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Report

In vitro activity of taxol and taxotere in comparison with doxorubicin and cisplatin on primary cell cultures of human breast cancers

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Summary

The *in vitro* activities of taxol and taxotere in comparison with cisplatin and doxorubicin were assessed in 30 primary tumor cultures from human breast cancers. Both taxanes were much more potent than cisplatin and doxorubicin. Taxotere was 3.1; 296, and 9.6-fold more cytotoxic than taxol, cisplatin, and doxorubicin respectively. The cytotoxic activity observed in our experiments confirms the potential clinical relevance of the two taxanes in the management of breast cancer.

Introduction

In recent years much efforts have been devoted to the search for new drugs with different molecular targets, other than DNA, to better control human tumor growth.

Taxol is a mitotic spindle poison that stabilizes microtubules and inhibits their depolymerization to free tubulin [1]. It was isolated and characterized in 1971 from the bark of the *Taxus brevifolia* [2], and has been demonstrated to possess broad activity in preclinical screening studies and antineoplastic activity in several tumors [3].

An analogue of taxol, taxotere has been recently obtained from a non cytotoxic precursor extracted from the needles of *Taxus baccata* L. [4, 5]. Like taxol, taxotere too promotes microtubule assembly and bundle formation *in vitro* [6], so being a potent inhibitor of cell replication. Taxotere was also active against a wide variety of transplantable murine tumors including B16 melanoma, and displayed no marked schedule dependency in B6D2F1 mice [7].

We evaluated here the *in vitro* efficacy of taxol and taxotere in comparison with cisplatin and doxorubicin on 30 primary cell cultures from human breast cancers.

Materials and methods

Primary cell lines

The primary cell lines were obtained from surgical material of primary breast cancers and treated as follows: the tumor tissue was minced and digested overnight with collagenase (1000 units/ml) and hyaluronidase (500 units/ml). The clumps of neoplastic cells obtained with the digestion were plated in

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plastic culture flasks to initiate primary cultures. The clumps were grown at 37° C in an enriched medium (MM) specifically developed for human mammary epithelium [8].

Five to seven days later a wide area of monolayer cells with extensive mitotic activity surrounded each clump.

The mammary epithelial origin and the malignant phenotype of the proliferating cells were studied using a panel of monoclonal antibodies (MOv15/MLuC1; MBr1; MBr19/MLuC2) [9, 10]. The proliferating cells were then utilized in the drug assay.

Receptor status

Estrogen and progesterone receptor assays were performed on the 30 fresh tumor samples used for the chemosensitivity assays according to the method of the EORTC Breast Cancer Cooperative Group [11].

Cellular kinetics

Immediately after surgery, the cellular kinetics evaluated as ^{3}H-thymidine labeling index (^{3}H-TdR LI) [12] was determined on small fragments sampled from the different areas of the tumor and incubated for 1 h at 37° C with the labeled precursor.

A kit obtained from Ribbon (Milan-Italy) was used for this first step. Autoradiography was performed on histological sections with an exposure time of 3 days at 4° C. Autoradiograms were developed in Ilford K5 emulsion gel for 5 min. at 18° C and fixed in Hypam Ilford. The samples were stained with haematoxylin and eosin at 4° C. The ^{3}H-TdR LI was determined by scoring between 1000 and 3000 cells on different specimens from the same lesion and was defined as the percentage ratio between the labeled and all the tumor cells.

In our previous work [13], the ^{3}H-TdR LI evaluated on a consecutive series of 281 primary breast cancers presented a median value of 3.1% (range 0.01–12.8%). The value of 3.1% was used in the present study as cut-off to define slowly and rapidly proliferating tumors.

Clonal assay (HECA)

The clonal assay was performed according to the method originally described by H.S. Smith [14–17].

Briefly, 2.5×10^4 UV irradiated human fibroblasts were seeded into 35 mm tissue culture dishes to provide a feeder layer. Two hours later the tumor cells were harvested from primary cultures that were dispersed to a single cell suspension and plated on the irradiated fibroblasts at either 350; 1000, or 3500 cell/dish with MM. Randomly selected dishes were observed for clumps immediately after seeding. Sixteen hours later, the medium was removed and fresh medium with the desired drug concentration was added.

Three drug concentrations were used and for each drug dose at least six dishes were prepared with the appropriate number of cells. At least four dishes were used as untreated control for every level of seeded cells. Four hours later the medium was removed and the dishes were washed with basal salt solution and then refed with MM containing 2.5×10^4 freshly trypsinized irradiated fibroblasts. The dishes were incubated until easily visible colonies (aggregates of 30 or more cells) were present.

The dishes were fixed with methanol and stained with May-Grunwald Giemsa. The colonies in each dish were counted and the number of colonies per 100 cells plated was determined. The number of colonies in the drug-treated dishes was divided by the mean number of colonies per 100 cells plated on control dishes.

The dose response curves were generated by calculating the means and standard deviations for each drug concentration and used to obtain the drug concentrations that were able to kill 50% (LD_{50}) and 90% (LD_{90}) of colony-forming cells.

Drug preparation and storage

Doxorubicin (Farmitalia Carlo-Erba) as lyophilized powder was diluted with sterile physiologic sa-

line solution at the concentration of 1 mg/ml, divided into aliquots, and frozen at – 70° C. Cisplatin (Bristol) was directly available at a concentration of 0.5 mg/ml. Taxotere was provided as powder by Rhone-Poulenc Rorer S.A. (Antony Cedex, France) and EORTC/PTMG (Preclinical Therapeutic Models Group). Taxol (NCI) was received from EORTC/PTMG. The two taxanes were diluted with ethanol (95%) to obtain stock solutions of 1 mg/ml and stored at – 20° C prior to use. The test solutions of the four drugs were prepared fresh for each experiment by diluting the stock solutions in culture media.

The concentrations of doxorubicin and cisplatin (doxorubicin: 2.5×10^{-2}, 5.0×10^{-2}, 7.5×10^{-2} μg/ml; cisplatin: 1, 2, 3 μg/ml) used in the assay were selected according to our previous studies [15–17] in which the clinical relevance of the HECA was found.

The doses of taxol and taxotere were selected starting from the reported peak plasma concentrations (PPC) as reference values: taxol 4.27 μg/ml [18–20] and taxotere 0.48 μg/ml [21–23]. These relatively high concentrations of the two taxanes resulted in almost complete cell kill. To obtain evaluable dose-response curves the HECA was done at the following concentrations: taxol: 1×10^{-3}, 1×10^{-2}, 1×10^{-1} μg/ml; taxotere: 5.0×10^{-4}; 1×10^{-3}, 5.0×10^{-3} μg/ml.

Results

The clinical and pathological characteristics of the patients (30 cases) are reported in Table 1.

The clonogenic assay was performed on primary untreated breast cancers comparing the *in vitro* activity of taxotere, taxol, doxorubicin, and cisplatin. According to our previous report [17] on a wider number of cases, the clonal efficiency of the assay was very high (median value: 17.6%; range 0.8 – 50.4%).

The values of drug concentration able to kill 50% (LD_{50}) and 90% (LD_{90}) of cells for each tested drug are reported in Tables 2 and 3.

By the Spearman rank correlation coefficient we did not find any correlation between the *in vitro* activity of either taxane and cell kinetics or receptor status.

Table 1. Clinical and pathological characteristics of the 30 patients from whom primary cell cultures were obtained

MEDIAN AGE (years)	62
MENOPAUSAL STATUS	
– Premenopausal	9
– Postmenopausal	21
HISTOLOGICAL TYPE	
– Ductal	22
– Inflammatory	2
– Lobular	3
– Others	3
LYMPH NODAL STATUS	
– N +	13
– N –	17
T-STATUS	
– T1	7
– T2	17
– T3	5
– T4	1
RECEPTOR STATUS	
– ER + PgR + (> 10 fmol/mg)	22
– ER + PgR –	1
– ER – PgR – (< 10 fmol/mg)	6
– ER – PgR +	1
LABELLING INDEX	
– > 3.1%	16
– < 3.1%	12
– Not evaluable	2

The LD_{50} values showed a different *in vitro* behavior of the four drugs with the non parametric Mann-Whitney test. The median LD_{50} values were 3.75×10^{-3}; 1.2×10^{-3}; 1.15×10^{-2}, and 3.5×10^{-1} μg/ml for taxol, taxotere, doxorubicin, and cisplatin respectively, we observed that taxotere is more potent, *in vitro*, than taxol ($z = 5.168$ $p < 0.001$), doxorubicin ($z = 6.462$ $p < 0.001$), and cisplatin ($z = 6.622$ $p < 0.001$) in every tested primary breast cancer.

The dose-response curves obtained with taxanes (Fig. 1) suggest the existence of critical concentrations of the drugs ($> 10^{-2}$ and $> 10^{-3}$ μg/ml respectively) able to strongly reduce the survival of the cells *in vitro*.

The analysis of the LD_{50} values observed with the drugs in 30 primary tumor cultures did not show a correlation between the *in vitro* activity of taxol and doxorubicin (Spearman rank correlation coefficient $r = 0.140$, $p = 047$) and between taxotere and

Table 2. LD_{50} values (μg/ml) obtained in the treatment of 30 primary breast cancer cell lines with taxol (TOL), taxotere (TER), doxorubicin (DOX), and cisplatin (PLAT)

N° of case	LD_{50} TOL	LD_{50} TER	LD_{50} DOX	LD_{50} PLAT
1	$> 10^{-2}$	2.0×10^{-3}	1.4×10^{-2}	6.5×10^{-1}
2	$> 10^{-2}$	2.0×10^{-3}	1.2×10^{-2}	1.24
3	8.0×10^{-4}	1.4×10^{-3}	4.3×10^{-2}	1.20
4	3.7×10^{-3}	8.5×10^{-4}	9.0×10^{-3}	2.4×10^{-1}
5	3.1×10^{-3}	8.5×10^{-4}	7.0×10^{-3}	4.4×10^{-1}
6	6.5×10^{-3}	1.5×10^{-3}	1.2×10^{-2}	3.5×10^{-1}
7	6.5×10^{-3}	1.5×10^{-3}	1.3×10^{-2}	7.0×10^{-1}
8	5.1×10^{-3}	9.0×10^{-4}	1.0×10^{-2}	2.2×10^{-1}
9	4.6×10^{-3}	2.8×10^{-3}	1.0×10^{-2}	2.4×10^{-1}
10	5.8×10^{-3}	1.1×10^{-3}	2.2×10^{-2}	5.5×10^{-1}
11	5.8×10^{-3}	1.1×10^{-3}	9.0×10^{-3}	3.0×10^{-1}
12	1.8×10^{-3}	8.5×10^{-4}	8.0×10^{-3}	2.0×10^{-1}
13	2.5×10^{-3}	1.2×10^{-3}	1.3×10^{-3}	2.0×10^{-1}
14	6.5×10^{-3}	1.5×10^{-3}	9.0×10^{-3}	3.5×10^{-1}
15	5.8×10^{-3}	1.3×10^{-3}	8.0×10^{-3}	3.5×10^{-1}
16	3.4×10^{-3}	8.5×10^{-4}	1.0×10^{-2}	4.5×10^{-1}
17	3.6×10^{-3}	1.1×10^{-3}	7.0×10^{-3}	4.0×10^{-1}
18	3.8×10^{-3}	1.1×10^{-3}	8.0×10^{-3}	2.5×10^{-1}
19	8.2×10^{-3}	1.2×10^{-3}	1.7×10^{-2}	6.0×10^{-1}
20	3.6×10^{-3}	1.2×10^{-3}	2.0×10^{-2}	2.5×10^{-1}
21	3.2×10^{-3}	1.2×10^{-3}	1.1×10^{-2}	2.5×10^{-1}
22	3.2×10^{-3}	1.2×10^{-3}	1.4×10^{-2}	4.0×10^{-1}
23	7.8×10^{-3}	1.5×10^{-3}	1.5×10^{-2}	3.5×10^{-1}
24	9.6×10^{-3}	1.3×10^{-3}	1.8×10^{-2}	3.5×10^{-1}
25	9.8×10^{-3}	2.4×10^{-3}	2.6×10^{-2}	1.1
26	3.3×10^{-3}	1.1×10^{-3}	9.0×10^{-2}	2.0×10^{-1}
27	0.7×10^{-3}	2.5×10^{-4}	1.5×10^{-2}	2.5×10^{-1}
28	8.5×10^{-4}	6.5×10^{-4}	2.6×10^{-2}	1.2×10^{-1}
29	5.0×10^{-3}	1.5×10^{-3}	9.0×10^{-3}	1.2×10^{-1}
30	3.0×10^{-3}	8.5×10^{-4}	9.0×10^{-3}	2.5×10^{-1}

Table 3. LD_{90} values (μg/ml) obtained in the treatment of 30 primary breast cancer cell lines with taxol (TOL), taxotere (TER), doxorubicin (DOX), and cisplatin (PLAT)

N° of case	LD_{90} TOL	LD_{90} TER	LD_{90} DOX	LD_{90} PLAT
1	$> 1.0 \times 10^{-2}$	$> 10^{-2}$	$> 7.5 \times 10^{-2}$	2.4
2	$> 1.0 \times 10^{-2}$	4.0×10^{-3}	5.7×10^{-2}	> 3.0
3	$> 1.0 \times 10^{-3}$	3.0×10^{-3}	$> 7.5 \times 10^{-2}$	> 3.0
4	1.4×10^{-2}	2.3×10^{-3}	3.1×10^{-2}	8.0×10^{-1}
5	4.3×10^{-2}	$> 5.0 \times 10^{-3}$	2.3×10^{-2}	1.4
6	$> 1.0 \times 10^{-1}$	3.5×10^{-3}	4.3×10^{-2}	1.16
7	$> 1.0 \times 10^{-1}$	4.3×10^{-3}	4.7×10^{-2}	1.76
8	4.5×10^{-2}	3.2×10^{-3}	3.3×10^{-2}	7.6×10^{-1}
9	3.4×10^{-2}	1.2×10^{-3}	3.4×10^{-2}	8.0×10^{-1}
10	6.0×10^{-2}	3.9×10^{-3}	5.0×10^{-2}	9.7×10^{-1}
11	4.2×10^{-2}	3.4×10^{-3}	3.1×10^{-2}	1.0
12	2.6×10^{-2}	3.7×10^{-3}	2.9×10^{-2}	6.5×10^{-1}
13	1.5×10^{-2}	2.8×10^{-3}	4.9×10^{-2}	6.5×10^{-1}
14	4.9×10^{-2}	3.6×10^{-3}	3.2×10^{-2}	1.2
15	3.0×10^{-2}	2.4×10^{-3}	2.8×10^{-2}	1.15
16	2.6×10^{-2}	2.2×10^{-3}	3.2×10^{-2}	1.5
17	2.6×10^{-2}	2.2×10^{-3}	2.6×10^{-2}	1.2
18	3.0×10^{-2}	2.1×10^{-3}	2.9×10^{-2}	8.5×10^{-1}
19	6.9×10^{-2}	2.4×10^{-3}	3.8×10^{-2}	2.0
20	2.1×10^{-2}	2.3×10^{-3}	5.7×10^{-2}	7.0×10^{-1}
21	9.8×10^{-3}	2.2×10^{-3}	4.0×10^{-2}	8.5×10^{-1}
22	2.5×10^{-2}	2.5×10^{-3}	5.3×10^{-2}	1.35
23	2.9×10^{-2}	2.9×10^{-3}	5.4×10^{-2}	1.1
24	3.3×10^{-2}	2.4×10^{-3}	6.4×10^{-2}	1.95
25	6.6×10^{-2}	1.0×10^{-2}	1.0×10^{-1}	5.0
26	1.3×10^{-2}	2.1×10^{-3}	5.2×10^{-2}	7.0×10^{-1}
27	7.4×10^{-3}	2.4×10^{-3}	6.5×10^{-2}	9.0×10^{-1}
28	8.4×10^{-3}	2.0×10^{-3}	4.0×10^{-2}	4.5×10^{-1}
29	8.6×10^{-2}	1.9×10^{-3}	3.4×10^{-2}	4.4×10^{-1}
30	1.2×10^{-2}	2.0×10^{-3}	3.5×10^{-2}	9.0×10^{-1}

doxorubicin ($r = 0.203$, $p = 0.278$). On the other hand, a clear correlation ($r = 0.626$, $p < 0.001$) was found between the LD_{50} values of taxol and taxotere.

Discussion

The *in vitro* HECA on breast cancer cells after short term culture has proved to accurately predict both sensitivity and resistance *in vivo* even though (a) there is a loss of heterogeneity, since only selected subpopulations of cells grow in this culture system; and (b) the cell cycle kinetics differs from the *in vivo* situation since all the cells do cycle after primary culture [14–17].

The interesting cytotoxic activity of taxol and taxotere against various malignant neoplasms reported by others [5, 7, 24–26] was confirmed in a series of 30 primary human breast cancer cell lines cultured *in vitro* using the clonogenic HECA assay.

The low LD_{50} values obtained with both taxanes were in agreement with the data reported by others in spite of different cell lines and assay systems [5, 7, 24–26].

In our study taxotere was more potent than the other drugs. In particular the comparison among the LD_{50} median values showed that taxotere was 3.1; 296, and 9.6 times more cytotoxic than taxol, cis-

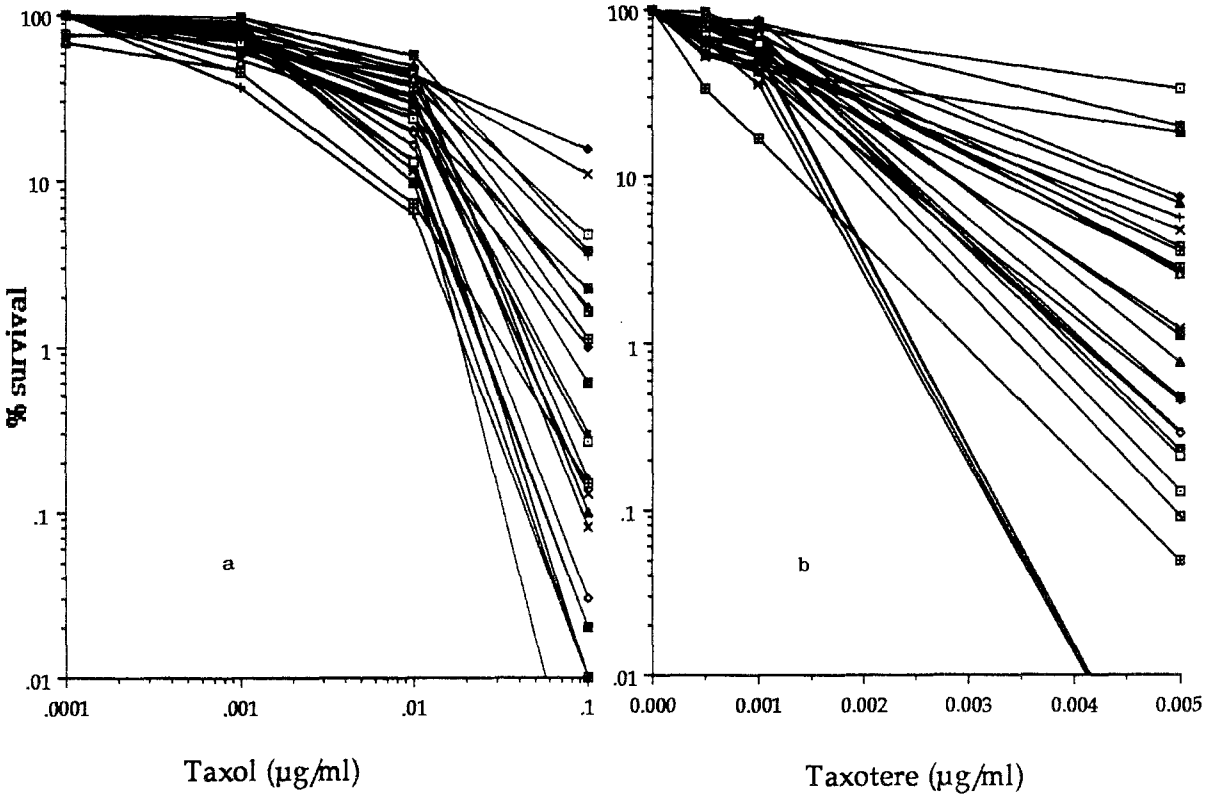

Fig. 1. Survival curves of 30 primary breast cancer cell lines to increasing doses of taxol (a) and taxotere (b).

platin, and doxorubicin respectively. These data confirm the results of previous works in which taxotere was 1.6 to 6.5 times more active than taxol.

Phase II trials with taxol or taxotere as first line or after adjuvant chemotherapy for metastatic breast cancer reported a significant antitumor activity of the two drugs with an overall response rate ranging from 57% to 73% [27–31]. On the basis of these clinical data and from the analysis of our results we have came to the following speculations:

a) the clear correlation between the LD_{50} values of taxol versus taxotere supports the possibility of a cross resistance between taxol and taxotere but only a partial cross resistance between taxol and doxorubicin and between taxotere and doxorubicin.

b) the activity exerted *in vitro* by both taxanes in respect to doxorubicin, which is considered very active as a single agent in breast cancer tumor [32], could be interesting in the management of this cancer because of their higher effect.

The *in vitro* activity observed by the use of the two drugs on primary breast cancer cell lines was obtained with very low concentrations (the ratio between LD_{50} median value obtained and the PPC is 1:1000 for taxol and 1:400 for taxotere). The median LD_{50} values for both taxanes were extremely low corresponding with the (dose-limiting) neutropenia caused by these two drugs observed in clinical studies [33] and in comparison with the median LD_{50} value for doxorubicin (the ratio between LD_{50} value obtained and doxorubicin PPC is 1:20).

Overall our *in vitro* results seem to confirm the potential clinical relevance of the two taxanes in the management of breast cancer even if the taxol and taxotere concentrations used in this study are not directly transferable to the *in vivo* situation. Moreover the observation that there is not cross-resistance *in vitro* between the two taxanes and doxorubicin seems to support a possible clinical use of one of these two drugs either after anthracycline failure or in association with doxorubicin.

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