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Evaluation of In Vitro Chemosensitivity Using Human Lung Cancer Cell Lines

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Abstract The use of well-characterized human lung cancer cell lines has allowed for new opportunities in preclinical and clinical drug evaluation. Development of semiautomated tests of in vitro cytotoxicity such as the MTT assay, which utilizes the formazan salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), has allowed for preclinical evaluation of novel chemotherapeutic agents and drug combinations. In addition, techniques such as this make possible the testing of sufficient data sets to allow determination of true biochemical drug synergy. Assessment of drug combinations which possess in vitro synergy or supraadditive effects can suggest chemotherapeutic regimens for further clinical testing. Using the MTT assay in conjunction with isobolographic analysis, it is possible to test commonly used regimens which are based on presumed or apparent in vivo drug synergy, such as the combination of etoposide and cis-platinum. This frequently prescribed combination was found to lack in vitro biochemical synergy when tested with human lung cancer cell lines, indicating that the observed clinical benefits of this drug combination may be due to factors in the tumor microenvironment, drug metabolism, or non-overlapping toxicities. Finally, although it remains to be determined if a significant role for in vitro drug testing will be found in direct clinical applications, preclinical drug evaluation during the drug development process using cultured tumor cell lines may ultimately allow for disease or patient specific therapies for testing.

Key words: chemosensitivity, MTT, synergy, lung cancer, supraadditive

The establishment and characterization of a series of human lung cancer cell lines has proven a remarkable resource for research into the molecular basis of pulmonary carcinogenesis [1,2]. However, the availability of these cell lines, and their biochemical analysis, has yet to generate similar advances in the clinical management of lung cancer [3]. This contrast is due to a variety of circumstances, not the least of which are the logistical difficulties inherent in conducting clinical trials of adequate statistical power. Nonetheless, the potential utility of these lung cancer cell lines in preclinical evaluation of therapies has been augmented by the development of in vitro screening methods that enable large-scale screening of multiple chemotherapeutic agents in a variety of combinations and doses. Using an assay based on the metabolic reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a wide variety of chemotherapeutic agents, as well as biological response modifiers and radiotherapeutic modalities, can be tested using an in vitro semiautomated method. Moreover, using techniques such as this, tests can be performed on large numbers of samples and parameters facilitating analysis of therapeutic combinations using appropriate statistical methods to evaluate for true synergistic effects, or effective novel biochemical modulations. In this review we will discuss some of the methodology and application of in vitro testing of therapeutic agents using human lung cancer cell lines.

BACKGROUND

A variety of methods have been used to test potential therapeutic agents in both the preclinical setting and in clinical trials of individualized patient specific therapies. The human tumor cloning assay (HTCA), a soft agar cloning assay first utilized in the 1970's, has a sensitivity of

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60–70% and a 90% predictive value for resistance in a variety of tumor samples tested [4]. This method, though, has proven to be labor intensive and thus difficult to use when screening multiple combinations of drugs [4,5]. In addition, in order to assess the prospective value of in vitro chemotherapeutic testing in a clinical trial, there would need to be available a test that allows for evaluation of not only single agents, but also combination of agents, using a technique that involves fewer manipulations to allow for easy evaluation of individual drug combinations and concentrations. To overcome these limitations, many alternative methods for in vitro evaluation of tumor or cancer cell line chemosensitivity have been developed. These have included techniques such as dye exclusion assays [6], an adhesive tumor cell culture system [7], and multicellular tumor spheroids [8]. All of these assays, however, have proven to be labor intensive when testing for drug sensitivities against single agents, and even more unwieldy when used in evaluating drug combinations. However, if testing for single agent or fixed dose sensitivity is desired, these methods have potential utility, particularly the easily performed dye exclusion assay which has been incorporated into clinical trials evaluating tumor specific drug sensitivities [9]. In vivo methods, such as the subrenal capsular assay [10] and nude mouse tumor culturing [11] offer the obvious benefit of using a system that allows for evaluation of potential host/drug interactions. However, such assays are, by their nature, excessively cumbersome and expensive to use for adequate sampling to fully evaluate either single agents, or combinations of chemotherapeutic drugs.

The tetrazolium salt MTT has allowed for an efficient microculture assay of drug sensitivity that can be performed in a semiautomated manner [12]. This test is based on the capability of living cells to reduce a tetrazolium salt to a blue formazan product that can be detected and quantitated by absorbance spectrophotometry at 570 nm. This technique takes 72 h to perform and can be conveniently done in 96 well microculture plates allowing for processing of multiple samples. This allows for sufficient testing of drug combinations to carry out adequate statistical analyses necessary to evaluate in vitro drug interactions, as well as to assess large numbers of new chemotherapeutic agents. Similar qualities have been identified in other semiautomated techniques such as the sulforhodamine B (SRB) assay [5], currently in use in the disease specific drug screening program of the National Cancer Institute (NCI) Developmental Therapeutics Program.

MTT ASSAY IN SINGLE AGENT EVALUATION

The development of the MTT assay was initially carried out in the EL_4G^- mouse lymphoma cell line by Mossman [12] to aid in the evaluation of lymphokine and mitogen mediated cell proliferation. However, the ease of processing large numbers of samples in a semiautomated manner presented an obvious method for evaluating chemotherapeutic cytotoxicity. In 1987 the MTT assay was used to assess chemosensitivity in the NCI-H460 and NCI-H249 lung cancer cell lines, as well as the Chinese hamster lung fibroblast V79 cell line [13]. An important modification of the MTT assay introduced in this study was the solubilization of the reduced formazan dye in dimethylsulfoxide and mineral oil, rather than in acid isopropyl alcohol as originally described. This modification added to the ease and speed with which multiple wells could be assayed when processing large numbers of samples. The MTT assay was found to correlate very well with a clonogenic assay when doxorubicin and cis-platinum were evaluated for cytotoxicity in the NCI-H249 and NCI-H460 cell lines. In the case of vinblastine cytotoxicity, the MTT assay demonstrated a greater sensitivity of these cell lines to this agent, then was observed using the clonogenic assay. Although no clinical correlations were possible in this study, the capability of the MTT assay to identify chemosensitive cell lines as accurately as clonogenic assays allowed for its potential development as a tool for preclinical testing and screening of novel therapeutic agents. Additional investigators have also used the MTT assay for evaluating chemosensitivity of established small cell lung cancer (SCLC) cell lines, as well as on several patient samples, with similar results revealing the potential for individualized therapies [14,15]. Probably the greatest utility, though, for the MTT assay in evaluating chemotherapeutic strategies in lung cancer, may be in the capability it gives researchers to generate the sufficient data, within a convenient time frame, necessary to successfully determine the supra- or subadditive effects of combined single agent.

EVALUATION OF MULTIPLE DRUG COMBINATIONS USING THE MTT ASSAY IN HUMAN LUNG CANCER CELL LINES

Successful medical treatment of a variety of metastatic or locally advanced malignancies has been based on the now common approach of using non-cross resistant drugs in combination chemotherapy regimens [16]. The number of solid tumor malignancies that have a significant, and potentially curable, response to combination chemotherapy remains modest. A partial list of these diseases includes a variety of lymphomas, germ cell neoplasms, and certain leukemias. Small cell lung cancer is among the diseases that is highly responsive, albeit rarely curable, to combination chemotherapy, or to multi-modality therapy involving radiotherapy and concurrent chemotherapy [3]. Although one advantage of combination chemotherapy involves the administration of drugs with non-overlapping toxicity and resistance profiles, another direct benefit can be of positive therapeutic drug interactions that exceed the response which is seen when administering the single agents alone. Apparent clinical drug synergy (supraadditivity) such as this has been documented in patients with several combinations of agents. These clinically observed supraadditive effects, however, have been difficult to adequately model in vitro because of the large number of data points necessary to assess if true supraadditivity exists at the cellular level between a given combination of drugs. A variety of methods, both statistical and intuitive, have been employed to assess if the effects of giving two or more agents concomitantly are supra- or subadditive [17,18]. Statistical methods often employ analyses incorporating isobolographic determinations at a predetermined iso-effective relative concentration of drug(s). One useful model based on isobolographic analysis utilizes the concept of an "envelope" of additivity, outside of which lies, at least in a graphic representation, either supra- or subadditive drug interactions [17]. This mode of analysis makes allowance for the non-linear dose-response curve that most chemotherapeutic agents demonstrate when used alone. A primary difficulty, however, with the envelope of additivity, is that in order to adequately ascertain if there is supra- or subadditivity in vitro, a large number of evaluable data points need to be generated. This requires a method such as the MTT or SRB cytotoxicity assays.

A primary example of two active compounds that are thought to have a supraadditive effect when given together is the combination of etoposide and cis-platinum in SCLC. This drug combination was first theorized to be synergistic based on studies using a common murine leukemia model in which 30% of animals tested achieved a cure using doses of etoposide and cis-platinum that, individually, resulted in no cures in the murine model system [19]. There was an apparent ability to achieve a greater in vivo response than might be predicted based on the response rate seen with the individual agents. However, this apparent supraadditive effect can only be inferred from the data presented, since in vivo responses to a drug are generally non-linear, and thus not directly evaluable by extrapolation of single dose-response data. The use of established lung cancer cell lines and an assay such as the MTT allows for the generation of sufficient data to determine if true drug supraadditivity exists at the cellular level. A direct example of the utility of this approach with the MTT assay was the isobolographic analysis of cis-platinum and etoposide synergy in four non-small cell lung cancer (NSCLC) and four SCLC cell lines [20]. To make this analysis, it was necessary to determine at least 24,000 individual measurements of in vitro cytotoxicity. The necessity for a semiautomated approach to achieve this is readily apparent. The conclusion reached in this study was that in none of the eight lung cancer cell lines (NCI-H23, NCI-H226, NCI-H441, NCI-H661, NCI-H841, NCI-H1092, NCI-H1284, and NCI-H774) was there appreciable in vitro drug synergy as measured by cytotoxicity. This study highlighted the utility of the combination of well-characterized human tumor cell lines and an easily performed, rapid test for cytotoxicity in determining in vitro biochemical drug sensitivity and synergy. However, the common observation from previous clinical experience that an apparent in vivo drug synergy occurs between these two agents highlights one of the major pitfalls in using this or any in vitro approach to evaluate or design potential clinical therapies. Actual in vivo synergy might easily be due to local effects in the tumor environment, drug metabolism, non-linear dose-effect curves, non-overlapping toxicities, or other effects, and not necessarily to direct biochemical or intracellular supraadditivity. Likewise, true in vitro synergy may not confer a clinical advantage if the syner-

gistic effects are exerted on normal tissues to the same extent as on tumors.

The use of this method of analysis using lung cancer cell lines has been extended to studying the combination of the antimetabolites methotrexate (MTX) and 5-fluorouracil (5-FU) [21]. These drugs, when given in sequence, are reported to demonstrate both in vitro and in vivo synergy [22,23]. However, despite incorporation into numerous clinical trials, there is no definitive evidence of clinically important synergy in this combination and its utility as a pharmacologic manipulation has been open to question. Using the previously described technique of isobolographic analysis, schedule dependent MTX/5-FU combinations were tested and analyzed for their cytotoxicity in a pair of NSCLC cell lines (NCI-H23 and NCI-H358). Schedules that exposed the cell lines to the purine analog 5-FU prior to or simultaneously with MTX resulted in antagonistic cytotoxic effects. Furthermore, a schedule that exposed the cell lines to MTX for only 8 h prior to 5-FU resulted in a similar outcome. It was not until a 24 h pretreatment with MTX was given to the cells that a true supraadditivity resulted. These findings suggest the optimal interval between MTX administration and 5-FU infusion is longer than the one usually employed in clinical trials. Although there may well be in vivo factors limiting clinical success with this strategy, the application of an assay such as the MTT, used in conjunction with established and characterized cancer cell lines, may suggest clinical schedules to pursue in further clinical investigations.

Additional drug combinations that are in the process of clinical evaluation have also been investigated for in vitro drug synergy in a similar manner. Drug synergy was documented between leucovorin and 5-FU, and leucovorin and 5-FU with cis-platinum, but not between cis-platinum and 5-FU alone, when tested against a panel of eight NSCLC lines [24]. The supraadditive effects of leucovorin on 5-FU have been demonstrated in vivo in colorectal malignancies, and continue to be studied in a variety of other diseases. The combination of cis-platinum, 5-FU, and leucovorin is also showing promise in the treatment of squamous cell carcinoma of the head and neck, and is also currently under evaluation in NSCLC [25–28].

Established lung cancer cell lines continue to allow for preclinical evaluation of novel drug combinations in conjunction with MTT assay. A panel of seven NSCLC cell lines was used to study the enhancement of 5-FU and 5-FUrd cytotoxicity by leucovorin [29], a biochemical modulation that has found increasing popularity in the clinical setting [30]. Enhanced cytotoxicity was observed in all cell lines tested, although it was greater with 5-FU than 5-FUrd. A similar analysis using a panel of NSCLC cell lines has been recently performed using the combination of 10-ethyl-10-deazaaminopterin (10-EDAM), a novel methotrexate analog, and dipyridamole [31]. 10-EDAM cytotoxicity was demonstrated to be increased by the addition of dipyridamole, an agent that presumably inhibits nucleoside salvage but has no anti-neoplastic properties of its own.

CLINICAL UTILITY

Although the introduction of semiautomated technologies to test combinations of drugs against lung cancer cell lines has resulted in increased understanding of true drug synergy in several clinically useful combinations, the translation of this capability to patient care is still problematic. A recent comparison and discussion of the relative merits of in vitro drug screening methodologies used by the NCI Developmental Therapeutics Program has been published [5]. The MTT assay was compared to the sulforhodamine B (SRB) assay, a protein binding assay that indirectly measures cell viability. Although the two methods yielded equivalent results when tested against a panel of 38 human tumor cell lines, the SRB method proved to be more convenient due to the lack of the timed incubation required for reduction of MTT.

The MTT assay, however, has proved to be a useful tool in conjunction with established lung cancer cell lines in adequately assessing true biochemical drug synergy. Whether using the MTT semiautomated or the SRB assay, future studies requiring the use of high volume and rapid methods for screening of new drug regimens will continue to play a significant role in drug development for use in solid tumor chemotherapeutic regimens. The unanswered questions regarding the clinical significance and possible pragmatic utility of determining individual tumor chemosensitivity should not detract from the insights drug testing has and can continue to offer into mechanisms of drug interaction and sensitivity.

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