Original Article

MET/PKCß expression correlate with metastasis and inhibition is synergistic in lung cancer

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Abstract

Background: Treatment of non-small cell lung cancer (NSCLC) remains a difficult task in oncology. Targeted inhibition of oncogenic proteins is promising. In this study, we evaluate the expression of MET and PKCß and in vitro effects of their inhibition using SU11274 and enzastaurin (LY317615.HCl) respectively.

Materials and Methods: Patient samples were analyzed by immunohistochemistry for expression of PKCß and MET, utilizing tissue microarrays under an IRB-approved protocol. Expression of PKCß and MET was evaluated in cell lines by immunoblotting. Treatment with SU11274 against MET and enzastaurin against PKCß was performed in H1993 and H358 cell lines, and cell proliferation and downstream signaling (phosphorylation of MET, AKT, FAK, and GSK3ß) were evaluated by immunoblotting. Statistical analysis was performed using SPSS 16.0.

Results: Expression of MET positively correlated with lymph node metastases (p=.0004), whereas PKCß showed no correlation (p=0.204). MET and PKCß expression were also strongly correlated (p<0.001). Expression of MET was observed in 5/8 cell lines (H358, H1703, A549, H1993, H2170; absent from H522, H661, or SW1573), whereas PKCß expression was observed in 8/8 cell lines. Cell proliferation was significantly impaired by treatment with SU11274 and enzastaurin, and their effects were synergistic in combination (CI=0.32 and 0.09). Phosphorylation of MET, AKT, FAK, and GSK3ß were strongly inhibited with both agents in combination.

Conclusions: Concomitant inhibition of MET and PKCß significantly increased cytotoxicity in vitro against NSCLC, disrupting important downstream signaling pathways. Further evaluation in animal models is warranted.

Keywords: c-MET, developmental therapeutics, lung cancer, protein kinase C

BACKGROUND

Lung cancer is the most common cause of cancer mortality in the United States and worldwide. The disease presents commonly in advanced stages, which renders prognosis poor. Non-small cell lung cancer (NSCLC) is the most common form of lung cancer, accounting for approximately 80% of lung cancer cases. Though multidisciplinary therapeutic approaches toward NSCLC have improved survival and morbidity, the survival rates for advanced NSCLC remains dismal even with novel chemotherapy.

Inhibitors against epidermal growth factor receptor (EGFR) tyrosine kinase and antibodies against the ligand-binding domains of EGFR (such as cetuximab) that have come to
clinical fruition produce only modest improvements in clinical outcomes.\[^{[5]}\] However, there are multiple other molecular abnormalities in lung cancer yet unexplored.\[^{[6]}\]

The protein kinase C (PKC) family of serine-threonine protein kinases has been implicated in several important cellular functions including proliferation, motility, invasion, and apoptosis.\[^{[7]}\] Of the various PKC isoforms, PKC\(\beta\) expression has been demonstrated in several human cancers, most notably B cell lymphomas.\[^{[8]}\] Its overexpression has been shown to be an adverse prognostic factor in diffuse large B cell lymphomas.\[^{[8]-[10]}\] This was evaluated in a gene expression study, where 6817 genes were evaluated in relation to refractoriness versus curability in diffuse large B cell lymphomas; patients whose tumors had higher expression of PKC\(\beta\)2 had worse five-year event-free survivals (36 vs. 49%, \(p=0.054\)).\[^{[8]}\] PKC\(\beta\) has been implicated in angiogenesis, making it an attractive target for therapeutic inhibition in cancer.\[^{[11]}\] Downstream, PKC can target the PI3K/AKT pathway and other signal transduction pathways.\[^{[12],[13]}\] Enzastaurin (LY317615.HCl) is an oral small-molecule acyclic bisindolylmaleimide inhibitor of PKC\(\beta\) currently undergoing phase I-III clinical trials that inhibits PKC\(\beta\) in the low nanomolar range. At higher dosages, it may inhibit other PKC isoforms, most notably PKC alpha. It is currently being studied in multiple myeloma,\[^{[14]}\] breast cancer,\[^{[15]}\] cutaneous T-cell lymphoma,\[^{[16]}\] thyroid cancer,\[^{[17]}\] colon cancer, glioblastoma,\[^{[18]}\] and non-small cell lung cancer.\[^{[18]}\]

The c-MET receptor tyrosine kinase (MET) was originally identified as the cellular homologue of the TPR-MET oncoprotein.\[^{[19]}\] MET is over-expressed in a number of malignancies, sometimes mutated (germline mutations/single nucleotide polymorphisms (SNPs) or somatic mutations), and at other times amplified. It has been shown to be a promising target in the treatment of cancer, with multiple compounds in pre-clinical and clinical development.\[^{[20]}\]

Combination therapies, especially those involving MET inhibition, have been increasingly studied as a treatment strategy in lung cancer.\[^{[21]-[24]}\] Given the current knowledge of roles of MET, a growth-factor receptor, PKC\(\beta\), an intracellular target in lung cancer and the efficacy of their inhibition as independent targets, we have investigated whether their dual inhibition leads to increased cytotoxicity against NSCLC in vitro. Intracellular signaling networks were also evaluated for effects of the combination therapies.

**MATERIALS AND METHODS**

**Tissue microarray and immunohistochemistry**

Paraffin-embedded, formalin-fixed tumor tissues from patients with NSCLC with associated clinical information (histological subtype, clinical stage, overall survival and vital status) were processed into a tissue microarray (TMA) under an Institutional Review Board-approved protocol (UCMC #9571 and #13473). Immunohistochemistry (IHC) was performed using biotin-free HRP-labeled polymer complex bound to secondary antibody (DAKO Cytomation, Carpinteria, CA), and performed according to previously published procedures.\[^{[25]}\] Negative controls were performed by substituting the primary antibody step with non-immune mouse immunoglobulins. Results were assessed semi-quantitatively (by three independent pathologists, MW/MT/AH) by light microscopy, and graded as negative (0), weak (1+), strong (2+), and very strong expression (3+). Sixty-nine patients were analyzed.

**Cell lines and culture**

NCI-H522 (adenocarcinoma), NCI-H1703 (adenocarcinoma), A549 (adenosquamous carcinoma), NCI-H1993 (adenocarcinoma), NCI-H2170 (squamous cell carcinoma), NCI-H661 (large cell carcinoma), SW1573 (bronchoalveolar carcinoma) and NCI-H358 (bronchoalveolar carcinoma) cell lines were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were cultured as per our established protocols.\[^{[26]}\]

**Reagents and antibodies**

Enzastaurin was provided by Eli Lilly (Indianapolis, IN). Fetal bovine serum (FBS) was obtained from Gemini Bioproducts (Woodland, CA). SU11274 was obtained from EMD Biosciences (San Diego, CA). Cell culture media, penicillin, and streptomycin were obtained from Cellgro (Boehringer Ingelheim, Heidelberg, Germany). Antibodies used included: anti-PKC\(\beta\)2, anti-Met, anti-phospho-FAK (Tyr925), anti-FAK, anti-phospho-AKT (Ser473) and anti-phospho-GSK3\(\beta\) (Ser9) (Santa Cruz, Santa Cruz, CA); anti-phospho-Met (Tyr130/1234/1235) (Invitrogen, Carlsbad, CA); \(\beta\)-actin monoclonal antibody (Sigma, St. Louis, MO). All other chemicals were purchased from Sigma (St. Louis, MO).

**Immunoblotting**

To examine protein expression in NSCLC cell lines under basal conditions, subconfluent cells were cultured in medium supplemented with 10% FBS. To detect the inhibition of cell transduction pathways, cells grown on 10-cm culture dishes for 24 hours were washed twice with PBS and incubated at 37°C with 2.5 \(\mu\)M enzastaurin, 2.5 \(\mu\)M SU11274, or enzastaurin and SU11274 in combination (or DMSO as a negative control) for different durations as described, in serum-free media. Whole cell lysates were collected and immunoblotting was performed following routine
protocols.[26] The same membranes were subsequently stripped and reprobed in a similar fashion with different primary antibodies. β-actin levels were used to control for equal loading amounts.

**Cell proliferation studies**

Cells were plated in 96-well plates at 5×10^3 cells per well in serum-containing media and grown for 24 hours. Drugs (or drug carrier) were added in serum-free media, and cells were incubated for 72 hours. Cell growth was estimated utilizing fluorometric readings after the addition of Alamar Blue (Invitrogen, Carlsbad, CA), a non-radioactive, non-toxic dye that is reduced and whose fluorescence is proportional to cellular metabolic activity. A HT Synergy Plus microplate reader (Biotek, Winooski, VT) was used to measure fluorescence (530-560 nm excitation wavelength and 590 nm emission). Drug synergism was estimated by the median-effect analysis[27] using the Calcusyn 3.0 software package (Biosoft, Camdridge, UK).

**Statistical analysis**

Comparisons between average means of protein expression were made by utilizing the Student’s t-test for independent samples. For evaluation of differences in frequencies among different categories, the Chi-square test was used. The statistical software used was SPSS, version 16.0 (SPSS Inc, Chicago, IL).

**RESULTS**

**Expression and correlation of PKCβ and MET in NSCLC tumor samples**

Evaluation of protein expression of PKCβ and MET in the TMAs was performed. Table 1 lists the characteristics of the 69 patients included in the analysis. Most patients were older individuals, with a slight male predominance. Forty-five per cent of patients had adenocarcinomas, followed by large cell carcinomas (23%) and squamous cell carcinomas (14%). Given that most samples were derived from curative lung resections, 55% of patients had clinical stage I disease. The expression of PKCβ and MET among patients whose tumors had not metastasized to lymph nodes (clinical stage I) versus those whose had (stages II-IV) was compared [Table 1]. MET expression was significantly increased in patients with positive lymph nodes (1.97 versus 1.36, p=0.009, Student’s t-test). PKCβ expression tended to be also higher in patients with positive lymph nodes; however, statistical significance was borderline (1.93 versus 1.47, p=0.114, Student’s t-test) [Figure 1]. In order to further evaluate this, we also grouped patients according to whether they had strong versus weak expression of PKCβ and MET and correlated these two variables [Table 2]. Again, MET expression positively correlated with the presence of positive lymph node metastasis (p=0.004, Chi-square), while PKCβ expression was not significantly associated with lymph node metastasis (p=0.204). The association between MET and PKCβ expression was also investigated in the same fashion. There was a strong positive correlation between PKCβ and MET expression in the NSCLC samples (p<0.001, Chi-square) [Table 2].

**MET and PKCβ Expression in NSCLC Cell Lines:**

In order to further investigate the potential therapeutic implications of concomitant PKCβ and MET inhibition, eight NSCLC cell lines were evaluated for protein expression by immunoblotting. MET expression was robustly observed in 5/8 cell lines (H358, H1703, A549, H1993, H2170), while PKCβ expression was seen in all 8 cell lines [Figure 2].

**NSCLC Cell Growth Inhibition with Enzastaurin and SU11274 Treatment:** Cell growth inhibition with various drugs was evaluated in H1993 and H358 cell lines. After treating these cells for 72 hours with SU11274, enzastaurin, or both drugs in combination in increasing concentrations, it was observed that the combination was synergistic in causing significant effects on cell proliferation in both cell lines. Combinatorial indices for H358 cells at ED50 was 0.32, and for H1993 was 0.09, therefore proving significant synergistic effect [Figure 3].

**Table 1: Characteristics of 69 Patients whose Samples were Included in the TMA Analysis**

| Age at diagnosis (years) | 63.5 (+/- 10.4) |
|--------------------------|-----------------|
| Gender                   |                 |
| Male                     | 40 (57.9%)      |
| Female                   | 29 (42.1%)      |
| Histology                |                 |
| Adenocarcinoma           | 31 (44.9%)      |
| Squamous cell carcinoma  | 10 (14.4%)      |
| Large cell carcinoma     | 16 (23.1%)      |
| NSCLC                    | 6 (8.6%)        |
| BAC                      | 4 (5.7%)        |
| Mixed                    | 1 (1.4%)        |
| Other                    | 1 (1.4%)        |
| Clinical Stage           |                 |
| I                        | 38 (55%)        |
| II                       | 9 (13%)         |
| III                      | 18 (26%)        |
| IV                       | 4 (6%)          |

**Table 2: Correlation of MET and PKC expression in 69 patient samples**

|                   | LN neg | LN pos | P=0.004 |
|-------------------|--------|--------|---------|
| MET Low           | 23     | 8      |         |
| MET High          | 15     | 23     |         |
| PKC Low           | 18     | 10     | P=0.204 |
| PKC High          | 20     | 21     |         |
| PKC Low           | 20     | 8      | P=0.001 |
| PKC High          | 11     | 30     |         |

According to LN Status and between each other. Low ≤ average, high > average expression. Statistical significance calculated using Chi-square test.
Effects on Cell Signal Transduction with Enzastaurin and SU11274 Treatment: In order to investigate the molecular effects of treatment with SU11274 and/or enzastaurin, immunoblotting with phospho-specific antibodies was carried out in H1993 cells treated with these drugs for the time periods shown [Figure 4]. Treatment of H1993 cells with SU11274 completely inhibited phosphorylation of MET, FAK, and AKT within two hours after the initiation of treatment; however, an incomplete reduction in phosphorylation levels of GSK3β was observed. Treatment with enzastaurin revealed mild reductions in phosphorylation of MET and incomplete abrogation of FAK, AKT, and GSK3β phosphorylation. When both SU1174 and enzastaurin were used concomitantly, complete elimination of phosphorylation of MET, FAK, and AKT was observed, with near-total abrogation of GSK3β phosphorylation [Figure 4].

DISCUSSION

There have been multiple examples of the utilization of a combination of targeted agents in lung cancer. Unfortunately, the accumulated experience has been largely unsuccessful in the treatment of this disease. Our work presents a combination that might prove to be biologically relevant, and perhaps have a reasonable toxicity profile to be investigated in the clinical setting. Herein, we have identified that MET and PKCβ are expressed in lung cancer, and their inhibition can be synergistic. Interestingly, also, metastasis is common in lung cancer, and lymph node metastasis correlated with both MET and PKCβ expression.

For nearly two decades, MET has been known to be commonly aberrant in lung cancer, whether overexpressed,\textsuperscript{[21,28,29]} amplified,\textsuperscript{[30-34]} mutated,\textsuperscript{[35-37]} or alternatively spliced,\textsuperscript{[34,38]} and it therefore remains the focus of intense investigation. Binding of the MET receptor by its ligand HGF leads to receptor dimerization and activation of its intrinsic tyrosine kinase, followed by internalization into clathrin-coated
vesicles, delivery to sorting endosomes, and degradation via the lysosomal pathway. Phosphorylation of MET at Y1230, Y1234, and Y1235 in the activation loop of the tyrosine kinase domain correlates with increased tyrosine kinase activity. In our inhibition experiments, phosphorylation of MET considerably decreased with SU11274 treatment.

MET activation can lead to autophosphorylation or phosphorylation of downstream intermediates and activation of signaling pathways. As an example, in small cell lung cancer (SCLC), activation of MET with HGF leads to phosphorylation/activation of several pathways involving cell proliferation/survival (ERK1/2, AKT), cell cycle (RB), and cytoskeletal proteins (paxillin, FAK).[^39]

MET has also been shown to signal synergistically with EGFR as part of a broad signaling network that cooperatively drives activation of these downstream pathways.[^22,40] Indeed, concomitant inhibition of MET and EGFR in erlotinib-resistant cells harboring the T790M mutation significantly increases lung cancer cytotoxicity above MET-targeted therapy alone in both in vitro and in vivo settings.[^21]

Inhibition of PKCβ with enzastaurin has been recently studied in thoracic malignancies. Our previous work has demonstrated the in vitro effect of enzastaurin against malignant pleural mesothelioma,[^41] and its synergistic activity when combined with cisplatin. In NSCLC, in vitro activity of enzastaurin and pemetrexed, a commonly used antifolate compound, has also revealed synergistic activity against SW1573 and A549 cell lines. Multiple biochemical pathways were shown to be affected, such as cell cycle control, apoptosis, and angiogenesis.[^42] In another recent publication, enzastaurin has been shown to be able to reverse acquired resistance to gefitinib, an EGFR small molecule inhibitor; while this study evaluated cell lines that are not NSCLC (colon cancer and prostate cancer), the mechanism observed may likely be observed in NSCLC.[^43] The efficacy of combination enzastaurin and other cytotoxic agents in NSCLC might be dependent upon the schedule by which these drugs are delivered. Morgillo et al, have investigated the antiproliferative effects of enzastaurin with two commonly used drugs in the treatment of NSCLC, gemcitabine and pemetrexed. A synergistic effect was only observed when enzastaurin treatment was undertaken following the delivery of either gemcitabine or pemetrexed, while an antagonistic effect was observed if enzastaurin treatment preceded the cytotoxic agents.[^44] A phase II clinical trial in which enzastaurin was used as a single agent as second- or third-line against NSCLC did not meet its primary end-point (an increase in progression-free survival of 20%); however, 13% of patients treated had progression-free survival greater than six months, signaling that perhaps a subset of patients with NSCLC might benefit from this drug.[^45]

We have shown here that MET and PKCβ tend to be coexpressed in NSCLC cell lines and tissues and that simultaneous inhibition of MET and PKCβ significantly decreased cell proliferation in in vitro assays. This finding was associated with decrease in activation of downstream effectors such as GSK3β, AKT and FAK. These data suggest that concomitant inhibition of MET and PKCβ may be an effective treatment strategy for NSCLC, especially for those patients whose tumors have developed prior tyrosine kinase resistance. These findings warrant further investigation in vivo to determine whether such a dual inhibition strategy is effective in reducing tumor progression.

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