In Vitro Sensitivity Assays in Cancer: A Review, Analysis, and Prognosis

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Tumors are complex systems consisting of heterogeneous cancer cells as well as normal cells with each exhibiting unique drug sensitivity spectra. There have been many attempts to design in vitro systems to determine drug response of tumors. The most widely used system is the clonogenic assay, which has demonstrated some clinical predictivity. However, the clonogenic assay has been shown to have negative aspects, including low frequency of evaluation, clump artifacts, lack of cytotoxic end-points and disruption of normal cell-cell interactions existing in a true tissue environment. Newer models are described utilizing cytotoxic as well as cell-proliferation end-points, and maintenance of three-dimensional tissue architecture in vitro. It is concluded that less artifactual, more realistic models can be used to select more tumor-specific drugs which themselves in turn will make in vitro chemosensitivity assays more useful for cancer patients.

Key words: tumor tissue culture, in vitro chemosensitivity, prediction of clinical response, cancer chemotherapy

THE PROBLEM

Human tumors are complex systems consisting of cancer cells often of multiple classes, supporting stroma, normal cells, and frequently lymphocytes. Each tumor is a highly individual, dynamic system. Even tumors that are classified under the same histotype are highly individualistic. Researchers have known this characteristic for a long time and have concluded that each tumor has its own individual drug response spectrum. The major problem has been how to devise an experimental system in which to determine the drug response spectrum of a tissue as complex as a tumor in contrast, for example, to determining in vitro drug response or antibiotic response spectra of simple single-cell organisms such as bacteria.

CLONOGENIC ASSAYS, ORIGINS AND POSITIVE POINTS

There have been many early attempts to design in vitro systems to culture tumor cells and tissues (1–16). A number of researchers attempted to grow myeloma and various other types of tumor cells in agar based on observations of Puck and Marcus (17) that normal fibroblasts would not grow in agar, and tumor cells would. Therefore, potentially selective systems for disaggregated tumor cells to grow in the absence of an overgrowth of fibroblasts were utilized. Clonogenic assays had their beginning with Puck and Marcus (17). The first attempts at agar assay of primary tumor cells were done with mouse myeloma (Park et al. [18]; Ogawa et al. [19]). Subsequently human myeloma (Hamburger and Salmon [20] as well as other tumor types (Hamburger and Salmon [20]; Courtenay and Mills [21]; Courtenay et al. [22]; Salmon et al. [23]) were cultured.

As Weisenthal and Lippman (24) point out, clonogenic assays were considered to be the “gold standard assay” in radiobiology by the time of the 1970s. Roper and Drewinko (25) demonstrated that clonogenic assays in established cell lines differed in results from non-clonogenic assays. Steel (26) described a stem cell model of human tumor growth and cells which could form clones in agar were therefore termed stem cells. Salmon et al. (23) then reported positive correlations between in vitro response of tumor cells in soft agar and clinical response. As Weisenthal and Lippman (24) stated, the latter three publications stimulated a world-wide interest in using clonogenic assays for clinical drug testing and preclinical drug screening (18,20–22,26–124).

Von Hoff (125) has recently reviewed these studies on clonogenic assays. Over 40 types of cancer have been cultured in the agar colony formation assay and evaluated for drug response. Over 14,000 specimens have been cultured in agar of which close to 5,000 have been evaluated for drug response. Von Hoff (125) has reviewed 54 different in vitro-in

Received August 9, 1990; accepted August 27, 1990.

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vivo clinical correlation trials from 35 institutions performed over the past 12 years. The combination of the clinical trials has resulted in 2,300 correlations. The sensitivity of the combined clonogenic assays [true positives/(true positives + false negatives)] is 79%. Specificity of the test [true negatives/(true negatives + false positives)] is 86%. The predictive value positive [true positives/(true positives + false positives)] is 69%. The predictive value negative [true negatives/(true negatives + false negatives)] is 91%. Von Hoff (125) points out the results are as impressive as the results reported for the estrogen receptor assay or for in vitro antibiotic sensitivity testing. In initial studies comparing clinical outcome when patients are treated with results of the assay vs. clinician choice there seems to be greater responses among patients treated by results of the assay (126). In related trials, of 21 patients with gastro-intestinal cancers, 3 out of 11 patients treated with drugs to which the tumors were insensitive died within two years of surgery, in comparison to the survival of 10 out of 10 patients for at least 4 years treated with drugs to which their tumors were sensitive in the clonogenic assay (187). That sensitivity assays may increase patient response and survival is a very important point (126,127). However, the number of evaluable patients is small in these efficacy studies. In addition, clonogenic assays have correlated well with assays in nude mice carrying the same human tumors assayed in vitro, which indicates clonogenic assays could, at least in some cases, replace animal studies for cancer drug evaluation (184–186).

PROBLEMS WITH CLONOCGENIC ASSAYS

However, the clonogenic assay technique has drawbacks. It was found that a very low percentage of disaggregated tumor cells, on the order of 1 in 10^4 or lower, would grow in agar-based medium. The cells that did grow were termed tumor stem cells (Salmon et al. [23]), as indicated above, although it was never made clear what the relationship was of cells which could grow in agar to particular malignant cell types. As Von Hoff (125) points out, in his laboratory in San Antonio, of approximately 14,000 patient tumors attempted to be grown, only 30% of the cases have yielded evaluable results. In addition, the assay takes a number of weeks to yield useful data. Second-generation techniques modifying the clonogenic assay, for example, using rat erythrocytes, low O_2 atmosphere and tubes (21,22), capillary cloning (109,123), and [3H]thymidine incorporation (128,129), have helped to increase the number of specimens that can be evaluated for drug sensitivity. Von Hoff (125) points out that growth is best in the clonogenic assay for cancers of the ovary, uterus, kidney, brain, and mesothelioma.

Cloning assays also have the problem of the use of multiple drugs in the testing process since it is difficult to add drugs sequentially to the culture system. Quality control has also been a problem with the cloning assays, in particular difficulties of distinguishing clones from cell clumps. However, these problems seem to have been somewhat reduced (125). Weisenthal and Lippman (24) argue, however, that aggregates which seem to be colonies pose a large problem in the clonogenic assay, although Von Hoff (125) states that this is no longer a problem. Weisenthal and Lippman (24) state that the clumps may create a bias in assays that predict drug inactivity in that the clumping could produce an artifact but coincidentally accurate true-negative rate in clinical correlation studies in which most patients have tumors that are chemo-resistant. In this light, Weisenthal et al. (130) noted that in the clonogenic assays, 60–65% of specimens were not sensitive to spirogermanium, sangivamycin, and homoharringtonine. Other assays have shown essentially all cell types, normal and neoplastic, are sensitive to these drugs, indicating that the clonogenic assays results were really false negatives, and according to the authors, probably due to clumping artifacts.

Weisenthal and Lippman (24) note, with regard to theoretical problems of the clonogenic assay, that cell disaggregation necessary for plating in the clonogenic assay results in a loss of normal cell interactions and three-dimensionality which may be critical in drug response (131,132). Spheroids (133–135) can be constructed from disaggregated cells to form three-dimensional structures. Similar to solid tumors, the interior may be hypoxic and at low pH, and interior cells may not be as accessible to metabolites and drugs as the exterior cells. Of critical importance, when drug response was compared by Bhuyan’s group (135) in spheroids to single-cell suspension, the spheroids were much more resistant to antimetabolites such as cytosine arabinoside and methotrexate, >11-fold and >125-fold, respectively, and somewhat more resistant to vincristine, doxorubicin, and other antibiotics. Alkylating agents affected both cellular configurations equally. These results emphasize the importance of three-dimensionality. Indeed, in recent experiments, Bhuyan’s group (136) has seen for certain drugs such as Piercetin C that colon carcinoma cells are sensitive as single cell cultures but are resistant in vivo and, importantly, are resistant as three-dimensional slices in vitro. These results emphasize the importance of three-dimensional intact-tissue culture (see below).

Reversibly non-dividing (G_0) cells are probably not assayed in clonogenic assays. Cells assayed are actively dividing and may be the most sensitive of the tumor cells to chemotherapy. Actual stem cells of the tumor that showed the capability for potential continual self-renewal in vivo could be mainly G_0 cells in vitro and may not be assayed because of their inability to be directly cultured in clonogenic assays. A tumor may be very enriched in G_0 cells, which would make it unassayable in the clonogenic assay. Tumor cells committed to differentiation and thereby lesser malignancy may selectively develop colonies in vitro (Weisenthal and Lippman [24]).
CLONOGENIC-LIKE ASSAYS

Kern and Weisenthal (137) have significantly modified the clonogenic assay. The main modifications are to, in addition to measuring colony formation, measure [3H]thymidine incorporation in the cells incubated in the agar-based medium. In addition, Kern and Weisenthal used products of drug concentration × time that were approximately 100-fold higher than those achievable clinically. They found for patients whose tumors showed a less-than-40% thymidine-incorporation inhibition or less-than-15% colony-forming inhibition that the assay is 99.2% specific and 43% sensitive in identifying the patients as drug-resistant. Only one of 127 patients with tumors showing extreme drug resistance responded to chemotherapy. Kern and Weisenthal feel a high specificity for drug resistance was possible because the drugs were tested at very high concentration × time exposures in vitro.

It should be noted that, as Kern and Weisenthal (137) state, 18–42% of the patients, depending on the tumor types, had specimens which showed this extreme drug resistance with essentially none of the patients responding to chemotherapy. This broad resistance was seen over a wide range of cancer types including those that were otherwise usually relatively high responders.

Kern and Weisenthal (137) point out the applications of their highly specific drug-resistance assay include avoiding the use of regimens on patients containing inactive agents, in particular for the individual agents of breast cancer, including CMF (cyclophosphamide, methotrexate, and 5FU), CAF (cyclophosphamide, doxorubicin, and 5FU) or CA (cyclophosphamide and doxorubicin). Kern and Weisenthal's assay (137) can also reduce treatment-induced morbidity in patients with highly drug-resistant tumors and can identify patients with highly resistant tumors as candidates for experimental therapy such as bone-marrow transplant and other experimental therapies. The Kern-Weisenthal assay (137) however suffers significantly due to its inability to accurately identify drug-sensitive of tumors.

Another modification of the clonogenic assay, the disc-diffusion, soft-agar, colony-formation assay, has been developed by Corbett and colleagues (138) and modified by Smith et al. (139) to screen drugs which are selective against solid tumors. In this assay selectivity is evaluated by co-culturing the L1210 leukemia line, which is sensitive to most currently used drugs, and a drug-insensitive solid tumor such as pancreatic or colon adenocarcinoma. The colonies of the two cancer types are morphologically distinct. The test agent is placed on top of the agar previously seeded with the two cancer types. The authors have found that if an agent has cellular selectivity for a solid tumor as opposed to leukemia selectivity in the disc-diffusion assay it is more likely to be active against different types of solid tumors in vivo. The assay needs clinical validation. On a theoretical basis, it is not clear what, indeed, generally distinguishes solid tumor cells and leukemia cells growing in the highly selective agar-based growth system where growth kinetics of both cell types could be similar.

Another modification of the clonogenic assay is to culture cells on an adhesive matrix (140). However, there is a question of what cell types adhere to the matrix, in particular whether fibroblasts are predominant. Another variation of the clonogenic assay used a medium developed specifically for mammary epithelium and irradiated fibroblasts as a substrate. This assay was able to accurately predict drug sensitivity as well as drug-resistance in breast cancer (141). If the high accuracy can be repeated independently, this assay should be used more extensively in breast cancer. A similar approach has been taken in colon carcinoma (142) which also seems promising.

CELL-TOXICITY END-POINT ASSAYS

Pavlik et al. (143) point out that the standard clonogenic assay, although showing promise and identifying drug resistance as stated above, has been only moderately successful in predicting clinical sensitivity at 40–70% of the time depending on the laboratory. Pavlic et al. (143) point out that one explanation for the failure of predicting sensitivity is that the clonogenic assay does not provide an estimate for potential recovery from the cytostatic effects of drugs which can lead to false-positive results. The clonogenic assay does not distinguish cell kill from a halt in cellular reproduction on a non-permanent basis, leaving cells viable.

The 1–2 log cell kill that is able to be measured in clonogenic assays may be insufficiently sensitive to predict in vivo drug sensitivity. In clonogenic assays sub-clinical concentrations may have to be used to avoid false positives. However, the sub-clinical low levels of drugs may preclude normal uptake by the cells of the drugs (24).

Weisenthal and Lippman (24) point out the time after drug exposure that end-points are measured and which end-points are measured are critical. These authors report that for HL60 promyelocytic leukemia cells viability decreased from 38% to 17% to 0% when assayed 2, 4, and 6 days, respectively, after 1-hour exposure to doxorubicin by means of a dye-exclusion assay. They emphasize the importance of waiting a sufficient time to make measurements following a cytotoxic insult.

The clonogenic assay does not determine tumor kill as mentioned above. In the studies of Pavlik et al. (143) viable cells were determined by the activity of intracellular esterases which convert a colorless fluorescein diacetate to green fluorescein. Non-viable cells are identified by their ability to take up propidium iodide which is taken up only by cells that have a non-intact cell membrane and fluoresces red. Pavlik et al. (143) demonstrate that the exposure of tumor cells to even very high concentration of an anticancer agent can inhibit in vitro proliferation while esterase activity and the ability to
exclude propidium iodide are persistently retained allowing the potential of recovery from drug exposure. However, when the viability of established cell lines used by Pavlik et al. (143) progressively declined on days 4 and 7 following drug exposure, recovery did not occur. When proliferative recoveries did occur, the viabilities remained elevated on days 4 and 7.

Pavlik et al. (143) found that in vitro drug sensitivity based on proliferation criteria was associated with high-viability estimates in 22% of determinations that they performed on primary tumor cell preparations. Pavlik et al. (143) argued that the cells’ potential recovery may explain the excessive false positives in chemosensitivity assays based on proliferation end-points.

In this light, dye exclusion as an end-point to measure cell viability or cell death allows one to measure the drug resistance of G0 as well as proliferating cells. As Weisenthal and Lippman point out (24), the key critical technical problems in using dye exclusion as an end-point are that sufficient time must elapse following drug treatment for lethally damaged cells to lose their membrane integrity. This would, of course, depend upon the target of the chemotherapeutic drug. It is important to note that Weisenthal and Lippman (24) point out that dye exclusion assays may be valuable in testing of neoplasms which proliferate poorly in culture. The assay developed by Weisenthal et al. (144) used disaggregated cells and the dye fast green as their reporting dye which is excluded from living cells. Clinical correlations with dye exclusion as an end-point compare favorably with clonogenic assays. However, the Weisenthal assay may be better suited to hematological tumors than to solid malignancies since it is based on measurements on cells in suspension.

The Weisenthal assay has been used by Gazdar et al. (127) to measure drug sensitivity in small-cell lung cancer. Only about 25% of patients entered in the protocols could have a determination done, and the time to grow sufficient cells for assay (median 37 days) was only sufficient for secondary treatment. However, there was a correlation between in vitro sensitivity and in vivo response. In addition, patients whose tumors had in vitro responses had a tendency to longer survival (127). It seems, however, for small-cell lung cancers, more efficient and rapid in vitro systems are necessary.

ASSAYS USING THREE-DIMENSIONAL TISSUES IN VITRO

With regard to the advantages of measuring cell viability by dye exclusion and also preserving at least some cell-cell interactions, Rotman et al. (145–147) propose an approach to in vitro chemotherapeutic assays in a procedure they term fluorescent cytoprinting. Specimens in this procedure are mechanically or enzymatically mildly digested into cellular clusters termed micro-organs which the authors claim are exclusively tumor cells. The micro-organs are exposed to fluorescein acetate. Those that are fluorescent are viable and are collected with a Pasteur pipette and explanted on a support of cellulose fibers impregnated with collagen. The drug toxicity is measured by comparing the fluorescence as seen microscopically before and after drug treatment. As the authors point out a key feature of fluorescent cytoprinting is that the cytotoxic effects are not measured against control cultures but against the base-line provided by the cytoprint of the same culture before drug addition. A high rate of evaluability has been demonstrated by these authors and high in vitro-in vivo correlations have been reported (Rotman et al. [145–147]). The end-point of macroscopic measurements of the “micro-organs” still leaves one to be concerned about what cell-types are present in the micro-organs. However, the idea of preserving cell-cell interactions and also using the drug-treated culture as its own control previous to drug treatment is appealing. However, it is not demonstrated that the cultures are under permissive growth conditions which would allow the possibility of some cells to cycle and would represent at least to some degree the in vivo situation.

Short-term assays based on incorporation of nucleic acid precursors into tumor fragments have been utilized in some clinical studies (148,149). The studies indicate a high correlation of 92% between in vitro clinical sensitivity and in vivo response with tumor metastases but with primary tumors only about 62%. These assays can have a high rate of false negatives. The methodology involves cutting the tumors into 1 mm³ fragments for incubation for 3 hours with drugs. During the last hour of incubation the tumor pieces are exposed to [³H]thymidine and [³H]uridine followed by a chase of labeled precursors from intracellular spaces at 4°C with 100-fold concentrated solution of unlabeled precursors. As Weisenthal and Lippman (24) point out, these studies are subject to numerous pitfalls relating to the artifactual alterations in intracellular pool sizes and salvage vs. de novo deoxothymidine monophosphate synthesis. As Weisenthal and Lippman (24) state, if labeling could be delayed for a number of generations after drug exposure, the artifacts would be less pronounced and more accurate estimates of the number of surviving proliferating cells could be obtained. However, the relatively non-physiological conditions used in this system would probably not support the long-term culture needed for delayed assay. However, recent studies of the short-term assay for germ-cell tumors indicated not only in vitro-clinical correlations (90% true-negative rate, 54% true-positive rate) but longer survival for the patients with tumors sensitive to drugs in vitro (148).

The foregoing, however, make it obvious that a next-generation of technology is necessary for in vitro drug response assessment. It would be imperative in the next generation that the technology allow the development of a model that more closely resembles the in vivo situation in vitro. Leighton (150) in 1951 brought tissue culture closer to the in vivo situation by introducing a three-dimensional sponge matrix system. Leighton’s system employed a cellu-
lose sponge matrix that was impregnated with a plasma clot. The system was modified by Leighton (151) by using collagen-coated cellulose sponges which would be suitable to measure growing carcinomas, many of which were able to lyse the clot. Leighton was able to grow the Walker carcinoma as well as several other tumors such as mouse mammary carcinomas and rat hepatomas and embryonic tissues. It was reported that the growth characteristics of the tumor and embryonic tissues were better in the collagen-coated cellulose sponges compared to either cellulose sponges or collagen sponges alone.

Sherwin and Richters (152) have termed the approach of maintaining in vivo-like tissue culture or in vivo-like tissue architecture in vitro, histoculture. Their approach was to explain 2-mm² tissues only about 1-mm thick, which would allow for optimal diffusion, which is very important as pointed out in the original tissue culture article by Alexis Carrel (153).

These ideas were further developed by Yoshida et al. (154,155), who cultured embryonic lung tissue on sponges derived from pigskin. This approach was also developed by Douglas et al. (156).

Three-dimensional colon-organ culture was initially advanced by Browning and Trier (157) and by Schiff (158). Their method kept fragments of human small intestine in contact but above the culture medium by explanting the tissue on a matrix partially above the medium level, or by rocking the culture, putting the tissue in and out of the medium. Autrup (159) used 95% O₂/5%CO₂ as an atmosphere to enhance survival. Autrup has extended the growth capability of colon extensively by using gelatin sponges as a growth support and the rocking method alluded to above allowing the maintenance of normal colonic mucosa for at least 28 days. Great care was taken to avoid ischemia. The mucosal layers of colon specimens are dissected away from the muscularis externa and put on the sponges to maximize the diffusion of nutrients and oxygen into the tissue by stretching the tissue on the sponges (Autrup [160]).

Hoffman et al. (161–165), have utilized the gel-supported histoculture approach to develop a next generation of in vitro chemotherapeutic assays. The system allows for the culturing of over 20 tumor types at an 80% or better evalability. The main advantages of this system:

- Cultures tissue in three dimensions, preserving native tissue architecture
- Uses morphological end-points
- Detects proliferation by histological autoradiography and polarization microscopy yielding very high resolution data, the collection of which can be semi-automated by digital pixel analysis
- Viability of tumor and non-tumor cells can be measured at high resolution by fluorescent-dye exclusion and inclusion detected in three-dimensions by confocal scanning-laser microscopy (Hoffman RM unpublished data)

Has a very high rate of evaluation for all tumor types—approximately, 80% or better
Multiple agents can be tested sequentially
Uses relatively small amounts of tissue—as small as 100 mg
Has very low frequency of false negatives and low frequency of false positives (Hoffman RM, Kubota T, Connors KM, unpublished data)
Normal as well as tumor tissue can be cultured in parallel to assess the tumor specificity of anti-neoplastic agents
Cytotoxic as well as biological drugs and activated immune cells can be assayed
Depending on end-point chosen, data can be obtained in 3–14 days

Recently the native-state method has been used for drug-response analysis of urological tumors with findings of a high culture success ratio, longevity in culture, maintenance of primary histopathology, and reproducible chemosensitivity response (166,167).

In addition to being able to predict chemotherapeutic response, the degree of proliferation of cells within the histocultures correlates with the stage and grade of breast and ovarian tumors (164) and distinguishes small-cell from non-small-cell lung tumors (165).

**PROGNOSIS OF SENSITIVITY ASSAYS**

What does the future hold for in vitro sensitivity assays in cancer? The cumulative results from clonogenic systems, suspension cell systems, and three-dimensional systems argue that the concept is already providing some clinical use with greatest accuracy in predicting in vivo resistance (Table 1). It is necessary to predict clinical sensitivity at greater accuracy in particular to identify effective antitumor agents. The monolayer human solid-tumor cell line drug-screening approach of the National Cancer Institute (168–179) seems quite artifactual in that monolayers are often more sensitive than three-dimensional in vitro tissue or in vivo tissue (134–136) and the cell lines are highly selected and may have little resemblance to their original tumor. The most promising approach is to use the most in vivo-like three-dimensional histoculture models along with multi-end-point analyses to select truly tumor-specific drugs (76). New subtle endpoints such as those developed by Ogura et al. (183) that measure drug response by intracellular morphological changes should also help predict drug response. The most important current need in cancer therapy is the development of cancer-specific drugs (Grindley [180]) which require relevant in vitro models that will identify them (Grindley [180]). With the discovery of highly effective tumor-specific drugs, the true-positive accuracy and usefulness of in vitro sensitivity assays will rise.
<table>
<thead>
<tr>
<th>Assay</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamburger-Salmon clonogenic assay (18–126)</td>
<td>Most correlations in clinical trials</td>
<td>Low tumor growth (30%); testing drug combinations difficult; clump artifacts. Complex system</td>
</tr>
<tr>
<td>Courtney-Mills modification of clonogenic assay (21, 22, 182)</td>
<td>Higher colony-forming percentage due to erythrocyte-enriched medium and low-0₂ incubation conditions</td>
<td></td>
</tr>
<tr>
<td>NCI Screening-Group modification of clonogenic assay (83)</td>
<td>Good reproducibility</td>
<td>Low tumor growth</td>
</tr>
<tr>
<td>Corbetti disc-diffusion, soft-agar-colony formation, two-tumor assay (138,139)</td>
<td>Can distinguish drug sensitivity of leukemic and solid tumor cells simultaneously to select for solid-tumor-specific drugs 80% of tumors can be assayed</td>
<td>Relies on cloning (see above)</td>
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<tr>
<td>Von Hoff, Maurer capillary modification of clonogenic assay (91,109,123)</td>
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</tr>
<tr>
<td>Kern radionuclide incorporation modification of clonogenic assay (46,54,137)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>National Cancer Institute solid-tumor-cell-line screen (168–179)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baker-Life-Trac clonogenic assay (140)</td>
<td>80% evaulability; long-term culture</td>
<td>Time-consuming; monolayer culture may give false positives; fibroblast overgrowth may be problem Complicated media and substrates; monolayer culture may give false positives</td>
</tr>
<tr>
<td>Smith mammary cell assay (141). Friedman colon cancer assay (142)</td>
<td>Allows expansion of number of cells from small biopsy enabling high frequency of evaulability</td>
<td></td>
</tr>
<tr>
<td>Weisenthal DISC assay (138,127)</td>
<td>Morphological end-point; measures cytotoxicity</td>
<td>Cannot evaluate cytostatic drugs; application limited to hematologic tumors or tumor cells which grow in suspension such as small-cell lung carcinoma Costly, large team effort and many animals required; tumor growth difficult to measure Many other mechanisms for drug resistance than P-170</td>
</tr>
<tr>
<td>Bodgen subrenal capsule assay (35)</td>
<td>In vivo assay, useful for new drug evaluation</td>
<td></td>
</tr>
<tr>
<td>P-170 glycoprotein assays (182)</td>
<td>Theoretical advantage of measuring the multi-drug-resistance gene product P-170 3-dimensionality of spheroids is a more in vivo-like model</td>
<td></td>
</tr>
<tr>
<td>Spheroids (133–135)</td>
<td>Tumor architecture preserved</td>
<td></td>
</tr>
<tr>
<td>Sivestri radionuclide incorporation assay (148,149)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotman fluorescence-organoid assay (145,147)</td>
<td>Rapid assay; measures same area of culture before and after drug treatment, thus allowing internal control</td>
<td></td>
</tr>
<tr>
<td>Hoffman native-state, three-dimensional histoculture assay (161–167)</td>
<td>Maintains tissue in vivo-like state; uses morphological end-points; measures cell proliferation or cell viability; distinguishes tumor from non-tumor cells; very high rate of evaluation for all tumor types; multiple agents can be tested simultaneously or sequentially; uses relatively small amounts of tissue; very low frequency of false negatives; low frequency of false positives; normal tissue can be cultured also to compare drug response with tumor to determine if experimental drugs exhibit tumor selectivity; data can be obtained in 3–14 days depending on end-point measured</td>
<td>Labor intensive</td>
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ACKNOWLEDGMENTS

The manuscript was expertly word-processed by Polly Jayne Pomeroy, whom we greatly appreciate.

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