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Review Article

In vitro assays of tumor chemosensitivity and chemoresistance

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CONTENTS

Abstract

There is a need for clinically useful drug response assays to individualize chemotherapy for cancer patients. This review summarizes the various assays developed to assess the drug sensitivity and/or resistance of primary tumor cells and the clinical validation of these various technologies. Significant predictive correlations between some *in vitro* drug response assays and cancer patient response and survival have been demonstrated. Prospective randomized clinical trials comparing survival between patients given a drug tested *in vitro* and patients treated with standard chemotherapy are, however, required to demonstrate the real impact of *in vitro* assay-assisted treatments on patient survival.

Introduction

Despite the consistent progress in recent years, cancer is curable only in some patients and there is a continued need for new antitumor drugs. In particular, a review of the incidence and mortality data for each type of cancer clearly indicates that the curability of some cancers (*i.e.*, lung, pancreas, stomach) remains particularly low and has not been significantly improved by the advances in cancer research (1).

The tremendous progress in molecular pathology, and particularly as regards methods for the identification of genetic lesions in cancer (2), genomic and proteomic analysis of tumors and high-throughput compound screening and structure-based drug design, has led to the identification of a large number of drug targets and molecules as potential new drugs that act on these targets. However, despite these advances, drug development remains a particularly long and difficult process (3, 4). In fact, it is not possible to proceed directly from *in vitro* cell culture-based assays to clinical trials (5, 6), and the development of new drugs requires a large number of *in vivo* studies in animal models to evaluate drug safety and activity, and to predict the possible spectrum of antitumor activity of a given compound. Although animal studies can not be eliminated entirely, the need remains for the development of *in vitro* assays to better predict drug tumor sensitivity and resistance (6).

In vitro cell culture assays are required not only for the screening of new drugs, but also for selecting an optimal individual drug therapy according to the pattern of *in vitro* drug resistance/sensitivity. This is particularly relevant given the heterogeneity of tumors. In fact, studies carried out on leukemia cells have clearly indicated that a specific acquired genetic abnormality is pathogenetically important for the development of each type of leukemia; however, the full progression to leukemic disease requires additional mutations that are different for the various patients (7). This tumor heterogeneity within each tumor histiotype represents an important variable in clinical treatment and suggests that the best therapeutic approach would be individualized drug therapy.

This review discusses the different types of *in vitro* tumor assays and their use to predict drug sensitivity, resistance, or both. In this context, it is important to emphasize that the review will cover only studies related to assays based on the growth of primary tumor cells and excludes assays based on tumor cell lines. Several studies (reviewed in 6) have compared cell cycle time and drug sensitivity (to chemotherapeutic agents such as carboplatin, cisplatin and paclitaxel, or tyrosine kinase inhibitors) in tumor cell lines or primary tumor cells grown

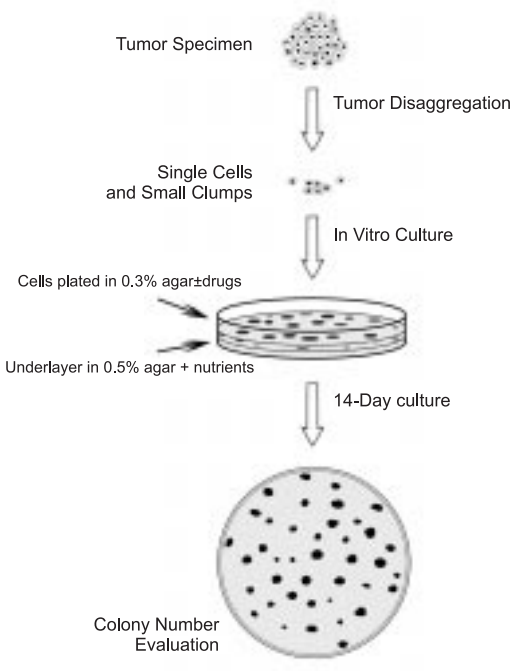

Fig. 1. Outline of the human tumor clonogenic assay.

in vitro in a clonogenic assay. In spite of the great heterogeneity of the cell lines and primary tumor cells, there was a common trend in the cell lines towards a higher growth rate and a reduced drug response compared to the primary tumor cells (6). The main role of cancer cell lines is in the initial steps of screening and characterization of new cancer drugs (8, 9).

Human tumor clonogenic assay

The so-called human clonogenic stem cell assay, or colony-forming assay, is the most widely studied chemosensitivity/chemoresistance assay. In a review of the literature in 1990, Von Hoff described 54 different *in vitro/in vivo* trials. The sensitivity of the test was 79%, the specificity 86%, the positive predictive value 69% and the negative predictive value 91% (10). The test, however, has major limitations due to the high number of tumor cells required and the low percentage of successful assays (40-70%).

The test is based on the evaluation of the capacity of single malignant cells to divide and form colonies in or on an agar-based matrix (11-13). (Fig. 1). In the first step of the assay, the tumor specimens are mechanically minced with scissors and subsequently incubated with an enzyme cocktail consisting of collagenase, deoxyribonuclease (DNase) and hyaluronidase. After filtration on sieves with a mesh size of 50 μM in diameter, the cells are plated. In the most commonly adopted clonogenic assays, the cells (usually 5 x 10^4 cells/well) are plated in 24-well dishes in culture medium containing fetal calf serum and 0.4% agar on top of a 0.5-0.75% agar cushion (14). The cells are either pre-exposed for a short time to drugs before culture or continuously exposed to drugs during the entire culture time of approximately 2-3 weeks. Cells that are killed by these drugs or damaged so that they are blocked in the cell cycle fail to form colonies. The evaluation of colony number in samples treated with drugs compared with untreated controls provides a measure of drug activity.

The human tumor clonogenic assay may be susceptible to major changes due to the identification and characterization in many tumors of the cancer stem cell fraction. *In vitro* and *in vivo* assays, including the human tumor colony assay, have provided evidence that only a small fraction of tumor cells exhibit extensive proliferative capacity *in vivo* and *in vitro* (15).

The existence of cancer stem cells was first demonstrated in the context of acute myelogenous leukemia (AML). The first conclusive evidence for the existence of leukemic stem cells came from the identification of a very rare leukemic cell population capable of producing leukemia after transplantation in a xenograft transplant system (immunodeficient mice) (16). Recent studies indicate that leukemic stem cells are not functionally homogeneous, but, like normal hematopoietic stem cells, comprise distinct hierarchically arranged leukemic stem cell classes (17). Leukemic stem cells may be purified on the basis of their peculiar properties related to the expression on their surface of the CD34 antigen (also a marker of normal hematopoietic progenitor/stem cells) and of high levels of the α-chain of the interleukin-3 receptor (IL-3Rα) (18). The latter antigen is often highly expressed in acute leukemias (19).

Similar observations have been reported in multiple myeloma, where myeloma stem cells are $CD138^-$ B-lymphoid cells with the ability to replicate and differentiate into malignant $CD138^+$ plasma cells (20).

Recently, evidence was also obtained of the existence of cancer stem cells in tumors of epithelial origin. A rare cell population, characterized by CD44 expression and lack of expression of CD24 ($CD44^+/CD24^-$), was identified in breast cancer cells that produce tumors after *in vivo* injection (21). Similar results have been observed for cancers of the central nervous system. Neural tumor cells that express the membrane CD133 antigen accounted for most of the brain tumor proliferative activity; in culture, these $CD133^+$ cells give rise to neural/glial cells identical to those of the original tumor (22). However, it remains to be proven if these $CD133^+$ cells form tumors in immunodeficient mice (22).

Finally, using xenograft models based on the injection of primary tumors, evidence was obtained for the existence in prostate carcinoma of tumor stem cells that survive transplantation and maintain pluripotentiality, as demonstrated by the capacity to generate progeny that differentiate along the epithelial secretory lineage in response to androgens and along the neuroendocrine cell lineage in response to androgen deprivation (23).

According to these findings, it was suggested that malignant tumors are comprised of both cancer stem cells, which have a great proliferative potential and continuously feed the tumor, as well as more differentiated cancer cells with limited proliferative potential. The improvement in the definition and identification of cancer stem cells may contribute to the development of therapies targeted to the unique properties of these cells to enable their selective killing. These findings could contribute to the development of improved clonogenic assays for cancer stem cells to be used to predict the sensitivity/resistance of these cells to drugs.

A large part of cancer research is focused on the identification of new therapies targeted to functionally relevant genes or pathways of crucial importance for cancer development. It is believed that in some tumors, standard chemotherapy is able to kill more differentiated cancer cells with limited proliferative potential, leading to tumor shrinkage, while a portion of the cancer cells survive and continue the process of tumor growth and progression after a period of remission. The stem cell model of cancer predicts that therapies whose target is expressed at the level of cancer stem cells are more likely to be successful in eradicating tumors (24).

ATP cell viability assay

The ATP assay measures the level of viability of tumor cells grown in the absence and the presence of drugs, through the determination of intracellular ATP in the culture cell population (25). Quantification of ATP levels has been shown to directly reflect the number of viable cells (25). Survival fractions can be accurately determined by calculating the ATP ratios of treated and untreated samples. The cell culture conditions used in this assay involve the use of 96-well round-bottomed polypropylene plates and serum-free medium, conditions favoring the growth of malignant cells and suppressing the growth of normal cells (25) (Fig. 2). The cells are grown for 6-7 days either in the absence or in the presence of the drugs. The advantage of this test resides in its high sensitivity, but it is limited by the possible contamination of tumor cells with normal nonmalignant cells.

Several studies have suggested a potential clinical utility of the ATP assay in the chemotherapy setting. Two studies have been carried out in ovarian cancer patients. Konecny *et al*. provided evidence that stage III primary ovarian cancer patients resistant *in vitro* to platinum have a poorer prognosis and a shorter survival than chemosensitive patients (26). These findings were confirmed in another study based on the analysis of 161 ovarian cancer patients showing that the likelihood of obtaining a clinical response in chemosensitive patients was double that observed in chemoresistant patients (27). In a third study, Sharma *et al*. observed an improvement in the response to therapy in a group of 46 pretreated patients with advanced ovarian cancer (28).

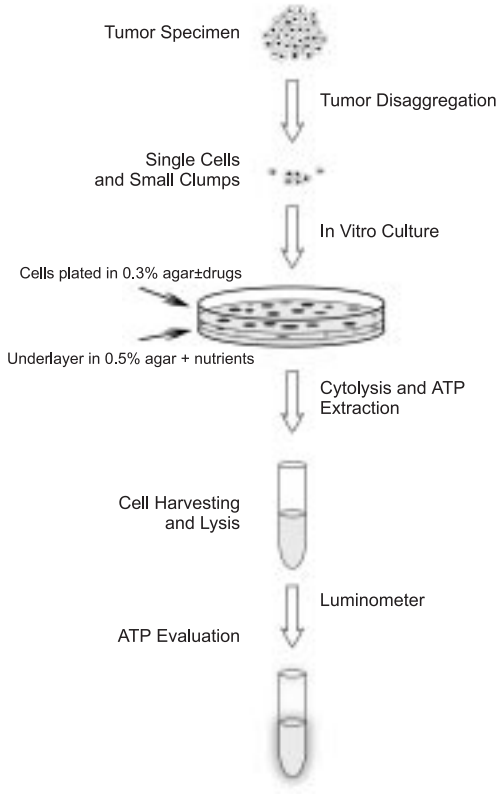

Fig. 2. Outline of the ATP chemosensitivity assay.

In vitro studies have provided evidence that the ATP assay may be performed in other types of tumors, such as breast cancer (29) and melanoma (30), and could represent a basis for the development of clinical studies.

MTT dye reduction assay

The MTT dye reduction assay is based on the cellular reductive capacity to metabolize MTT dye to a highly colored formazan product. This reduction capacity depends on mitochondrial succinate dehydrogenase activity and represents a good measure of mitochondrial function and cell viability. Tumors can be assayed in the MTT test as small organoids or fragments grown on collagen gel sponges, or cell aggregates grown in adherent cultures (31). The MTT test is widely used in cancer cell lines to test drug activity, but it is rarely used in primary tumors. Its main limitation is related to the cell culture conditions that do not favor selective tumor growth.

In spite of these limitations, the MTT histoculture drug response assay was successfully used to predict drug resistance in gastric cancer, showing in stage III and IV patients a better survival among patients sensitive to mitomycin C and fluoropyrimidine compared to chemoresistant patients (31).

Recent studies have provided evidence that the MTT assay may be used to predict tumor resistance in head

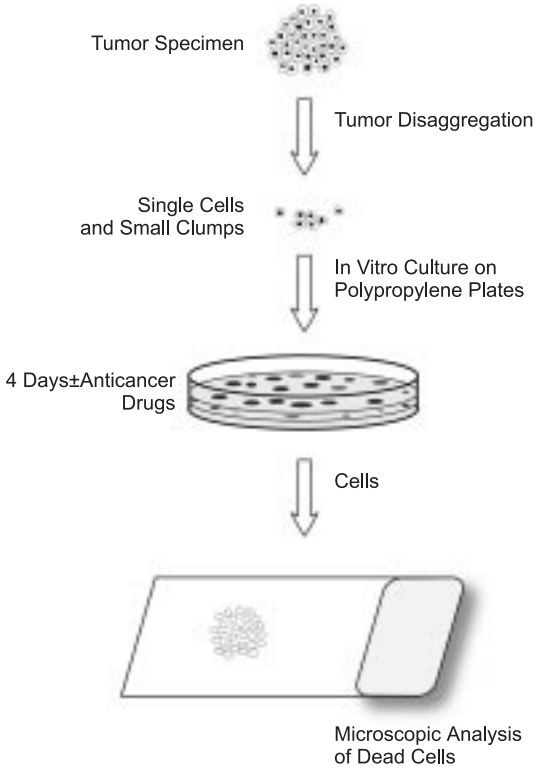

Fig. 3. Outline of the differential staining cytotoxicity (DiSC) assay.

and neck cancer. In these studies, tumor tissues were histocultured on Gelfoam sponge gel in 24-well cultures, demonstrating tumor growth in the large majority of cases. The 2-year cause-specific survival was significantly greater for patients sensitive to 5-fluorouracil (5-FU) or cisplatin, or both agents, than for chemoresistant patients (32). These findings were confirmed in an independent study showing a good correlation between chemosensitivity and clinical response (33).

Differential staining cytotoxicity assay (DiSC)

This assay was originally developed by Weisenthal *et al.* (34) to measure the drug sensitivity of hematological malignancies. This assay is based on the ability of vital cells to exclude dyes *in vitro*. Tumor cell suspensions are grown in liquid suspension culture in the absence and in the presence of drugs for 4-6 days in polypropylene-coated tubes, cell culture conditions that limit the growth of normal cells (Fig. 3). Fast green and nigrosin stains are used to identify dead cells and Wright-Giemsa for the identification of living cells. The evaluation of the ratio of living tumor cells to dead tumor cells, analyzed by optical microscopy, provides a measure of drug-induced cytotoxicity. The main clinical applications of the DiSC assay are related to the study of chronic lymphocytic leukemia (CLL). The analysis of 389 patients clearly showed that treated patients, but not untreated patients, develop pleiotropic drug resistance (35). The use of this test in CLL patients may have a favorable impact on treatment: CLL patients resistant to fludarabine in the DiSC assay are also resistant to treatment with this drug (36). However, the real impact of the DiSC assay on CLL patient survival has not yet been demonstrated in controlled clinical trials.

Extreme drug resistance assay (EDRA)

The EDRA measures the activity of chemotherapeutic agents in inhibiting the proliferation of small tumor cell aggregates suspended in a low-density layer of agarose that overlays a solid layer of agarose (37, 38). This methodology was described in detail by Fruehauf and Bosanquet (39) and by Fruehauf (40), and was studied in detail in several types of tumors and its clinical utility validated in ovarian, breast and certain brain cancers.

The EDRA involves the evaluation of the effect of drugs on the proliferation of tumor cells suspended in a low-density layer of agarose (0.3%) that overlays a layer of more solid agar (0.4% agarose) (Fig. 4). Agar culture is employed based on the observation that agar suppresses the proliferation of the stromal components of the tumor, while supporting the proliferation of tumor cells. Before *in vitro* growth, tumor tissue is mechanically and enzymatically (collagenase and deoxyribonuclease) disaggregated. During this procedure, it is particularly

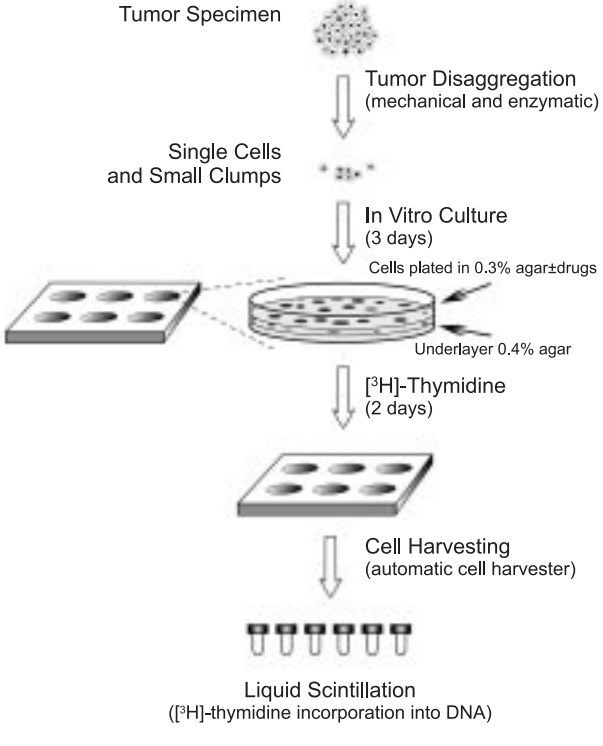

Fig. 4. Outline of the extreme drug resistance assay (EDRA).

important that small tumor aggregates are maintained after *in vitro* tumor dispersion, since these small aggregates are mainly responsible for *in vitro* tumor growth.

Tumor cells are grown in the absence or in the presence of antitumor drugs, added at a concentration corresponding to the peak plasma concentrations of these drugs after *in vivo* administration. However, since the *in vitro* exposure to these drug concentrations occurs for a markedly longer time than achieved *in vivo* after bolus i.v. administration, the *in vitro* drug exposure in the EDRA is higher (ranging for different drugs from 6 to 20 times) than that achieved *in vivo* (38). Therefore, the EDRA is carried out in the presence of suprapharmacological drug concentrations.

Cultures are then incubated for 3 days, during which time the agar culture is selectively permissive only for cancer cells. After these initial 3 days of culture, the cells are pulsed for an additional 2 days with [^{3}H]-thymidine, which is incorporated into the DNA of dividing cells. At the end of the culture, agarose cell suspension cultures are liquefied at 95 ^{o}C and the cells are then harvested onto glass fiber filters. After several washings with distilled water, the filters are incubated with scintillation fluid and radioactive decay is measured in a scintillation counter to determine the level of DNA synthesis in tumor cells grown in the absence (control) or in the presence of different drugs. The level of inhibition of DNA synthesis directly reflects and correlates with the activity of the drug, while the absence of an inhibitory effect of the drug on tumor cell proliferation indicates a condition of extreme drug resistance of the tumor to a given drug.

A review of published *in vitro* EDRA results for 4,263 patients where correlations with treatment responses were available showed that the rates of clinical response were significantly associated with *in vitro* results, with an overall sensitivity of about 85% and an overall specificity of about 80% (39). Importantly, the EDRA was found to have great accuracy (> 95%) for predicting drug resistance, while it had only 72% accuracy for predicting chemosensitivity (40).

This assay was explored in detail in two types of tumors: ovarian cancer and breast cancer. The EDRA was performed in about a thousand cases. In both of these tumors, clinical studies have provided evidence that the EDRA improves the rate of response to therapy and survival.

Ellis *et al.* reported their experience in breast carcinoma. Of 144 tumor specimens analyzed, tumors were successfully cultured for assay in 101 (70%) cases. Of these, 7-15% exhibited EDR to cyclophosphamide and 5-FU, and 35% to paclitaxel. There was an association between 5-FU and cyclophosphamide resistance and estrogen receptor negativity. Similarly, there was a trend towards an association between paclitaxel resistance and HER-2/neu overexpression (41).

Metha *et al.* studied a group of breast cancer patients undergoing treatment with either cyclophosphamide, methotrexate and 5-FU or doxorubicin and cyclophosphamide. Their tumors were evaluated *in vitro* for drug sensitivity/resistance by the EDRA and therapy was blinded to the EDRA results. The results of the study showed, after stratification of the patients according to their EDRA scores, that patients with low EDRA scores (*i.e.,* patients whose tumors were resistant to drugs) exhibited a shorter survival compared to patients with high EDRA scores (*i.e.*, patients whose tumors were not or were moderately resistant to drugs) (42).

The EDRA appears to be particularly useful for predicting drug resistance in ovarian cancer. A first cohort of 79 patients was examined *in vitro* for drug response: 17 of 79 patients (22%) exhibited extreme drug resistance to cisplatin and carboplatin. Both the progression-free survival and the overall survival were significantly shorter in patients with EDR than in patients with low or no drug resistance (43). These observations clearly indicate that platinum resistance is an important determinant of survival for patients with ovarian cancer, in line with numerous clinical studies indicating that platinum response is the principal determinant of clinical outcome.

In another study, the impact of EDR-sorted therapy on clinical outcome was evaluated (44). Fifty women were treated with chemotherapy based on EDRA guidance and were compared with 50 well-balanced control patients who were treated empirically. According to the results of the EDRA, the patients were subdivided into platinum-sensitive and platinum-resistant groups. The overall response rate was higher in patients with EDR-directed therapy compared with patients treated empirically. The overall survival was significantly longer in the first group of patients compared to the second group of patients. The EDR-assisted therapy did not significantly modify the clinical outcome of platinum-resistant patients.

The analysis of large numbers (> 5,000) of ovarian cancer specimens allowed the determination of the frequency of drug resistance for the different drugs and provided evidence that there is a relationship between drug resistance and the histiotype (45). The frequency of EDR to the different drugs was: cisplatin 10%, carboplatin 16%, cyclophosphamide 16%, doxorubicin 40%, gemcitabine 21%, paclitaxel 22% and topotecan 13%. Some differences in the frequency of chemoresistance among the different histological subtypes was observed; mucinous tumors were more resistant to cisplatin compared to papillary serous tumors, while endometroid tumors were less resistant than papillary serous tumors, and clear cell and undifferentiated tumors were usually less chemoresistant than papillary serous tumors.

Recent studies have shown that brain tumors may be grown *in vitro* under the conditions of the EDRA. A high percentage of glioblastomas, astrocytomas and meningiomas displayed very high drug resistance, a finding in line with the poor sensitivity of these tumors to antitumor drugs (46). The EDRA in patients receiving irinotecan for recurrent glioma showed a better clinical outcome in patients sensitive *in vitro* compared to resistant patients (47).

The EDRA was also used to evaluate the combined effects of radiation and chemotherapy on cervical

carcinoma. This assay provided evidence that up to 90% of cervical carcinoma specimens may be grown *in vitro* and that radiation and chemotherapy displayed a synergistic effect on these tumor cells (48).

The hollow fiber model

The hollow fiber model is a short-term *in vivo* assay in which cells growing in polyvinylidene fluoride (PVDF) "hollow fibers" are placed in various body compartments of mice. These fibers are permeable to substances with a molecular weight of < 500,000. Compounds are usually administered over a 4-5-day period, the fibers are then removed from the mice and the effect of drug action is assessed by a colorimetric assay with a tetrazolium-based dye (MTT) (49). This assay was used in a screening program for new anticancer drugs and was shown to be a good predictor of *in vivo* drug activity and to reduce the need for *in vivo* studies using tumor xenografts (50, 51).

Fluorescent cytoprint assay

This test measures cell viability in cultures of small clumps of tumor cells grown on sheets of cellulose-collagen (52). Briefly, after mild tumor dissociation, the tumor clumps are first incubated with fluorescein acetate and then implanted on a solid support made of cellulose fibers impregnated with collagen. A baseline fluorescent cytoprint is recorded initially prior to drug exposure and 4 days after drug addition. Computer analysis of the two pictures provides an objective means of evaluating drug effects on the tumor cells. An important limitation of the system is that it can not distinguish between the effect on tumor cells and on nonmalignant cells cocultured together with the malignant cells.

In vitro studies using ovarian and breast cancer biopsies suggested that this method may be better for detecting drug resistance than drug sensitivity (53, 54).

Conclusions

In general, numerous studies have shown that *in vitro* drug predictive assays are associated with several problems, mainly related to the choice of the clinically relevant drug concentrations to be used *in vitro,* interference of the culture conditions with the physiological environment for tumor cells that exists in the patient, heterogeneity of tumors and possible nonspecific effects caused by the cell culture system on cancer cells. Therefore, the relationship between *in vitro* drug response and a patient's response to chemotherapy is complex and difficult to interpret.

However, despite these limitations, several general conclusions can be drawn: 1) *in vitro* assays based on the selective growth of primary tumor cells have an important role in the process of drug development and in determining optimal chemotherapy; 2) human tumor assays can not be performed on all tumor types, *i.e.*, the rate of successful *in vitro* tumor growth varies for different tumors; 3) *in vitro* assays are better for predicting chemoresistance than chemosensitivity and patients with *in vitro* chemoresistance have a poor prognosis; and 4) although many studies suggest that *in vitro* chemosensitivity/chemoresistance assays may have a positive impact on the treatment of some neoplasias, controlled double-blind studies are required to prove the real impact of assay-guided chemotherapy on patient survival.

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