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Evaluation of Taxol® in head and neck squamous carcinoma multicellular tumor spheroids

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Taxol cytotoxicity was evaluated in human head and neck squamous carcinoma cell lines growing as multicellular tumor spheroids (MTS) and compared with monolayered culture using conventional clonogenic assays. End points were respectively the concentration inhibiting 50% of the cellular growth (IC_{50}) in clonogenic assays and the concentration required to induce a 50% decrease in the MTS volume (ID_{50}) or number in the overall spheroid population (SCC_{50}). A significant difference was observed when the cells were exposed for 10 days to Taxol as a consequence of the different growth kinetics of the spheroids. After 16 day exposure of spheroids to Taxol, no difference remained between ID_{50} and IC_{50}. In addition, a significant correlation was found between individual spheroid sensitivity to Taxol (ID_{50}) and the spheroid population sensitivity (SCC_{50}). Both parameters (ID_{50} and SCC_{50}) defined in cell models appear useful for the evaluation of chemosensitivity of three-dimensional structures known to be closer to *in vivo* tumor models.

Key words: Chemosensitivity, head and neck, multicellular tumor spheroid, squamous cell carcinoma, Taxol.

Introduction

Taxol, a diterpene with a natural chemotherapeutic activity as initially described in 1971,[1] has been isolated from the bark of the Pacific Western yew, *Taxus brevifolia*. Because of its reported efficacy in the treatment of drug refractory tumors including ovarian, lung, breast, and head and neck,[2] Taxol has received considerable clinical interest. Although the exact mechanistic basis for the antitumoral action of this drug is still unclear, several studies *in vitro* have showed that Taxol is an antimitotic agent which is active in the G_2/M phase of the cell cycle where it binds tightly to the β-tubulin subunit of microtubules.[3] The microtubules of cells treated with Taxol are resistant to depolymerization and form abnormal cytoplamic bundles and nuclear asters,[4] thus blocking cell division.[5,6] These microtubular changes affect cells mostly in G_2 and M, preventing completion of cell division, which results in accumulation of cells in G_2 and M.[7,8] Many of the arrested cells are doomed to die;[9] however, the proportion of the Taxol-arrested cells capable of further survival is unknown.

Several studies have characterized the *in vitro* cytotoxicity of this agent evaluated by clonogenic or tritiated thymidine assays,[10] colorimetric assays,[11] or flow cytometry.[12]

A question is often asked: can the human tumor cells, in experimental conditions *in vitro*, simulate the basic characteristics of tumors *in vivo*? Although it is difficult to extrapolate between these two systems, multicellular tumor spheroids (MTS),[13] obtained by the aggregation of individual tumor cells *in vitro*, provide a model which mimics tumors in at least two respects. First, as MTS growth progresses, the numbers of cells that are proliferating decreases and the proportion of non-proliferating (quiescent) cells increases.[14] The quiescent cells regain their proliferative capacity after dispersal from the MTS system and transfer to a more favorable growth environment.[15] Second, MTS undergo necrosis, the initiation of which appears to be controlled by nutrient and oxygen deprivation.[16] Hypoxic necrotic and aerobic cells are located in the central areas of MTS surrounded by several layers of actively growing cells.[14,17,18] MTS, established from tumor cell lines or, less frequently, from primary tumor specimens, thus constitute a useful model of solid tumors *in vitro*[19] and provide a suitable method for screening chemotherapeutic agents.[20,21]

Most studies showed that MTS were less sensitive

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to drugs than monolayer cells,[20,22,23] although observations were reported concerning some drugs such as fluorouracil, thiotepa, platin derivatives and nitrosoureas,[24] where the MTS were more sensitive than monolayer cells. These variations would be explained by more or less important penetration and distribution of some agents in the inner cells of spheroids.[25] To our knowledge, no studies have been carried out with Taxol in MTS.

The goal of the study was thus to evaluate the cytotoxic effects of Taxol in MTS obtained from human head and neck squamous carcinoma cell lines. The clonogenic assay, considered as the optimal method since its description by Puck and Marcus in 1955,[26] was chosen as reference.

Materials and methods

Cell lines

Four human squamous carcinoma cell lines were used. HTB43 and CCL17 cell lines were established from tumor biopsies of the hypopharynx and oral cavity epidermoid carcinoma, respectively. The doubling times of these cell lines were 20 and 18 h, respectively. They were provided generously by Dr A Hanauske (University of Munich, Germany). The CAL27 and CAL33 cell lines were both established from oral cavity carcinoma, and their doubling times were 18 and 16 h, respectively. They were provided generously by Dr J-L Fishel (Centre Antoine Lacassagne, Nice, France). Among these cell lines, only HTB43 and CAL27 formed MTS.

The cell lines were continuously maintained as monolayer cultures in RPMI 1640 medium (Gibco, Cergy-Pontoise, France) supplemented with 10% inactivated fetal calf serum (Dutsher, Brumath, France), penicillin (500 UI/ml), streptomycin (5 μg/ml) and glutamine (0.3 mg/ml). Cell cultures were passaged every 7 days to ensure exponential growth.

Chemicals

Taxol (paclitaxel, NSC 125973; Sigma, St Quentin Fallavier, France) was received as a sterile lyophilized powder. Stock solutions (10 mM) were prepared in ethanol and stocked at −20°C. Dilutions were prepared in sterile water just before each experiment. The final concentration of ethanol never exceeded 0.5%. Ethanol at that concentration is not cytotoxic.[27]

Clonogenic assays

As a reference for the evaluation of Taxol cytotoxicity, clonogenic assays were performed in the four cell lines. The cells were harvested from exponential phase culture by trypsinization, counted and plated in 6-well plates (diameter 35 mm; Costar, Brumath, France). Optimal seeding densities of each cell line were determined to obtain the best plating efficiency. An initial cell number of 600 cells/well was found suitable for all cell lines. One milliliter of each cellular suspension was distributed in each well, diluted in 1 ml culture medium. Before being treated, the cells were incubated for 24 h to ensure their exponential regrowth and, then, Taxol was added (200 μl) in serial dilutions from 0.01 to 10 nM. Cultures were incubated at 37°C with 5% CO_2 in air for 10 days. Following incubation with Taxol, the colonies were fixed with ethanol and stained with crystal violet (1%). Only colonies containing at least 50 cells were scored and survival was calculated by comparing the number of colonies in treated wells with untreated control wells. Each dose level was plated in triplicate and experiments were repeated three times for each cell line. Drug concentration inhibiting the cellular growth by 50% (IC_{50}) was calculated using the median-effect principle.[28]

MTS

HTB43 and CAL27 cell lines were harvested from exponential phase culture by trypsinization, counted and plated according to the modified method of Yuhas *et al.*[29] and that we have already described.[19] Briefly, 6-well plates were coated with 0.5% agarose (Prolabo, Paris, France) in order to prevent cell attachment to the bottom of the wells and received 2 ml of cellular suspension containing 5×10^4 cells/ml. After 4–5 days, MTS with a diameter of about 250 μm were transferred to individual wells in 12-well test plates (Costar) coated with 0.5% agarose. Then, 100 μl of Taxol was added, with final concentrations ranging between 0.01 and 10 nM. For 16 days, MTS were incubated at 37°C in 5% CO_2 in air in the presence of Taxol. The growth of MTS on test plates was determined twice per week by measuring the diameter of individual spheroids using a calibrated scale in the eyepiece of an inverted microscope. The diameter was converted to volume, assuming spherical geometry.

Spheroid growth curves were plotted from the relative volume variations versus time after treatment. The concentration required to inhibit the MTS

growth by 50% (ID_{50})[19] was calculated mathematically from dose–response curves constructed by plotting the normalized volume ratio of treated MTS/control MTS versus Taxol concentration. MTS were considered to have been controlled or 'cured' if they failed to regrow by the end of the observation period (16 days) following the treatment. The 'cured' proportion of MTS was calculated as the fraction of non-regrowing MTS relative to the total number of MTS originally present in the treated population.[19,30] Then, spheroids control curves were plotted and the concentration required to reduce the number of MTS by 50% as compared with the control (SCC_{50}) was calculated mathematically. This parameter was calculated in the same way as the SCD_{50} parameter (spheroid control $dose_{50}$) in radiobiological experiments.[19,30,31] Each experiment was assessed in triplicate. Eight to 12 spheroids were used for each drug dilution and the results were expressed as mean value (± SD).

Statistical analysis

All the results are presented as mean values of three independent experiments and were compared using Student's unpaired *t*-test. Correlations were analyzed by Wilcoxon's test. A significance level of $p < 0.05$ was used throughout.

Results

Evaluation of Taxol cytotoxicity in monolayer cell culture (clonogenic assay)

Figure 1 shows the survival curves of each cell line after treatment with Taxol. Evaluation of Taxol cytotoxicity was estimated by the calculation of IC_{50} values described in Table 1. IC_{50} values varied from 0.20 (± 0.03) to 1.05 (± 0.42) nM but were not statistically different ($p > 0.05$).

Growth characteristics of untreated MTS

Growth curves of untreated spheroids (Figure 2; control curves) obtained from HTB43 and CAL27 cell lines were characterized by a linear phase with correlation coefficients of 0.978 and 0.998, respectively. The slope of each curve, with respective values of 0.209 and 0.409, indicated that the MTS growth of the HTB43 cell line was slightly faster than that of MTS of the CAL27 cell line. The doubling times (± SD), graphically calculated from the growth curves, were 7.72 (± 1.39) days for CAL27 and 6.02 (± 2.21) days for HTB43. Mean initial diameters (± SD) were 249 (± 26) and 281 (± 39) μm for CAL27 and HTB43 spheroids, respectively.

Growth characteristics of Taxol-treated MTS

Growth curves of Taxol-treated MTS plotted as log(volume/initial volume) versus time are illustrated in Figure 2. From these curves, a growth delay of Taxol-treated MTS was observed as compared with untreated control. For the HTB43 cell line, growth delay increased proportionally to Taxol concentrations from 0.01 to 4 nM. At higher concentrations, antitumoral effect was observed, above 10 nM for HTB43 and 4 nM for CAL27, and the MTS volume decreased continuously without regrowth.

From clonogenic survival curves, no difference in cytotoxicity was observed between these cell lines with IC_{50} values of 0.64 (± 0.68) and 0.59 (± 0.20) nM for the HTB43 and CAL27 cell lines, respectively. However, a variation in growth pattern was noticed from one spheroid to another within the same MTS population. Four phenomena were observed that characterized the evolution of MTS growth during the drug treatment. (1) The volume of treated MTS increased in parallel with the control with a time delay. (2) The volume of treated MTS, after a small start phase of cell proliferation, remained stable and did not grow up to the end of the test. (3) The volume of treated MTS decreased immediately followed by a regrowth. (4) Finally, treated MTS failed to grow and their volume decreased, and they could not be observed due to cell dissociation. All these observations led a heterogeneous evolution of MTS relative volume during the assay, illustrated (Figure 3) by the coefficient of variation (CV). Whereas relative volume CV values of untreated MTS were more homogeneous and stable with a global mean of 36%, CV values of Taxol-treated MTS were much more heterogenously varying with the concentration and the time of exposure, and reached 100% 16 days after treatment in both cell lines.

MTS chemosensitivity to Taxol

ID_{50}. Dose–response curves of MTS are illustrated in Figure 4 and showed a 90% volume regression with

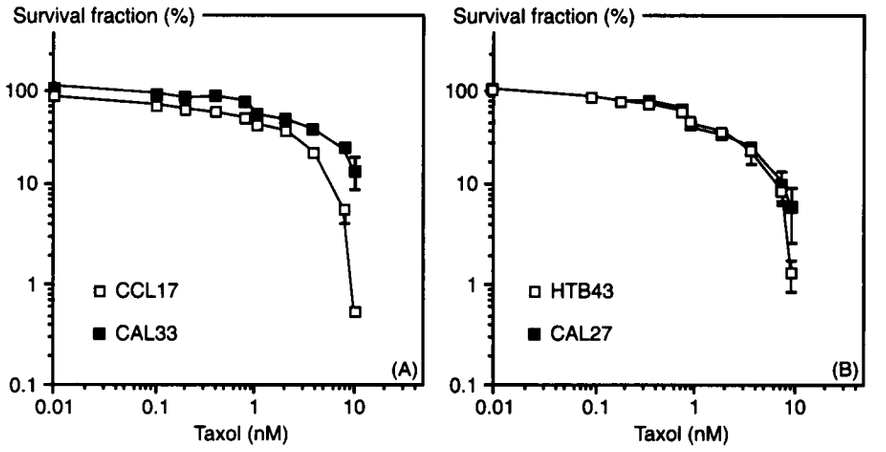

Figure 1. Clonogenic survival curves of human head and neck squamous carcinoma cell lines (A, CCL17 and CAL33; B, HTB43 and CAL27) treated with Taxol in continuous contact for the assay. The surviving fraction was compared with that of untreated cells. All points were determined in triplicate. Standard error bars are shown when greater than the size of the symbols.

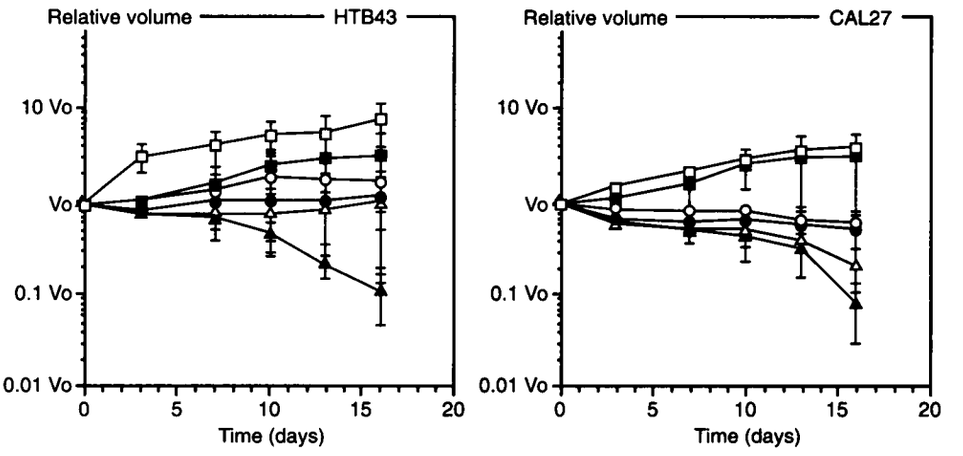

Figure 2. Growth curves of human head and neck squamous carcinoma multicellular spheroids (A, HTB43; B, CAL27) exposed to Taxol. Results are expressed as relative volume variation (volume/initial volume). Each point is the mean value (± SEM) of eight to 12 spheroids. Standard errors bars are shown when greater than the size of the symbols. (□, Control; ■, 0.01 nM; ○, 0.4 nM; ●, 1 nM; △, 4 nM; ▲, 10 nM.)

10 nM of Taxol, 16 days after treatment. Taxol doses required to reduce MTS volume by 50% (ID_{50}) decreased with time of exposure and then stabilized from day 13. A statistically significant difference was observed between IC_{50} values (Table 1) and ID_{50} values (Table 2) at day 10 ($p < 0.05$), illustrating a higher sensitivity of both cell lines to Taxol when growing as monolayers. No statistical difference was observed between IC_{50} parameter values obtained from clonogenic assays and ID_{50} values at day 16 ($p > 0.05$).

SCC_{50}. Figure 5 shows the percentage of controlled ('cured') MTS as a function of Taxol concentration for the HTB43 and CAL27 cell lines. From these curves defined by a logarithmic equation, SCC_{50} parameter values were calculated. As we observed for the ID_{50} parameter, spheroid control curves stabilized from day 13, suggesting the stabilization of MTS volume and number at the end of the assay. Mean values (± SD) of SCC_{50} were 0.88 (± 0.65) nM and 0.75 (± 0.11) nM for the HTB43 and CAL27 cell lines, respectively.

A significant correlation was observed between SCC_{50} and ID_{50} parameter values ($p < 0.01$, $r = 0.979$) illustrating that Taxol cytotoxicity in MTS

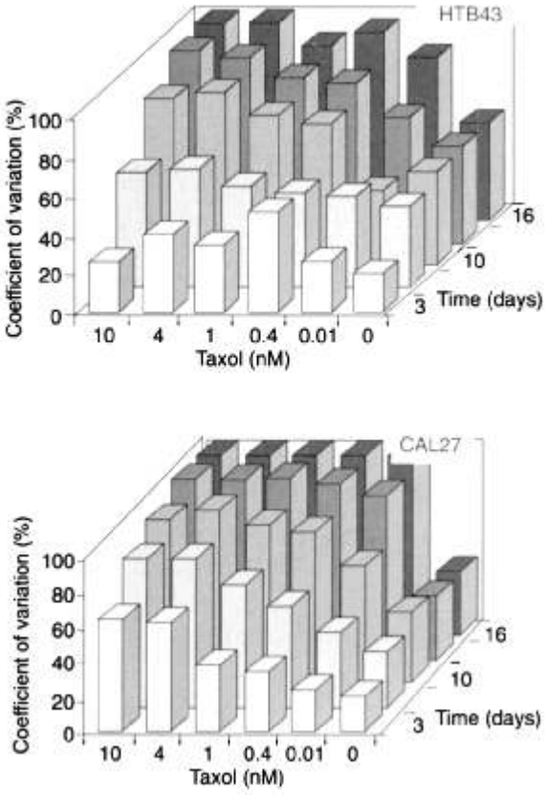

Figure 3. Distribution histograms of coefficients of variation (CV) of multicellular spheroid growth versus taxol concentration and versus time. Each experiment was repeated three times.

can be estimated from either the control of individual MTS volume or from the number of MTS remaining from the initial population. No correlation was observed between SCC_{50} and IC_{50} parameter values.

Discussion

MTS are essentially used to evaluate the radiosensitivity of tumor cell lines or cells from tumoral fragments. Therefore, we attempted to use this model to evaluate the cytotoxicity of Taxol in two head and neck squamous carcinoma cell lines (HTB43 and CAL27). At first, the evaluation of cytotoxicity was performed with a clonogenic assay. A time- and dose-dependent survival relationship with Taxol was demonstrated *in vitro*.[32,33] With a longer incubation time, the number of cells in the mitotic phase increases and lead to death via apoptosis.[9] We analyzed Taxol cytotoxicity in human squamous carcinoma cell lines in continuous contact

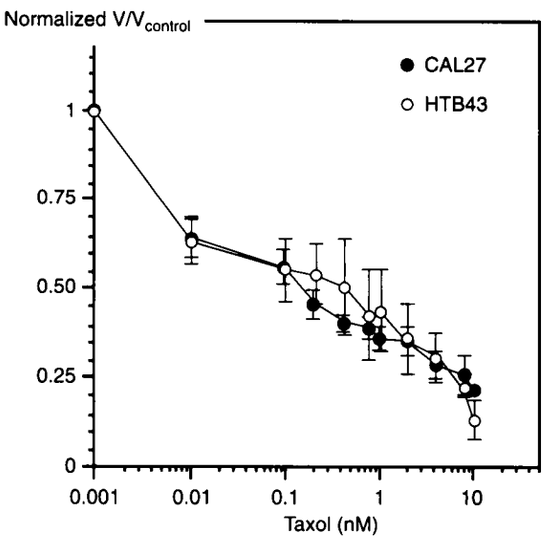

Figure 4. Dose–response curves of Taxol-treated head and neck squamous carcinoma multicellular spheroids (HTB43 and CAL27) expressed as normalized volume ratio ($V/V_{control}$) versus Taxol concentration. The ratio was calculated at 16 days after treatment. Each point represents the average volume (± SD) of eight to twelve spheroids.

Table 1. Taxol cytotoxicity in the four head and neck squamous carcinoma cell lines growing as monolayers

	Cell line			
	CAL33	CAL27	HTB43	CCL17
IC_{50} (nM)	1.05 (0.42)	0.59 (0.20)	0.64 (0.08)	0.20 (0.03)

IC_{50} values were calculated from clonogenic assays and are mean values of three independent assays in triplicate (SD).

Table 2. Taxol cytotoxicity in the two head and neck squamous carcinoma cell lines growing as multicellular spheroids

	Taxol exposure duration (days)				
	3	7	10	13	16
CAL27	76.90 (16.00)	8.91 (2.51)	1.20 (0.23)	0.22 (0.09)	0.17 (0.03)
HTB43	not determined	12.84 (9.08)	4.33 (4.46)	0.63 (0.66)	0.29 (0.32)

ID_{50} values of spheroids exposed for 4–16 days to Taxol are mean values of three independent experiments with eight volumes determination (SD).

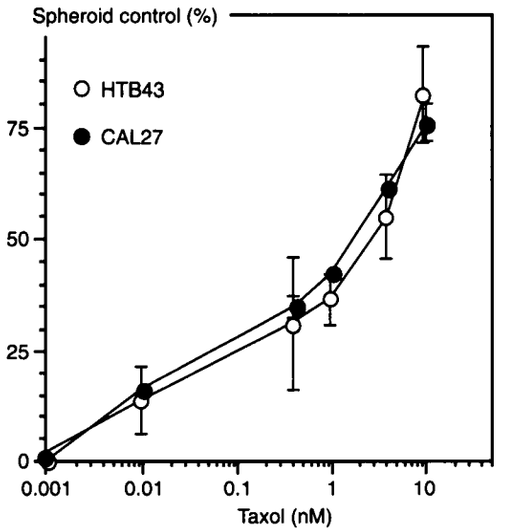

Figure 5. Spheroid control as expressed by percentage cured multicellular spheroids of HTB43 and CAL27 tumor cell lines as a function of Taxol concentration. The Taxol concentration values required to reduce the spheroid number by 50% as compared to control (SCC_{50}) were calculated at 16 days after treatment. Each point represents the mean (± SEM) of three experiments.

during the assay period. IC_{50} parameter values of Taxol for four cell lines ranged between 0.20 and 1.05 nM. The very low IC_{50} (below 0.001 nM) obtained by Braakhuis *et al.*[11] using a colorimetric assay, sulforhodamine B test, in three human head and neck tumor cell lines, was lower than we obtained.

The MTS was also used because this tumor model simulated, in part, *in vivo* conditions, e.g. oxygen and cell cycle kinetic heterogeneity.[14] These factors are known to influence tumor responsiveness to chemotherapeutic agents.[20] Analysis of growth curves plotted from HTB43 and CAL27 spheroids showed a linear phase characterized by a mean coefficient of correlation of 0.988. However, three phases were observed in the MTS growth.[17] The first was exponential in which the MTS proliferated up to a mean diameter of 50–200 μm. The second phase was a growth phase in which the number of cells proliferating decreased and the proportion of non-proliferating (quiescent) cells increased. During this phase, where there was a balance between proliferating and non-proliferating cells, the MTS growth was linear—this was observed in our study. When the cells became deprived of O_2, glucose and other substrates, and when toxic metabolic waste products accumulated, there were steep gradients in these metabolites and necrosis would occur in the center of spheroids. MTS volume remained stable or decreased.[14,17] Mean doubling time of spheroids was 6.8 days during this linear phase, whereas it was 20 h when the cells were in monolayers. Schwachofer *et al.*[30] found no relationship between doubling time of cells cultivated either in monolayers or in MTS. No correlation was found between the doubling time and the diameter of MTS in the two cell lines we studied. A high heterogeneity (reaching 100%) was observed, and was related to Taxol concentration and incubation time. This could be explained by the fact that this analysis was performed in spheroid populations which had diameters varying between 281 (± 39) μm (HTB43) and 249 (± 26) μm (CAL27). Hypoxia and necrosis began to develop in the MTS center from a diameter of 200–300 μm. Around necrotic areas, quiescent cells were localized[34] and it was reported that quiescent cells were less sensitive to taxoids than mitotic cells.[32] The growth might be more or less affected as a function of quiescent cell number which depended on the size of the MTS. This hypothesis would involve high variations concerning the response to antineoplasic drugs in a MTS population with a heterogeneous size.[20] These variations increased when the Taxol concentration was high.

Moreover, another hypothesis might be expressed concerning the incorporation of Taxol which might be more important inside spheroids. Bhuyan *et al.*[24] compared the cytotoxicity of cisplatin and tetraplatin in cells growing as monolayers and in MTS obtained from three ovarian tumor cell lines. They showed that spheroids were significantly more sensitive than cells in monolayers. They quantified platin accumulation inside MTS and found that this higher sensitivity was due to higher intracellular levels of the drug in the MTS cells. Durand[35] observed that the intracellular level of tetraplatin in the inner cell layers of spheroids was higher than in the outer cell layers. On the other hand, Erlanson *et al.*[36] reported relationships between the penetration, fixation, accumulation and diffusion of drugs such as Ara-C and actinomycin C in spheroids formed from gliomas and colon tumors. According to these factors, spheroids could be more chemosensitive.

As regards the chemosensitivity of squamous carcinoma cell lines HTB43 and CAL27 spheroids, ID_{50} values were similar (0.29 and 0.17 nM, respectively). When we compard the ID_{50}, calculated at day 16 post-treatment, with IC_{50} values obtained from clonogenic assays, no significant difference was noticed. Nevertheless, the absence of difference between these parameters was observed only from

day 13 post-treatment. Thus, the time factor had to be taken into account. The doubling time for HTB43 and CAL27 spheroids was 6 days. This showed that at least one cell cycle was necessary to notice Taxol effects,[32] it was only between day 10 and day 13 post-treatment that Taxol effects could have been demonstrated. This is the period where the IC_{50} and ID_{50} did not differ any more, although IC_{50} takes into account only individual cells; however, ID_{50} takes into account a group of cells corresponding to the structured tumor entity.

The SCC_{50} parameter was used in the literature under the same SCD_{50} (spheroid control dose$_{50}$) in terms of radiosensitivity parameter,[19,31] but was never used to evaluate the chemosensitivity of spheroids. Usually this was estimated by the growth delay assay[22] or more frequently by the clonogenic assays after disaggregation of MTS.[37]

These two techniques were not used in our evaluation. The growth delay was determined by calculating the difference between treated and control MTS, i.e. the time necessary to reach the initial volume. However, according to Sutherland,[14] this parameter took into account only the proliferating cells of MTS, leaving the inner cell layers of spheroids. This inconvenience could be avoided by analyzing small size spheroids (about 100 μm) containing only proliferating cells.[34] In our study, the MTS had a diameter larger than 100 μm. As for the clonogenic assay, two main problems were considered. First, the cells were more fragile after disaggregation of MTS by trypsinization and it was difficult to obtain a single cell suspension from spheroids, which is a fundamental criteria in optimization of the clonogenic assay.[34] Second, the cell suspensions obtained from spheroids did not represent the MTS any more, which had lost their structure.[34] Therefore we considered that the drug concentration necessary to reduce the initial population of MTS by 50% (SCC_{50}), assimilated to TCD_{50} (tumor control dose 50) in animals,[30] was more relevant for the estimation of the chemosensitivity of MTS as it was shown with SCD_{50} in radiobiology.[17] No statistical correlation was observed between SCC_{50} and IC_{50} values. Nevertheless, an increased number of cell lines will probably be needed to obtain a correlation, as Poirreau-Schneider[38] reported by comparing the survival fraction determined by clonogenic assay and the controlled spheroids fraction.

A significant correlation was found between ID_{50} and SCC_{50} values ($p = 0.01$; $r = 0.979$), and confirmed our previous results achieved in ovarian cell lines.[39]

In conclusion, the cytotoxicity of Taxol was found to be slightly different in multicellular spheroids, probably depending on the different growth kinetics and cell cycle phase involvement of the cells. Therefore, MTS appeared to be very useful for the *in vitro* analysis of human tumor chemosensitivity since they contained not only proliferating cells but also quiescent cells and extracellular matrix components which are not found in monolayer cultures and which may influence drug effects. The results of this study support the finding that the chemosensitivity of human tumor cell lines can be studied in terms of MTS 'control' considering the concentration required to reduce the initial MTS number, SCC_{50}, assimilated to SCD_{50} and TCD_{50} in animals, and obtained by extrapolation of spheroid control curves. This parameter correlates with ID_{50} as already found with SCD_{50} and ID_{50} by evaluating the radiosensitivity in ovarian tumors MTS.[19] MTS derived from human tumors appears to offer a tool for the *in vitro* analysis of human tumor chemosensitivity when exposed to drugs, simulating in part the conditions found in the tumor *in vivo*.

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