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Adaptation of a chemosensitivity assay to accurately assess pemetrexed in ex vivo cultures of lung cancer

Sarah L. Suchy,[1] Rodney J. Landreneau,[2] Matthew J. Schuchert,[2] Dakun Wang,[1] Paul R. Ervin Jr.[1] and Stacey L. Brower[1,*]

[1]Precision Therapeutics; Pittsburgh, PA USA; [2]Division of Thoracic and Foregut Surgery; Heart, Lung and Esophageal Surgery Institute; University of Pittsburgh Medical Center; Pittsburgh, PA USA

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Abbreviations: aAUC, adjusted area under the curve; ATCC, American Type Culture Collection; BEGM, bronchial epithelial cell growth medium; DRM, drug response marker; D-PBS, Dulbecco's phosphate buffered saline; EC_{50}, half-maximal effective concentration; EGF, epidermal growth factor; FBS, fetal bovine serum; FDA, Food and Drug Administration; MPM, malignant pleural mesothelioma; NSCLC, non-small cell lung carcinoma; pCR, pathological complete response; RI, response index; RPMI, Roswell Park Memorial Institute; SF, survival fraction

Purpose: Pemetrexed is the only FDA approved treatment for mesothelioma and is a second line agent for treatment of non-small cell lung carcinoma (NSCLC). Pemetrexed is inhibited by folate and its analogs, which are components of many culture media, making it challenging to study pemetrexed in vitro. In order to accurately evaluate pemetrexed's effects in vitro, the protocol for a standard chemosensitivity assay, the ChemoFx drug response marker, had to be modified.

Experimental Design: Novel rinse and media change steps were assessed and then added to the assay protocol in order to observe pemetrexed activity. The intraday and interday stability of pemetrexed were also established under the adapted protocol. Then, the modified protocol was used to examine pemetrexed in 65 ex vivo lung cancer specimens.

Results: Substituting 5% RPMI + EGF for BEGM allowed pemetrexed to exert its anticancer activity in the ChemoFx DRM. ChemoFx classified 6.2% of the lung specimens as responsive, 9.2% as intermediate responsive and 84.6% as non-responsive to pemetrexed.

Conclusions: Adapting the ChemoFx protocol allowed for the accurate evaluation of pemetrexed anticancer activity in ex vivo lung specimens. ChemoFx evaluation may provide an indication of a patient's clinical response to the drug prior to pemetrexed treatment. Having this information when treatment options are being considered could avoid wasted time, unnecessary costs and needless side effects that are the result of an inappropriate chemotherapy regimen.

Introduction

Lung cancer is the leading cause of cancer deaths worldwide.[1-3] Two forms of cancer that affect the lungs, mesothelioma and non-small cell lung carcinoma (NSCLC) can be exceptionally difficult to treat.[4-7] It is estimated that 3,000 cases of mesothelioma are diagnosed each year in the United States, with incidence of the disease predicted to peak within the next 10 years.[8,9] Eighty percent of lung cancers are classified as NSCLC, and NSCLC is the leading cause of cancer-related deaths in the United States.[1,10] Both diseases are heterogeneous in origin and progression, with chemotherapy providing some improvement but little to improve mean survival time.[11-15] Because relatively few treatments are effective against these highly aggressive cancers, a clearer understanding of the effectiveness of a given therapeutic option prior to an individual's treatment would greatly benefit those suffering from mesothelioma and NSCLC.

Pemetrexed, a folate analog, functions as an antifolate compound that blocks multiple enzymes involved in the production of nucleotides within cells.[16-19] In 2004, the FDA approved pemetrexed as a first line drug for malignant pleural mesothelioma (MPM), and it is still the only FDA approved treatment for mesothelioma, often in combination with cisplatin or carboplatin.[9,14,16,20] Even with pemetrexed treatment, most MPM patients' disease still progresses and retreatment with pemetrexed is sometimes recommended.[9,21] The clinical response rates in the literature for pemetrexed in mesothelioma span from 0–15%.[14,16] Also in 2004, pemetrexed was FDA approved as a second line agent for non-squamous NSCLC.[16,22] The clinical response rates for pemetrexed in NSCLC cases range from 4–23.3% in the literature, with an average response rate of 12.5%.[22-29]

Assessment of pemetrexed is complicated by folate, also known as vitamin B9, and other folate analogs interfering with the drug's activity in vitro and in vivo.[30-35] In patients,

*Correspondence to: Stacey L. Brower; Email: sbrower@ptilabs.com
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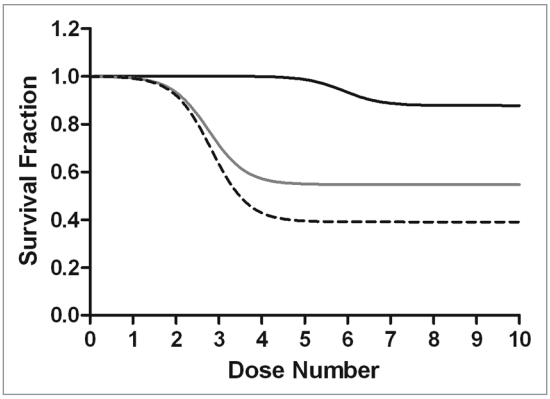

Figure 1. ChemoFx dose-response results for A549 cells treated with pemetrexed. The control wells, in black, are cells plated and treated in BEGM medium; the gray line signifies cells plated in BEGM but rinsed and treated in 5% RPMI; the black dotted line represents cells plated in BEGM but rinsed and treated in 5% RPMI + EGF.

supplementation with folic acid, the synthetic form of folate and vitamin B12 have helped ease side effects including myelosuppression, nausea, rashes and oral sores, without jeopardizing the drug's antitumor actions.[14,16,20,24,36] Examining pemetrexed in vitro has been a challenge as most cell culture media contain varying amounts of folate, frequently at levels higher than what is physiologically relevant; therefore, pemetrexed's efficacy in vitro can differ greatly depending on the amount of folate and folate analogs in the culture media and within the cells themselves.[30,31,34,37] Similarly, vitamin B12 has been shown to fluctuate widely in standard cell culture medias.[38] B12 is present in both sera and in basal media, and the amount of sera B12 can differ based on which species is used and how the serum is handled.[38] In their basal forms, Dulbecco's modified Eagle's medium (DMEM) contains no B12, while RPMI-1640 contains B12 at 3.7 nM, and McCoy's 5A modified medium contains B12 at 1.43 μM.[38] Consequently, careful consideration of media and media components must be made in order to observe pemetrexed's activity in vitro.

The ChemoFx drug response marker (DRM) (Precision Therapeutics, Inc.) is an in vitro chemosensitivity assay that evaluates ex vivo tumor samples for responsiveness to a given chemotherapeutic compound(s).[39-42] Patient samples are tested over a range of compound concentrations, and dose-response curves are produced from the survival fractions in each well after treatment. The dose-response curves are then used to grade an individual's tumor response to the compound(s) as responsive, intermediate responsive or non-responsive. ChemoFx can indicate the likelihood of treatment success with a given chemotherapeutic regimen, suggesting the suitability of a given therapy for a particular patient prior to treatment. Data from ChemoFx may be clinically relevant, as ChemoFx outcomes have been linked to patient outcomes in retrospective studies in both breast and ovarian cancers.[40-42] A recent study reported a 33% higher mean overall survival in primary ovarian cancer patients that were treated with a compound to which their tumors were classified as responsive by ChemoFx when compared with patients that were treated with a compound to which their tumors were classified as non-responsive.[42] In a breast cancer study, tumor samples from patients whose tumors were classified as responsive to docetaxel/capecitabine by ChemoFx were three times more likely to reach pathological complete response (pCR) than those whose tumors were labeled non-responsive by ChemoFx when all patients received docetaxel/capecitabine treatment.[41]

The purpose of this study was to customize an established, reproducible and sensitive in vitro assay to evaluate pemetrexed's chemotherapeutic effect on cell lines and ex vivo patient samples. An assay that can accurately assess pemetrexed's actions on individual patient samples, prior to the commencement of pemetrexed therapy, would be a valuable tool for clinicians.

Results

Adaptation of the standard ChemoFx protocol. Using pemetrexed in the standard ChemoFx protocol for lung cancer with samples plated and treated in bronchial epithelial cell growth medium (BEGM) revealed little cytotoxic activity by the drug over the range of doses examined (**Fig. 1**, A549 cells, control).[39] A rinse with phosphate-buffered saline (D-PBS) was introduced to dilute the BEGM, and 5% Roswell Park Memorial Institute 1640 medium (RPMI) replaced BEGM after the rinse. The result was a dose-dependent cytotoxic effect by the drug, which was significantly different than what was seen in the BEGM medium (**Fig. 1**). It was observed that pemetrexed's cytotoxic activity could be enhanced in the assay with the addition of epidermal growth factor (EGF) to the RPMI (**Fig. 1**).

Addition of folic acid to RPMI + EGF medium to test assay modification. To demonstrate that the change in drug response was specific to folate or folate analog levels, the assay was completed using a cell line that demonstrates in vitro response to pemetrexed (A549; see **Fig. 1**), cultured in BEGM, rinsed with D-PBS and treated with pemetrexed in 5% RPMI + EGF medium, which contained varying amounts of folic acid (**Fig. 2**). The purpose was to determine if the cytotoxic effect in the RPMI medium could be reversed by the addition of folic acid. Folic acid levels added ranged from 0 mg/L (0 mM, control)–500 mg/L (1.1 mM), with dose 0 wells serving as controls which contained folic acid but not pemetrexed. The magnitude of pemetrexed response in the ChemoFx DRM was correlated with the concentration of folic acid present. The lower the amount of folic acid in the 5% RPMI + EGF treatment medium, the greater the drug's activity, with 0 mg/L (0 mM) folic acid showing the most activity. When folic acid levels were increased beyond 200 mg/L (0.45 mM), the magnitude of response was diminished (portions of data not shown). Cells treated with folic acid alone (dose 0 for each folic acid-containing treatment) did not display any significant response or trend as compared with cells lacking both folic acid and pemetrexed treatment (dose 0 for the "Control" treatment, which did not contain any folic acid). These results indicated that the change in pemetrexed response between treatment in BEGM vs. treatment in 5% RPMI + EGF could be contributed to folate and folate analog composition differences between the two culture media.

Intraday and interday stability of pemetrexed in RPMI medium. Next, intraday and interday stability testing needed to be performed with pemetrexed in the 5% RPMI + EGF medium. ChemoFx quality control standards are already in place to test a drug's stability in the treatment medium over the course of hours and weeks. These standards needed to be applied to pemetrexed in the adapted protocol. Pemetrexed doses were prepared in the 5% RPMI + EGF treatment medium and were used to treat A549 cells originally cultured in BEGM medium at five time points, over the course of 8 h (intraday stability) (**Fig. 3**). Quality control criteria for the assay require that dose-response curves from each time point produce Log EC_{50} concentrations that differ by less than 0.5. Pemetrexed in 5% RPMI + EGF medium met those criteria at each time point over the time course, so pemetrexed was validated as stable in the new medium at room temperature over the course of 8 h.

Interday stability of pemetrexed in 5% RPMI + EGF medium was performed with drug preparations that were frozen for 12 wk prior to use in the assay. A fresh drug preparation was compared side by side with two different samples that had been thawed from stocks that were frozen 12 wk before the assay was completed (**Fig. 4**). The samples produced nearly identical dose-response curves, and the preparations met the quality control criteria with Log EC_{50} values that differed less than 0.5. Therefore, pemetrexed was validated as stabile in the new medium for up to 12 wk frozen.

Pemetrexed treatment of ex vivo lung samples with the adapted ChemoFx protocol. The adapted ChemoFx protocol was then applied to 65 ex vivo lung carcinoma samples treated with pemetrexed. The dose-response curves for each sample were analyzed and classified. ChemoFx found 6.2% (4/65) of the specimens to be responsive, 9.2% (6/65) of the specimens to be intermediate responsive, and 84.6% (55/65) of the specimens to be non-responsive (**Fig. 5**).

Discussion

In this study, the standard ChemoFx protocol was altered to accommodate a specific drug, pemetrexed. BEGM is a proprietary culture medium, so there is no way to know for certain the concentrations of folate and folate analogs that it contains. Pemetrexed activity could not be observed in the ChemoFx DRM when BEGM was used during the treatment, but pemetrexed activity was observed if RPMI was used instead. By incorporating a patented rinse and media change procedure, the assay demonstrated significantly enhanced responses to pemetrexed (**Fig. 1**).[43] The modified protocol was then applied to cell lines and ex vivo lung carcinoma samples in order to investigate pemetrexed's actions in vitro. Although 16 distinct cancer cell lines were originally evaluated for their in vitro response to pemetrexed (data not shown), only A549 was classified as "responsive" and, thus, was used throughout the remainder of the study.

To demonstrate that the protocol change somehow involved folate composition differences between the two media, exogenous folic acid was added to the RPMI medium to see if the drug's activity in the updated protocol could be reversed. The reversal of drug response seen in the NSCLC cell line A549 as

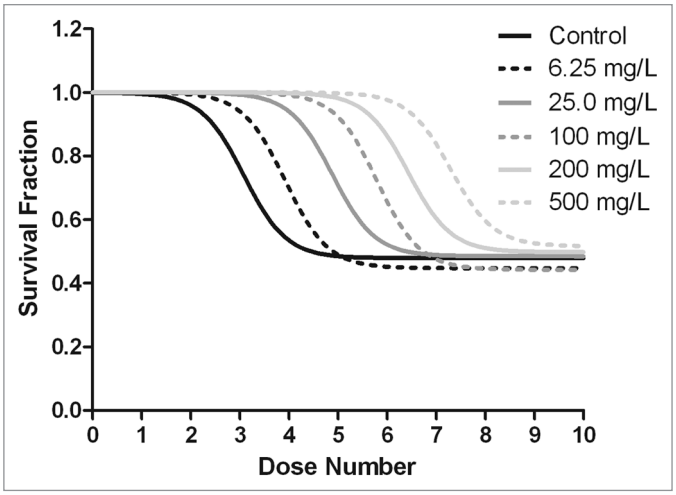

Figure 2. Increasing concentrations of folic acid reverse in vitro pemetrexed activity in 5% RPMI + EGF medium. Varying amounts of folic acid were added to 5% RPMI + EGF medium. A dose-dependent effect was illustrated with the lowest folic acid concentrations showing the greatest in vitro potency of pemetrexed and the highest folic acid concentrations demonstrating the least.

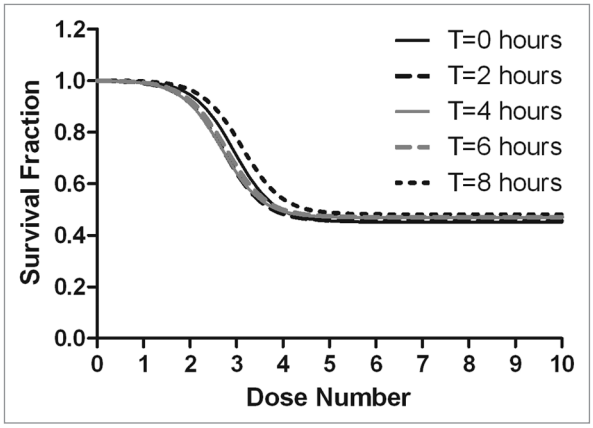

Figure 3. Intraday stability testing of pemetrexed by ChemoFx. A549 cells were plated in BEGM, followed by the rinse and media change to 5% RPMI + EGF prior to treatment. Pemetrexed, diluted in 5% RPMI + EGF, was then added to the wells over the span of 8 h (at 2 h intervals) to create the dose-response curve. Pemetrexed was found to be stable in 5% RPMI + EGF medium over the span of 8 h.

folic acid levels were increased confirmed that drug performance was directly affected by folate in the treatment medium. This finding in a NSCLC cell line is consistent with pemetrexed's mechanism of action, and the clinical observation that patients with supplementation with folic acid and B12 have helped ease side effects. Further studies must be conducted to confirm that the reversal of response by folic acid is observed in patient-derived primary cultures as well. Because BEGM's composition remains proprietary, it is not possible to say exactly what folate component(s) contributes to the differences in pemetrexed activity in the ChemoFx DRM, but it was demonstrated that adding

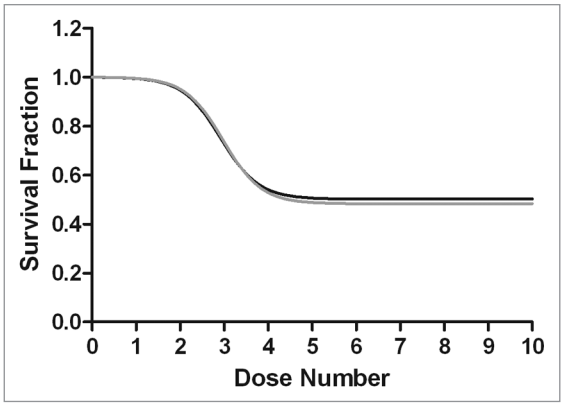

Figure 4. Interday stability testing of pemetrexed by ChemoFx. A549 cells were plated in BEGM, followed by the rinse and media change to 5% RPMI + EGF prior to treatment (black line). Pemetrexed, diluted in 5% RPMI + EGF, was prepared and frozen for a minimum of 12 wk before it was thawed and used in the assay (gray line). Frozen pemetrexed was shown to be stable in 5% RPMI + EGF media for up to 12 wk in -80°C.

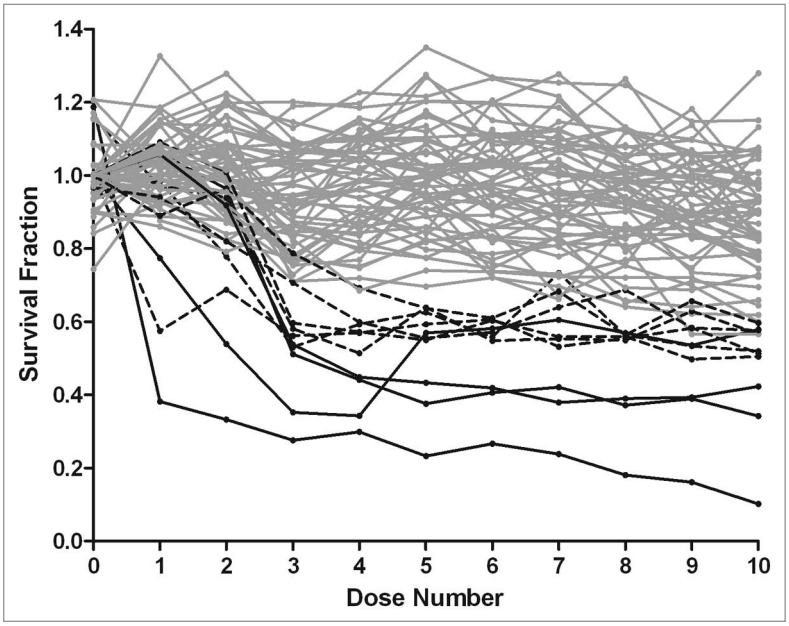

Figure 5. ChemoFx dose-response results for pemetrexed in 65 lung specimens using the rinse media change protocol. Non-responsive specimens, 84.6% (55 of 65), are in solid gray; intermediate responsive specimens, 9.2% (6 of 65), are in dotted black and responsive specimens, 6.2% (4 of 65), are in solid black. Each point represents the average of three replicates.

folic acid to the RPMI produced an environment that mimicked what was seen in the BEGM medium.

The intraday and interday stability tests proved that the modified protocol still met the quality control criteria set for the standard assay protocol. These data indicated that pemetrexed was reliably stable in the RPMI medium over the course of hours and weeks. The data also illustrated that the altered assay produced significant, reproducible results consistent with results produced by the standard assay.

The modification in the ChemoFx protocol allowed pemetrexed to be accurately evaluated on ex vivo lung samples. The power of the ChemoFx DRM is that the outcomes may identify mesothelioma and NSCLC tumors that would respond positively to pemetrexed therapy, before that therapy is commenced. Evaluating a regimen's suitability for a given patient prior to treatment can save wasted time and money, as well as unnecessary side effects.[44-46] The pemetrexed response rates generated by ChemoFx here (15.4% of patient samples showed at least some response to pemetrexed) were very comparable to literature reports of clinical response rates to pemetrexed in NSCLC and mesothelioma.[22-27] Collectively, the data presented here suggest that ChemoFx may serve as a tool to assist oncologists in determining whether or not pemetrexed is an appropriate treatment to be considered for a given NSCLC or mesothelioma tumor.

This study demonstrates the successful adaptation of a standard chemosensitivity protocol to evaluate pemetrexed activity in vitro. Using the updated protocol, which passed all quality control tests that the standard assay must pass, cell lines and ex vivo lung samples could be reproducibly assessed for pemetrexed response. As stated previously, ChemoFx outcomes have been correlated with patient outcome in breast and ovarian cancers.[40-42] Consequently, it is not unreasonable to suggest that the ChemoFx results for pemetrexed could be indicative of clinical outcomes in mesothelioma and NSCLC patients as well. In patients with mesothelioma and NSCLC, chemotherapy is not successful in limiting tumor progression in a significant number of patients; many patients suffer through side effects for weeks before learning that the treatment has done nothing to stop tumor progression.[47-49] Therefore, obtaining an indication of a patient's response prior to pemetrexed treatment is significant because it allows the physician and patient to weigh the likelihood of pemetrexed response against quality of life considerations.[50]

Materials and Methods

Test agents and vehicles. Pemetrexed was synthesized as a highly pure powder by 2A Pharma (2A-302986). The drug was reconstituted in RPMI with 5% FBS and EGF and was serially diluted in that medium to create 10 test concentrations for the cell lines and primary cultures.

ChemoFx DRM. The cell line and primary lung cell cultures were processed and assayed by the ChemoFx DRM. Briefly, cells were seeded in 384-well plates and were incubated with pemetrexed for 72 h at 37°C. Any cells remaining in the wells after the incubation were fixed with ethanol, stained with DAPI (Molecular Probes, Inc., D-3571) and counted using a proprietary automated microscope system (Precision Therapeutics, Inc.).[39] At each concentration, the survival fraction (SF) was calculated. The formula for calculating the SF is as follows: SF = mean cell count $_{dose}$ × mean cell count $_{control}$. The cell counts were obtained by averaging three

replicates for each concentration for the cell lines and the primary lung cell cultures. The dose-response curves were generated from the SF calculated for each concentration. The adjusted area under the curve (aAUC) was calculated for each dose-response curve as previously described.[41] The assay results were then classified as responsive (assay score ≥ 7.31), intermediate responsive (assay score 6.71–7.30) or non-responsive (assay score ≤ 6.70).

Cell lines. The lung carcinoma cell line A549 (ATCC, CCL-185) was seeded at 40,000 cells in T25 flasks (Greiner Bio-One international AG, 690-175) in BEGM medium (Lonza, CC-3171) containing 5% FBS (Hyclone, SH30071.03) and was allowed to culture for 1 wk to approximately 70–90% confluence. The cells were trypsinized and seeded into 384-well plates (Corning, 3712) at 320 cells per well. The cells were incubated for 24 h at 37°C in 5% CO_2. Using the liquid handler, the BEGM medium was removed and the cells were first rinsed with Dulbecco's PBS (Gibco, 14190) and then changed to RPMI 1640 medium (Mediatech, 10-040-CV) containing 5% FBS and 10 ng/mL recombinant human EGF. The cells were treated with pemetrexed-containing RPMI medium with 5% FBS and EGF and were assessed for sensitivity using the ChemoFx DRM.

Folic acid supplementation. Pemetrexed was prepared in RPMI medium with varying concentrations of folic acid (Sigma F7876-25G, batch no. 118K0864). The folic acid-containing medium was used to treat each of the 11 doses (doses 0–10) for each preparation; as such, dose 0 of each folic acid treatment contained the indicated concentration of folic acid but did not contain any pemetrexed. The final folic acid media concentrations tested on A549 cells were: 501 mg/L (1.1 mM), 401 mg/L (0.91 mM), 301 mg/L (0.68 mM), 201 mg/L (0.45 mM), 101 mg/L (0.23 mM), 51 mg/L (0.12 mM), 26 mg/L (0.059 mM), 13.5 mg/L (0.031 mM), 7.25 mg/L (0.016 mM), 4.13 mg/L (0.0094 mM) and 2.5 mg/L (0.0057 mM) (portions of data not shown). A control (no additional folic acid) was also assayed to measure the baseline response to the drug. RPMI medium already contains 1 mg/L (0.0023 mM) of folic acid normally, which is why the above concentrations were all increased by 1.0.[51]

Intraday stability testing. Pemetrexed was prepared in the 5% RPMI + EGF medium, serially diluted and then used in the assay to treat A549 cells immediately and in 2-h increments thereafter, up to 8 h post-preparation. The dilutions were maintained at room temperature for the entire time course. To pass quality control and be verified as stabile, each time point had to produce Log EC_{50} values that differed by less than 0.5.

Interday stability testing. Pemetrexed was prepared at assay concentrations in the 5% RPMI + EGF medium and frozen at -80°C. After a minimum of 12 wk, the dilutions were thawed and compared side by side with fresh drug preparations on A549 cells in the ChemoFx DRM. To pass quality control and be verified as stabile, the samples had to produce Log EC_{50} values that differed by less than 0.5.

Patient tumor specimens. Primary cell cultures were established using specimens acquired from the following sources: National Disease Research Interchange, Cooperative Human Tissue Network, Forbes Regional Hospital, Jameson Hospital, Saint Barnabas Medical Center, Hamot Medical Center, Windber Research Institute and University of Rochester Medical Center. All specimens used in the experiments were de-identified and were considered exempt (IRB protocol no. RD-109), informed consent was waived and limited data were available for each specimen. Tumor type was provided as lung carcinoma.

After surgical removal, the specimen was placed in a 125 mL bottle containing sterile McCoy's shipping medium (Mediatech, 10-050-CV) and shipped to the research laboratory at Precision Therapeutics, Inc. Primary cultures were initiated by mincing each specimen into 1 mm^3 explants, which were then seeded into the culture flasks containing 2% BEGM for lung specimens. Sixty-five confluent, primary lung cultures were trypsinized and seeded into 384-well plates at 8,000 cells/mL. The cells were incubated for 24 h at 37°C in 5% CO_2. The cells were then rinsed with D-PBS and the medium was changed to RPMI 1640 medium containing 5% FBS and 10 ng/mL EGF. The cells were treated in the RPMI medium with 5% FBS and EGF and assessed for sensitivity using the ChemoFx DRM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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