In vitro efficacy of chemotherapeutics as determined by 50% inhibitory concentrations in cell cultures of mammary gland tumors obtained from dogs

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**Objective**—To determine the 50% inhibitory concentration (IC-50) of carboplatin, cisplatin, and doxorubicin in cell cultures of mammary gland tumors obtained from dogs and to assess whether in vitro efficacy was within the range of clinically relevant concentrations.

**Sample Population**—30 mammary gland tumors excised from dogs.

**Procedure**—Cell cultures were established from the 30 tumors. Cultures then were treated with carboplatin, cisplatin, or doxorubicin. Growth inhibition of cultures was assessed via DNA measurement 24, 48, and 72 hours after treatment. The IC-50 values were calculated by use of linear interpolation.

**Results**—Cultures varied in their pattern of susceptibility. Doxorubicin induced significantly lower IC-50 values than the platinum derivatives. Cisplatin and carboplatin had comparable effects. The IC-50 values for carboplatin and doxorubicin were in the range of clinically relevant concentrations, but only part of the cisplatin cultures had IC-50 values within clinically relevant concentrations. We did not detect differences in the in vitro susceptibility among subtypes of tumors (ie, adenocarcinoma, solid carcinoma, malignant mixed tumor).

**Conclusion and Clinical Relevance**—The IC-50 values determined in this study allowed assessment of in vitro drug efficacy of chemotherapeutics in cultures of mammary gland tumors obtained from dogs. Variations in susceptibility were evident and emphasize the importance of assessing susceptibility and resistance patterns for each tumor. Prospective studies to assess the pattern of resistant mutations within tumor subtypes may be required.

**Materials and Methods**

**Sample population**—Cell cultures were established from 30 mammary gland tumors that were aseptically excised from dogs. None of the dogs had received treatment for the mammary gland tumors. These tumors represented 3 histologic subtypes (group 1, adenocarcinoma; group 2, solid carcinoma; group 3, malignant mixed tumor); each group consisted of 10 tumor samples.

**Procedure**—Samples were disaggregated, using a combination of mechanical disruption and short-term enzymatic treatment with collagenase. Explant cell suspensions were cultivated in RPMI 1640 culture medium supplemented with 20% fetal calf serum (FCS), 8% penicillin-streptomycin (5,000 U/ml and 5,000 µg/ml, respectively), and trace elements, as reported by McKeehan et al. Suspensions were cultured in 25-cm² culture flasks and incubated at 37°C, 98% relative humidity, and 10% CO₂. When confluence was attained, primary cell cultures were subjected to a standard trypsinization process. Cells then were counted electronically and transferred to a 75-cm² culture flask. When confluence was attained in these flasks, cultures were subjected to trypsinization. Cells then were allotted to aliquots that were used for cell characterization and assessment of susceptibility to various chemotherapeutics. Cultured cells were characterized by use of immunohistochemical techniques, using monoclonal antibodies (anti-vimentin, anti-pancytokeratin [PAN], and anti-pancytokeratin AE1/AE3).

Each culture was treated with carboplatin, cisplatin, or doxorubicin (50% inhibitory concentration (IC-50)) of carboplatin, cisplatin, and doxorubicin in cell cultures of mammary gland tumors obtained from dogs and to assess whether in vitro efficacy was within the range of clinically relevant concentrations.
doxorubicin. Six cultures were used to test efficacy of each chemotherapeutic. Chemotherapeutics were administered at 8 concentrations ranging from 0.05 to 100 µM. For this portion of the study, 5 X 10^6 cells were suspended in 50 ml of RPMI 1640 medium with 20% FCS and the supplements described previously. Cells then were seeded in wells of a 96-well culture plate. For each of the 3 chemotherapeutics and each time point (24, 48, and 72 hours), a 96-well plate was prepared. Wells in the outside row of each plate contained medium only and, thus, served as protection against evaporation. Wells in 1 row of each plate contained only 50 µl of medium that did not contain any chemotherapeutic (blank measurement). These microcultures were incubated for 24 hours, using conditions described previously, and the in vitro treatments subsequently were added. Wells in 1 plate were treated with 50 µl of RPMI 1640 medium containing 1 of the chemotherapeutics at concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, 10, 50, and 100 µM respectively. All wells in a row received the same concentration of chemotherapeutic. Microculture plates then were incubated for 24, 48, or 72 hours.

Drug efficacy was assessed at 24, 48, and 72 hours via DNA measurement, using a nucleic acid stain in accordance with instructions provided by the manufacturer. The untreated cells also were measured, thus serving as a negative-control sample. We used a nucleic acid stain that has high sensitivity for double-stranded DNA, which allows for accurate DNA quantitation and, therefore, assessment of cell proliferation.

Following addition of stain to the microcultures, a fluorometric measurement at dual wavelengths of 485 and 538 nm was performed. The extinction value for the blank measurement (ie, extinction value for the culture medium) was subtracted from values for all treated wells, and a mean value was calculated from the wells in each of the 6 cultures treated with the same concentration of chemotherapeutic. Results of the assay for each concentration were calculated as a percentage of the untreated control wells.

The IC-50 values were calculated for each tumor culture, using linear interpolation as described by Kurbacher et al. Mean IC-50 were calculated for each chemotherapeutic at a specific time point for all 30 tumors. Mean IC-50 of each chemotherapeutic was calculated for each tumor group (n = 10 tumors/group) to compare effectiveness of each chemotherapeutic among tumor groups.

Statistical analysis—Because of the small sample size and the fact that the data were not normally distributed, as determined by use of the Kolmogorov-Smirnov test, statistical analysis was performed by use of a nonparametric test. The IC-50 values were compared among the 3 chemotherapeutics and the 3 tumor groups, using the Wilcoxon matched-pairs test. In this analysis, mean IC-50 values of 2 chemotherapeutics or tumor groups were compared in pairs at 1 time. Values of P < 0.05 were considered significant.

Results

During immunohistochemical characterization of cells, all cultures had positive results for cytokeratin (PAN) and anti-cytokeratin AE1/AE3 antibodies. Cultures differed in the degree of positive reactivity. In addition, vimentin-positive cells also were found in all cultures. All 3 chemotherapeutics had differing degrees of time- or dose-dependent inhibitory effects, or both, in the cultures tested. Chemotherapeutics inhibited DNA production in cultures of tumor cells (Fig 1). Comparison of in vitro efficacy of the chemotherapeutics revealed that doxorubicin induced the most potent suppression of DNA production, followed in order by cisplatin and carboplatin.

Susceptibility and resistance patterns of each tumor culture also were assessed. Patterns differed among tumors, as determined on the basis of differences in DNA measurements for maximum and minimum values (Fig 2). Mean IC-50 values were calculated (Table 1). There was a time-dependent relationship of mean IC-50 values for carboplatin, cisplatin, and doxorubicin (Fig 3). After 24 hours, only 1 of the tumor cultures treated with carboplatin had an IC-50 value for inhibition of DNA production (87.5 µM). After 24 hours, IC-50 for tumor cultures treated with cisplatin ranged from 19.5 to 91.8 µM (mean IC-50, 48.5 µM), and there was only a slight difference among the histologic tumor types. The IC-50 values of tumors in group 2 (solid carcinomas) were significantly (P = 0.01) less than IC-50 values of tumors in group 3 (malignant mixed tumors). After 24 hours, cultures treated with doxorubicin had IC-50 values that ranged from 0.22 to 3.13 µM (mean, 0.84 µM). The IC-50 values for group 2 were significantly (P = 0.02) less than for the other groups.

Forty-eight hours after addition of carboplatin to the tumor cultures, IC-50 values ranged from 22.1 to 99.6 µM (mean, 33.5 µM). However, susceptibility did not differ significantly among the tumor groups. After 48 hours, cisplatin-treated cultures had IC-50 values that ranged from 4.24 to 78.9 µM (mean, 27.3 µM), and tumors in group 2 had significantly (P = 0.005) greater in vitro susceptibility to cisplatin, compared with tumors in group 3. After 48 hours, tumor cultures treated with doxorubicin had IC-50 values that ranged from 0.042 to 1.9 µM (mean, 0.34 µM); however, susceptibility among the 3 tumor groups did not differ significantly.

After 72 hours, cultures treated with carboplatin had IC-50 values that ranged from 3.4 to 93.9 µM (mean, 30.48 µM), but values did not differ significantly among tumor groups. After 72 hours, cultures treated with cisplatin had IC-50 values that ranged from 0.3 to 85.3 µM (mean, 18.7 µM). Cultures of tumors in group 1 had significantly (P = 0.036) greater susceptibility to cisplatin, compared with that for tumors in group 2. After 72 hours, cultures treated with doxorubicin had IC-50 values that ranged from 0.338 to 0.87 µM (mean, 0.28 µM); however, susceptibility did not differ significantly among tumor groups. Effects induced by the chemotherapeutics in each tumor group differed significantly among the drugs in
all 3 tumor groups for the 3 time points (Table 2). The IC-50 value in the doxorubicin-treated cultures was significantly lower than that in the carboplatin- and cisplatin-treated cultures in all 3 tumor groups after 24, 48, and 72 hours. The IC-50 value of cisplatin-treated cultures also was significantly lower than that of carboplatin-treated cultures in nearly all cases, except for tumors in group 2 after 48 and 72 hours and tumors in group 3 after 24, 48, and 72 hours. Therefore, the difference in susceptibility of each culture to carboplatin and cisplatin was apparent.

### Discussion

In vitro drug susceptibility testing is of importance in the development and examination of therapeutic
Quantification of DNA has been described as a method for assessing in vitro cell proliferation in non-clonogenic assays. The nucleic acid stain used in the study reported here is a fluorometric test with high sensitivity for double-stranded DNA, which allows accurate assessment of in vitro cell proliferation and drug-induced growth inhibition. The ability to use this assay in microculture plates in a semiautomated fashion increases its efficiency. The limitations, however, are that in measuring DNA, this assay reflects the number of cells but cannot be used to determine whether these cells are metabolically active or have retained their ability to divide.

It is important to characterize cultured cells for in vitro susceptibility testing, because it determines whether these cells represent the tumor explant or whether other cells (e.g., fibroblasts) have overgrown the culture and will influence the value of the susceptibility results. In the study reported here, all cultures had positive results for cytokeratin antibodies in the immunohistochemical testing, confirming the epithelial nature of the cultured cells. The cultures also had positive results for vimentin. Coexpression of cytokeratin and vimentin is considered a common finding in cultured cells and is believed to be attributable to rapid proliferation in vitro and the loosening or loss of cell-to-cell contact. However, the possibility of contamination with mesenchymal cells cannot be excluded. The use of flow-cytometry for characterization of cultured cells as described by Prop et al is more accurate and should be considered for future studies.

Determination of IC-50 values for dose-effect testing of a substance has been used to assess efficacy of chemotherapeutics during in vitro susceptibility testing in human medicine as well as veterinary medicine. Determination of IC-50 values in an in vitro setting allows comparison of the efficacy of various drugs among neoplasms of the same histologic type as well as the susceptibility of various neoplasms to a specific chemotherapeutic. In addition, the IC-50 value is seen as a possible way to compare, to a certain degree, the effective in vitro concentration with the plasma concentration attainable in vivo. Direct clinical relevance of in vitro testing, however, is a controversial area in the field of oncology. A number of studies have provided good correlations of in vitro chemosusceptibility results with clinical outcome in patients with various neoplastic diseases, including breast cancer. Certain authors stress the negative-predictive value of chemosusceptibility testing of tumors, enabling clinicians to possibly avoid unnecessary toxic effects in their patients. However, there are also a number of studies in which in vitro chemosusceptibility results yielded insufficient predictive value for the patients’ clinical course of treatment. Other authors stress the necessity for careful interpretation of in vitro results as well as the need for a greater number of prospective trials on correlations between in vitro and in vivo test results.

In the study reported here, IC-50 values of the tumor cultures differed distinctly, depending on the chemotherapeutic used for in vitro treatment. The IC-50 values of doxorubicin-treated cultures were attained at concentrations significantly lower than those for cultures treated with platinum derivatives, confirming the results of other authors regarding potency of this chemotherapeutic in mammary gland tumor cells of dogs. On the other hand, comparison between mean IC-50 values for carboplatin and cisplatin revealed that these substances had similar in vitro efficacy in a large proportion of measurements.

Comparison of mean IC-50 values with respect to tumor group did not reveal consistent differences among the 3 histologic tumor types tested. However, a greater number of cultures should be tested to adequately assess drug susceptibility and its correlation with a certain histologic type of tumor. A more differentiated interpretation of in vitro drug efficacy is possible by comparing IC-50 values with in vivo plasma concentrations of the tested agent after administration of the substances at therapeutic dosages.

In our study, IC-50 values for carboplatin-treated cultures were 87.3, 53.4, and 30.48 µM after 24, 48, and 72 hours, respectively. In dogs, peak plasma concentrations of 200 µM are attained following IV administration of 300 mg of carboplatin/m² of body surface area (BSA). This indicates that the IC-50 values for carboplatin during chemosusceptibility testing of mammary gland tumors of dogs are within the range of clinically relevant concentrations. However, it must be mentioned that plasma concentrations are not maintained for a period comparable to the drug exposure times used in this in vitro assay. Therefore, a direct translation of in vitro efficacy to in vivo situations cannot be made.

In contrast to our results, Knapp et al reported a IC-50 value of 6 µM for carboplatin in their chemosusceptibility testing of cultures of melanomas obtained from dogs. This emphasizes the clear differences in susceptibility of various tumors to a specific chemotherapeutic.

In our study, mean IC-50 values for cisplatin were 48.5, 27.25, and 18.78 µM, depending on the time point. Following IV administration of a dose of cisplatin (2 mg/kg of body weight or 60 to 70 mg/m² of BSA), peak plasma concentration in dogs is 23 µM, which decreases to 1.5 µM within 24 hours. Analysis of these values indicates that only part of the IC-50 values for cisplatin-treated cultures of mammary gland tumors of dogs were in the range of attainable in vivo plasma concentrations. In contrast to our results, Knapp et al reported a IC-50 value of 1.6 µM in melanoma cells obtained from dogs, again revealing the difference in susceptibility patterns among various tumor types.
IC-50 tumor cultures treated with doxorubicin had values that differed significantly. After 24 hours, mean IC-50 value was 0.84 µM. After 48 and 72 hours, mean IC-50 values were 0.34 and 0.28 µM, respectively. In dogs, peak plasma concentrations of 3.5 µM are attainable following IV administration of doxorubicin at a dosage of 30 mg/m² of BSA, indicating that the IC-50 values for the study reported here were within the range of clinically relevant concentrations. However, these plasma concentrations quickly decrease to < 1 µM and are not maintained for 24 hours, an exposure time that we used in the in vitro assay, which limits our ability to interpret these results with regard to in vivo conditions.

It also must be emphasized that the cultures in our study had a certain degree of variation in IC-50 values induced by the tested chemotherapeutics. Characterization of a chemotherapeutic by a mean IC-50 value does not take into account the drug’s efficacy in each of a number of cultures. This heterogeneity stresses the importance of determining susceptibility or resistance patterns of each culture for each chemotherapeutic. The ability to assess the susceptibility to each drug represents 1 of the major justifications for the use of in vitro chemosusceptibility assays.

Time- and dose-dependent efficacy of carboplatin, cisplatin, and doxorubicin were evident in cultures of mammary gland tumors obtained from dogs. Mean IC-50 values of carboplatin and doxorubicin were in the range of clinically relevant concentrations, whereas mean IC-50 value for cisplatin did not reach the range of clinical peak plasma concentrations by 72 hours. Limitations of the chemosusceptibility assay used for our in vitro treatments were the exposure times of 24, 48, and 72 hours, which do not correspond to the period that the concentrations of these chemotherapeutics are maintained in vivo. Therefore, a direct comparison of results of this in vitro assay to in vivo situations is not possible. Prospective studies that involve use of treatment regimens designed in accordance with in vitro chemosusceptibility results, using systemically administered chemotherapeutics or even novel approaches such as intraslesional and liposome-encapsulated agents that increase drug concentrations in tumor tissues above plasma concentrations, are necessary to accurately determine a direct correlation between in vitro and in vivo results. Only when we can compare clinical outcome of patients treated in accordance with in vitro test results with clinical outcome of patients treated empirically will we be able to assess the predictive value and clinical applicability of this assay for dogs with mammary gland tumors. The in vitro efficacy for the chemotherapeutics in our study was encouraging and justifies the performance of prospective studies in dogs with mammary gland tumors. The ability to determine differences in the susceptibility of tumor cultures makes this assay a useful screening method for in vitro cytotoxicity and allows for assessment of specific in vitro susceptibility and resistance patterns.

References
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