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Report

Use of an ATP-based chemosensitivity assay to design new combinations of high-concentration doxorubicin with other drugs for recurrent ovarian cancer

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Liposomal doxorubicin (Caelyx/Doxil) has been shown to be active in around 20% of recurrent ovarian cancers. As yet, there is little clinical data on combinations of existing agents with liposomal doxorubicin, despite considerable clinical experience with soluble doxorubicin in combination. In this study, we have used an ATP-based tumor chemosensitivity assay to determine the relative efficacy of high concentrations of doxorubicin tested in combination with cisplatin, treosulfan, 5-fluorouracil (5-FU) or vinorelbine against cells obtained from recurrent ovarian tumor tissue. The results show little enhancement of the efficacy of high concentrations of doxorubicin by 5-FU, cisplatin, or treosulfan. However, vinorelbine + liposomal doxorubicin showed additive inhibition, and this combination is worthy of further testing in clinical trials. [© 2002 Lippincott Williams & Wilkins.]

Key words: Caelyx, chemotherapy, combination, Doxil, liposomal doxorubicin, ovarian cancer.

Introduction

Anthracyclines are active against a wide variety of human cancers, but their cardiotoxicity has largely prevented dose intensification. Liposomal preparations (Caelyx/Doxil; Schering Plough/Sequus) allow much higher intra-tumoral concentrations to be achieved without any increase in toxicity.[1–6] We have previously used an ATP-based tumor chemosensitivity assay (ATP-TCA) to show that the doxorubicin dose–response in many patients plateaus below 100% cell inhibition, suggesting that there might be a subset of anthracycline-resistant cells which even the higher concentrations produced by liposomal encapsulation does not affect.[7]

Doxorubicin is not a new drug and has been widely used in combination with alkylating agents, antimetabolites and spindle-active agents. There is less data on the combination of liposomal preparations with other drugs, but current results indicate that similar combinations are likely to be effective.[8]

In this study we have again used the ATP-TCA to determine the concentration responsiveness of tumor-derived cells to concentrations of doxorubicin achievable with liposomal preparations, but have looked at the addition of other drugs in combination with doxorubicin. The ATP-TCA is based on the measurement of ATP by the firefly luciferin–luciferase reaction. ATP is the major intracellular source of energy for metabolism. When cells die their ATP is rapidly degraded by ATPases, so that loss of ATP can be used as a sensitive indicator of cytotoxicity.[9] The ATP-TCA results show good correlation with clinical

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outcome,[10,11] and there is evidence that this will translate into clinical benefit for assay-directed therapy.[12] The assay has been used to assess new agents and to design new combinations as an aid to the planning of clinical trials.[7,13–15]

Liposomal doxorubicin has proved particularly useful as a single agent in recurrent ovarian cancer[6,16–20] and we therefore chose to look at this tumor type. Treosulfan, vinorelbine and cisplatin were tested in combination with doxorubicin, which was used at 5 times the test drug concentration (TDC) used for the standard drug formulation to account for the increased intra-tumoral concentration achievable with liposomal preparations. It is not possible to test liposomal doxorubicin directly in our assay as serum-free media do not hydrolyze pegylated liposomes and a previous study has shown no effect of liposomal doxorubicin in cell lines maintained in such media.[21]

Materials and methods

Tumors

A total of 123 recurrent ovarian tumors were tested, with local ethics committee approval for the use of tissue or cells not required for diagnosis. These were all previously treated with carboplatin alone or carboplatin + taxanes first line, followed in nine cases by an anthracycline-containing regimen. The median age of the patients was 58 years (range 35–80). All patients had FIGO stage 3b or 4 disease at recurrence: 29% (29 of 101) showed clinical resistance to platinum and had relapsed during or within 6 months of primary platinum-based therapy. Histological type was stated on the submission form in 76 cases: 50 were of serous histological type, nine were endometrioid, four were clear cell, three were mucinous and 10 were poorly differentiated.

ATP-TCA

The ATP-TCA was performed as previously described.[7,9,15] Samples were transported to the laboratory in transport medium consisting of Dulbecco's modified Eagle's medium (Sigma, Poole, UK; D5671) as previously described.[7] Briefly, the tumor samples were first subjected to gentle enzymatic dissociation by collagenase (0.75 mg/ml; Sigma; C8051) to produce a single-cell suspension. The cells were then cultured in serum-free media (Complete Assay Medium; DCS Innovative Diagnostik Systeme, Hamburg, Germany) in 96-well polypropylene plates (Corning-Costar, High Wycombe, UK) with or without the test drugs at six dilutions, allowing four drugs to be tested with triplicate wells for each data point. Combinations were made by direct addition of the two drugs at the same concentration used for the single agents. Two controls were included in 12 wells each: a maximum inhibitor (MI) which killed all the cells present giving a zero ATP count and a no drug control (M0) consisting of medium alone. The cells were cultured for 6 days at 37°C with 5% CO_2. Following incubation, a commercial detergent-based extraction reagent was used to lyse the cells and inhibit the ATPases contained within the cells (DCS Innovative Diagnostik Systeme). ATP quantification took place by adding the luciferin–luciferase counting reagent to the cell lysate. The amount of light produced was measured in a microplate luminometer (Berthold MPLX). The results are expressed as the percent inhibition achieved at each concentration tested, calculated as: % inhibition = $1 - (\text{test} - \text{MI})/(\text{M0} - \text{MI}) \times 100$. Several indices of efficacy such as IC_{50} and IC_{90} can be calculated from the data, but we have previously found that a natural logarithmic sum index ($Index_{SUM}$) calculated by the direct addition of the percent survival at each concentration provides the best discrimination between drugs and tumors to show the heterogeneity of the activity observed.[9,11,22] We have also calculated the area under the concentration–inhibition curve ($Index_{AUC}$) and the percentage of tumors achieving 95% inhibition. Combination effects were assessed by the method of Poch *et al.*,[23] as previously used with the ATP-TCA in other studies.[13,14]

Drugs

The drugs used in the assay were obtained as vials for injection and made up according to the manufacturer's instructions. Doxorubicin, treosulfan and vinorelbine were stored in aliquots at −20°C, while cisplatin was kept at room temperature. The 100% TDC used were derived from pharmacokinetic data, adjusted for protein binding to approximate the concentration clinically achievable in the patient. For cisplatin the 100% TDC was 3.0 μg/ml, for treosulfan 20 μg/ml, for 5-fluorouracil (5-FU) 45 μg/ml, for doxorubicin × 5 2.5 × μg/ml and for vinorelbine 12 μg/ml. Combinations were made up by adding both drugs at their 200% TDC to the wells at the

beginning of the assay: sequential studies were not performed. Not all drugs or combinations were tested in every case.

Data analysis

Data from the luminometer were transferred to a spreadsheet (Excel 97; Microsoft) and calculations to derive the indices performed using a template. The calculated and descriptive data were entered into an Access 2000 database (Microsoft) for further analysis and analyzed using non-parametric statistics (QLSTAT; Addinsoft, Paris, France).

Results

The results show that doxorubicin $\times 5$ achieves $>99\%$ inhibition at clinically achievable concentrations in 61 of 124 (49%) of the ovarian tumors tested (Table 1). Ten ovarian tumors showed complete resistance. Of these, only three had previous exposure to anthracyclines. For the other individual agents tested, there was a sigmoidal concentration–response curve for both cisplatin and 5-FU, with a somewhat flatter curve for vinorelbine (data not shown).

For the purposes of comparison between drugs and tumors, an $Index_{SUM}$ of <300 was taken as *ex vivo* sensitivity and >350 as resistance, as previously published.[22,24] Values between these two points were regarded as equivocal. On this basis, 76% (90 of 118) of the ovarian tumors tested showed resistance to cisplatin, with only 16% (19 of 118) showing sensitivity. In contrast, 84% (103 of 123) showed sensitivity to doxorubicin $\times 5$, 37% (37 of 106) to treosulfan and 77% (41 of 53) to vinorelbine (Table 1). Only six tumors were tested with 5-FU, although four showed sensitivity.

The combinations tested are only slightly better than doxorubicin $\times 5$ alone, as shown in Figure 1 and Table 1. All produce $>90\%$ sensitivity based on an $Index_{SUM}$ of <300 (doxorubicin $\times 5$ with cisplatin = 98%, with treosulfan = 98%, 5-FU = 90%, with vinoreline = 100%). The best effect is obtained with the combination of doxorubicin $\times 5 +$ vinorelbine, which is better than doxorubicin $\times 5$ alone in 51 of 53 paired observations (Mann–Whitney *U*-test: $p<0.0001$ on $Index_{SUM}$), and better than doxorubicin $\times 5 +$ treosulfan in 39 of 41 paired observations ($p<0.0001$). The example shown in Figure 2 shows the advantage of the combination over the individual agents in terms of inhibition, but the effect is additive

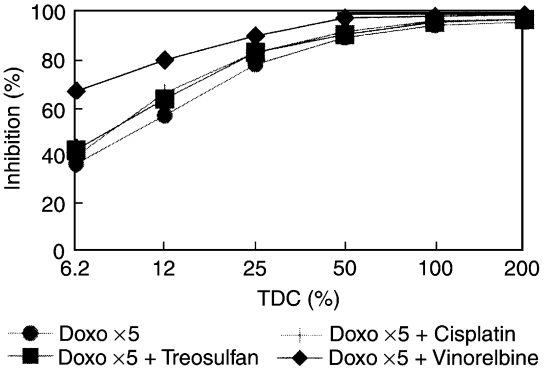

Figure 1. Median effect of doxorubicin $\times 5$ against ovarian cancer compared with the combinations tested.

Table 1. Results for single-agent doxorubicin tested at 5 times the standard concentration to reflect the increased intratumoral concentration achievable with the liposomal preparation

Drug/ combination	*n*	Age	$Index_{AUC}$	IC_{90}	IC_{50}	$Index_{SUM}$	$>95\%$ Inhibition
Doxorubicin $\times 5$	123	58 (35–80)	18014 (7085–19565)	35 (6–297)	12 (3–165)	139 (3–505)	83 (102/123)
Cisplatin	118	58 (35–80)	7686 (266–17144)	257 (76–1429)	135 (4–794)	445 (113–940)	4 (5/118)
Treosulfan	106	59 (35–80)	12657 (433–18121)	185 (33–978)	62 (4–544)	343 (91–846)	44 (47/106)
5-FU	6	51 (48–64)	16672 (0–18936)	85 (18–269)	15 (4–166)	151 (46–1338)	67 (4/6)
Vinorelbine	53	61 (40–80)	15508 (6145–19158)	146 (6–242)	6 (3–140)	183 (15–502)	72 (38/53)
Doxorubicin $\times 5 +$ cisplatin	52	60 (36–80)	18615 (12267–19488)	21 (6–246)	9 (3–52)	92 (8–314)	88 (46/52)
Doxorubicin $\times 5 +$ treosulfan	41	61 (44–80)	18725 (12857–19491)	21 (6–205)	8 (4–72)	92 (8–346)	98 (40/41)
Doxorubicin $\times 5 +$ 5-FU	11	58 (48–69)	17455 (10696–19234)	40 (10–292)	9 (4–46)	119 (21–361)	55 (6/11)
Doxorubicin $\times 5 +$ vinorelbine	53	61 (36–80)	19013 (16746–19488)	12 (6–86)	4 (3–28)	37 (0–209)	98 (52/53)

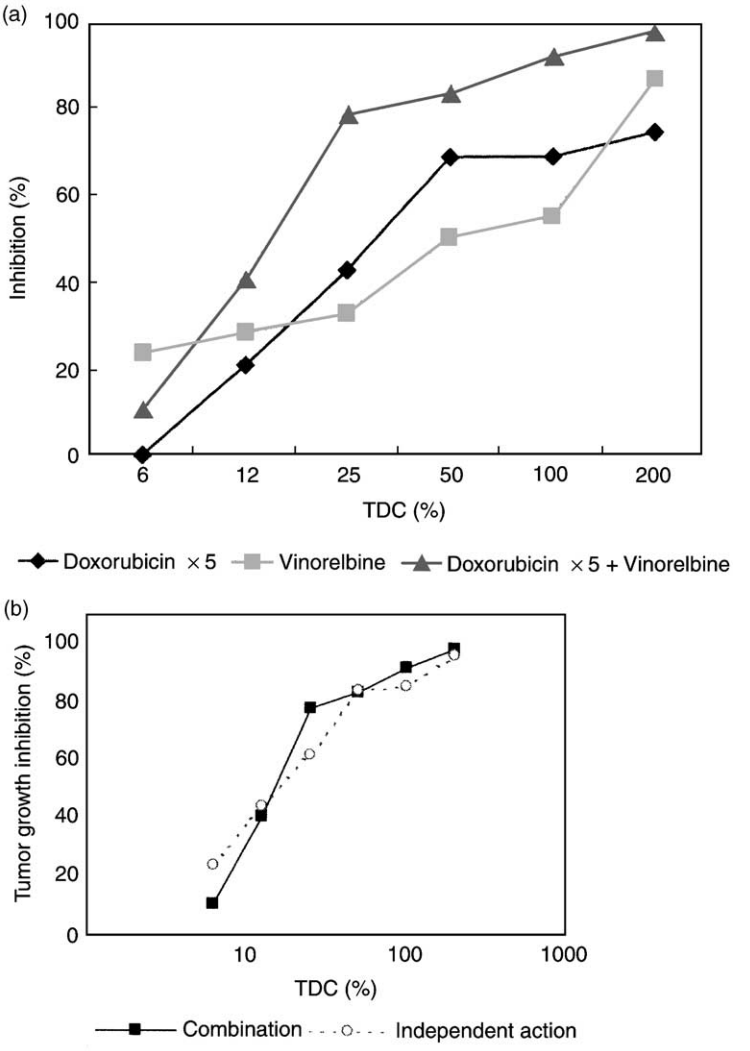

Figure 2. (a) Comparison of the activity of doxorubicin alone and in combination with vinorelbine in a tumor showing resistance to doxorubicin and vinorelbine. (b) Combination analysis[23] confirming an additive effect of the two drugs in this instance.

rather than synergistic when analyzed by the method of Poch *et al.*,[23] in which the observed effect at each concentration tested is compared with the expected effect (Figure 2).

Discussion

The proportion of tumors apparently sensitive to doxorubicin × 5 exceeds the response rates obtained in clinical trials of liposomal doxorubicin in recurrent ovarian cancer.[6,16–20] The largest of these obtained a 20% response rate.[18] The reason for this discrepancy may be overestimation of the likely concentration to which tumor cells are exposed. Our use of 5 times the standard TDC for doxorubicin was based on available data from animal studies.[2] It is worth noting that a recent study of human subjects presented at the 2001 San Antonio Breast Cancer Symposium also suggests a higher and more sustained enhancement of doxorubicin levels in tumors from breast cancer patients treated with liposomal doxorubicin.[25] However, in addition to the pharmacokinetic considerations, rapid acquisition of resistance by a small subset of tumor cells during treatment could also explain our results. We are pursuing an explanation, but on the basis of these data, we have now reduced the concentration tested in the ATP-TCA for liposomal doxorubicin to 3

times the normal TDC. Correlation data comparing ATP-TCA results with clinical outcome from follow-up of those patients in this series treated with liposomal doxorubicin are awaited.

We did not explore combinations in any detail in our previous study, though we noted that the addition of gemcitabine was unable to augment the response to any great extent,[7] a finding that has now been explored clinically in a small phase I study.[26] While this study did show nine of 27 responses, it should be noted that six patients had not received prior chemotherapy. We know of no rationale for adding these two drugs together, other than empirical evidence of activity of both single agents in multiple tumor types. Equally, we did not try to combine topoisomerase I and II inhibitors, as topotecan + liposomal doxorubicin was found to be toxic.[27]

As most of the patients had all had previous exposure to taxanes, we did not test this in combination with doxorubicin $\times 5$, though it should be noted that recent results combining liposomal doxorubicin with docetaxel are encouraging.[8] We chose instead to examine the effect of doxorubicin on cisplatin resistance and in combination with another alkylating agent to which resistance is less common in this setting, treosulfan. The results for the combination of doxorubicin $\times 5$ with treosulfan, 5-FU and cisplatin are disappointing, with little improvement in efficacy compared with single agent doxorubicin $\times 5$ (Table 1 and Figure 1). The lack of effect with cisplatin suggests that anthracyclines do not greatly influence resistance to cisplatin.

In contrast, the combination of vinorelbine and liposomal doxorubicin showed additive effects. This combination has been tried in the clinic in metastatic breast cancer and the data has been presented at an international meeting, although it is not yet published.[28] The combination was found to be safe, with acceptable toxicity, but showed a disappointingly low response rate (18%) in the small number of patients ($n = 22$) treated. Our data suggests that this combination is worthy of further consideration and should be tested clinically in recurrent ovarian cancer.

There are a number of other combinations that need to be studied. Some of the possible combinations are already being tested in the clinic on an empirical basis, e.g. the combination of liposomal doxorubicin with Herceptin.[29] It would be particularly interesting to examine the role of Caelyx/Doxil in combination with taxanes in ovarian cancer samples from previously untreated patients, as we have previously shown combinations of anthracyclines with taxanes to be very active in this tumor type.[11–13]

Conclusion

It is clear from this and previous studies that the use of ATP-based chemosensitivity testing can assist the development of new regimens and has the potential to speed up their introduction to the clinic.[29] Vinorelbine + liposomal doxorubicin is clearly of considerable interest and we await further clinical data.

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