029	0.256	0.7767
0.08	800	0.088	0.9451	0.474	0.70129	0.24	0.8639

CI indicates combination index, *Fa* indicates fraction affected

Table 4 Dose–effect relationships of drugs alone and drug combination in human cancer cell lines

Cell line	Single drug and combinations	CI value at			DRI value at		
		ED30	ED50	ED70	ED30	ED50	ED70
SH-SY5Y	Cisplatin				8.008	7.033	6.177
	Probenecid				2.287	4.002	7.005
	Mix 1:10	0.562	0.392	0.305			
	Paclitaxel				3.335	4.606	6.363
	Probenecid				6.364	16.632	43.47
	Mix 1:10000	0.457	0.277	0.18			
T47D	Cisplatin				5.423	3.061	1.727
	Probenecid				1.616	1.637	1.658
	Mix 1:10	0.803	0.938	1.182			
	Paclitaxel				1.878	2.913	4.518
	Probenecid				4.162	4.149	4.135
	Mix 1:10000	0.773	0.584	0.463			
HeLa	Cisplatin				2.488	1.882	1.423
	Probenecid				2.893	4.017	5.579
	Mix 1:10	0.748	0.78	0.882			
	Paclitaxel				4.246	4.408	4.576
	Probenecid				14.75	23.774	38.32
	Mix 1:10000	0.303	0.289	0.245			

CI combination index was calculated by Chou and Talalay's CI equation for effective dose (ED) 30, 50 y 70 _ CI < 1, CI $= 1$, and CI > 1 indicate synergism, additive effect, and antagonism, respectively. DRI dose reduction index determines the magnitude of dose reduction allowed for each drug when given in synergistic combination, compared with the concentration of a single agent that is needed to achieve the same effect level

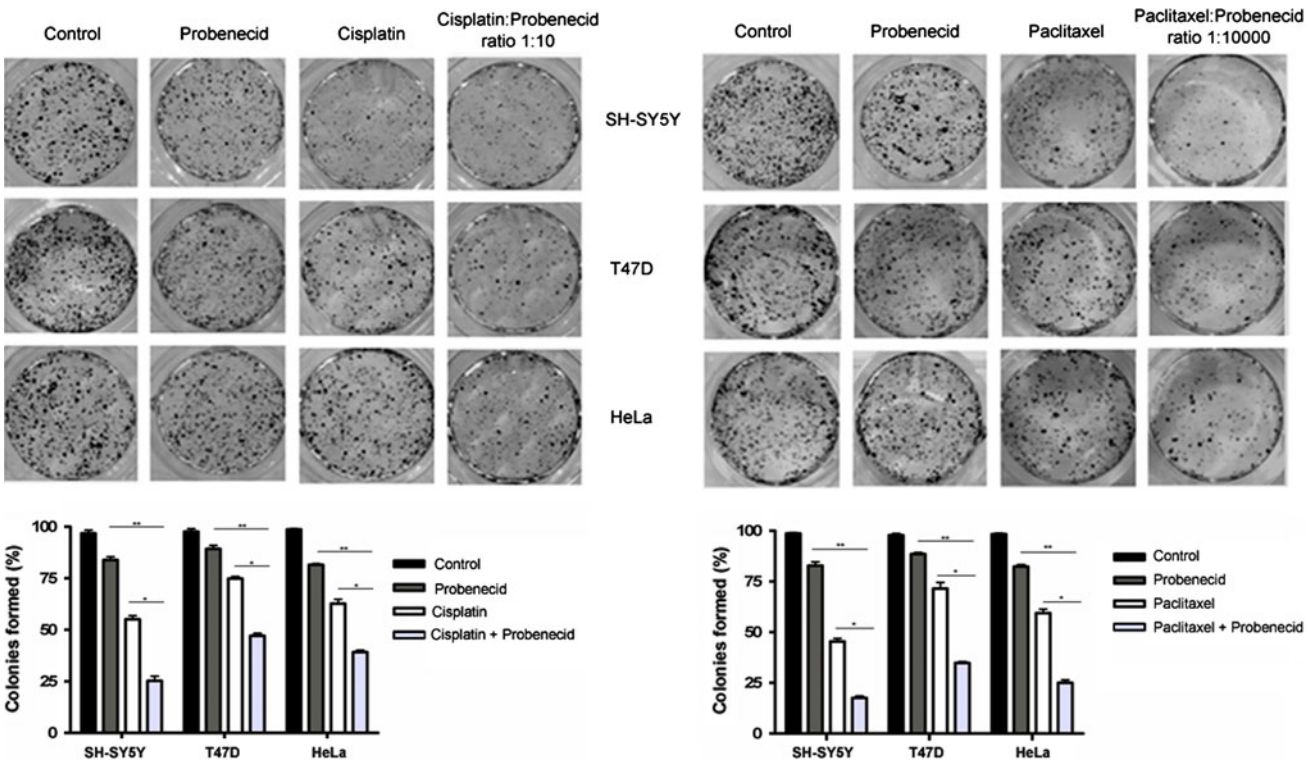

Fig. 3 Colony-forming assay showing cancer cell lines were treated with probenecid or cisplatin or paclitaxel as single agents or in combination at fixed ratio. The number of colony formed was calculated using ImageJ software. Images are representative of three separate experiments. Values were expressed as mean ± SEM obtained at different treatments. $^{**}P < 0.001$, $^{*}P < 0.01$

probenecid alone had a minimal growth-inhibitory effect. Cisplatin or paclitaxel in combination with probenecid resulted in synergistic growth-inhibitory activities at different dose levels. The effect was greater in SH-SY5Y, followed by HeLa and T47D cells. Differences in the sensitivities of the drugs alone and in combination may be due to phenotypic or genetic differences between the cellular types [48–52], and this may explain its relative resistance to treatment. We observed substantial decreases in the effective dose 50 (ED50) of cisplatin (1.88-fold to 7.03-fold) and paclitaxel (2.91-fold to 4.60-fold) in combination with probenecid. This dose reduction, due to the synergism of drug combination, has potential clinical advantages, as optimal therapy may be achieved at lower dose levels and possibly with reduced toxicity. Further analyses using a larger cohort of cell lines in therapeutically relevant antineoplastic combinations will be needed to establish a clinically relevant synergy.

Presently, the mechanism of action for the synergistic effect of probenecid with chemotherapeutic agents is unclear. However, several reports indicate that probenecid is an effective modulator of diverse transport channels, which can be implicated in chemoresistance [26–32, 53]; some of these transporters are regulated by ROS [54–56]. In addition, probenecid acts as a glutathione conjugate export pump inhibitor [57, 58]. The role of glutathione in distinct cell processes, including apoptosis, has been mainly ascribed to its potent antioxidant properties. In this regard, it is noteworthy that the protection by GSH is eliminated by probenecid [59–61]. Many antitumor agents, such as cisplatin and paclitaxel, exhibit antitumor activity via ROS-dependent activation of apoptotic cell death [62–66]. Cisplatin or paclitaxel in combination with probenecid could exceed the metabolic capabilities of the glutathione system, and this could increase the anticancer activity mediated by ROS accumulation in cancer cells and cause enhanced cell death via oxidative stress.

In summary, our results demonstrate that combinations of probenecid with cisplatin or paclitaxel in SH-5YSY, T47D, and HeLa cell lines have a better effect than either antineoplastic agent alone. Our results indicate that the use of probenecid in combination with chemotherapeutic agents in some types of cancer may improve therapy. Further in vitro and in vivo studies are needed to assess the underlying cellular and molecular mechanisms responsible for these effects. If confirmed, this may lead to increased efficacy of existing chemotherapies coupled with reduced

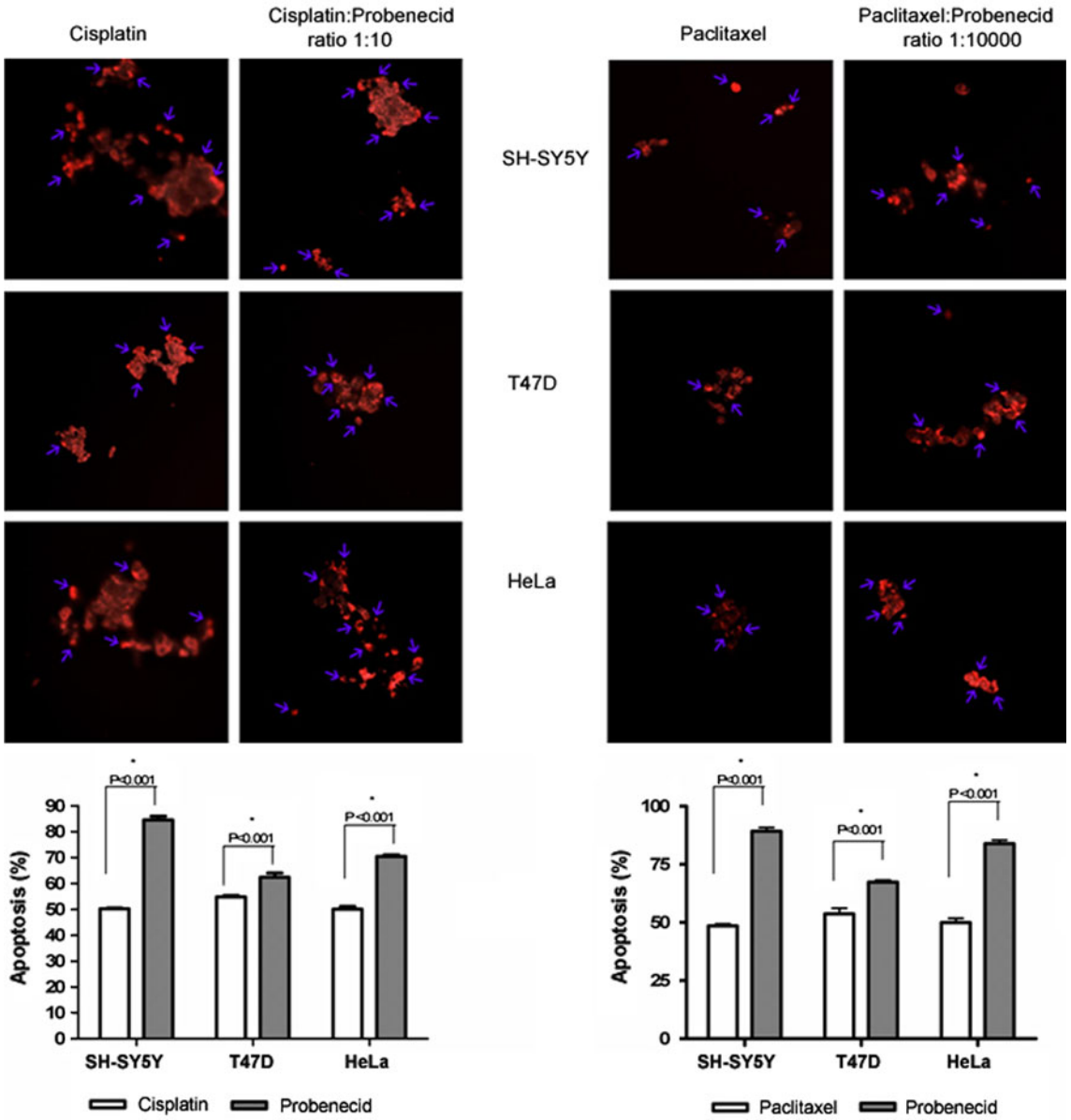

Fig. 4 Apoptosis assessment in cancer cell lines. The induction of apoptosis was determinate with an analysis of nuclear morphology. *Left side* is cells treated with cisplatin alone, and in combination with probenecid at fixed ratio 1:10. *Right side* is cells treated with paclitaxel alone and in combination with probenecid at fixed ratio 1:10000. Cells were viewed under fluorescence microscopy at ×40 magnification. $*P < 0.001$

toxicity to normal tissues in the treatment of neuroblastoma, breast, and cervical cancers.

Conflict of interest None.

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