Combination MET inhibition and Topoisomerase I inhibition block cell growth of Small Cell Lung Cancer

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ABSTRACT
Small cell lung cancer (SCLC) is a devastating disease and current therapies have not greatly improved the 5-year survival rates. Topoisomerase (Top) inhibition is a treatment modality for SCLC; however, the response is short lived. Consequently, our research has focused on improving SCLC therapeutics through the identification of novel targets. Previously, we identified MET to be overexpressed and functional in SCLC. Herein, we investigated the therapeutic potential of combinatorial targeting of MET using SU11274 and Top1 using 7-ethyl-10-hydroxycamptothecin (SN-38). MET and TOP1 gene copy numbers and protein expression were determined in 29 patients with limited (n=11) and extensive (n=18) disease. MET gene copy number was significantly increased (>6 copies) in extensive disease compared to limited disease (p=0.015). Similar TOP1 gene copy numbers were detected in limited and extensive disease. Immunohistochemical staining revealed significantly higher Top1 nuclear expression in extensive (0.93) versus limited (0.15) disease (p=0.04). Interestingly, a significant positive correlation was detected between MET gene copy number and Top1 nuclear expression (r=0.5).
In vitro stimulation of H82 cells revealed HGF-induced nuclear co-localization of p-MET and Top1. Furthermore, activation of the HGF/MET axis enhanced Top1 activity, which was abrogated by SU11274. Combination of SN-38 with SU11274 dramatically decreased SCLC growth as compared to either drug alone. Collectively, these findings suggest that combinatorial inhibition of MET and Top1 is a potentially efficacious treatment strategy for SCLC.
INTRODUCTION

In the year 2012, there will be an estimated 226,160 new cases and 160,340 deaths from lung cancer (1). Approximately 13% of prevalent lung cancer cases are small cell lung cancer (SCLC) (2). Though SCLC comprises a small percentage of the total overall lung cancer cases, it presents as a very aggressive disease with a median overall survival from the time of diagnosis of 2-4 months. Patients usually present with metastatic disease and have an overall 5-year survival of approximately 6% (3). Unfortunately, the median survival has not improved significantly over the last 25 years (4,5). A number of abnormal events have been identified as being important in the pathogenesis of SCLC. These include chromosome 3p abnormalities (6-8), aberrations in the tumor suppressor genes TP53 and RB1 (9), and the abnormal expression of proto-oncogenes MYC (10, 11) and BCL2 (12-14). Proto-oncogene receptor tyrosine kinases (RTKs) are key regulators of important biological functions, which have also been implicated in the etiology of multiple tumor types (15-18).

We have previously reported the RTK, MET, to be overexpressed and functional in SCLC (16). The structure and function of MET has been well characterized (for review see [19]). The MET receptor is a disulfide-linked heterodimer with a molecular weight of 190 kD. The 140 kD-chain spans the membrane and possesses cytoplasmic tyrosine kinase activity and can be detected in its 170 kD precursor form (16). It consists of an intracellular portion composed of tyrosine kinase (TK) domain, a juxtamembrane (JM) domain, a transmembrane (TM) domain, and the extracellular portion comprised of semaphorin (SEMA) domain, the plexins, semaphorins, and integrins (PSI) domain, and the immunoglobulins, plexins, and transcription factors (IPT) repeat domain. Hepatocyte growth factor/scatter factor (hereafter referred to as HGF) is the cognate ligand for MET (20). HGF binds to the SEMA domain of MET, which induces autophosphorylation of the intracellular tyrosine residues within the TK domain. This stimulation of MET by HGF leads to a plethora of biological and biochemical effects in the cell. In fact, the HGF/MET axis plays an important role in embryological development, growth, motility, invasion, metastasis, angiogenesis, wound healing, and tissue regeneration (21).

Novel therapeutic modalities that target MET kinase activity have focused on the development of small molecule inhibitors, such as SU11274 (22-24). This compound is 50-times more selective for MET compared to other tyrosine kinases (24). In vitro studies using SU11274 demonstrated selective inhibition of MET tyrosine kinase activity in lung cancer cell lines expressing MET (25). Moreover, MET autophosphorylation at the activation loop, tyrosine phosphorylation, and downstream phosphorylation events were all inhibited by SU11274, highlighting the potential therapeutic benefits of MET inhibition on the growth of lung tumor and metastasis.

The standard therapy for SCLC is etoposide, a Topoisomerase II (Top2) inhibitor, and cisplatin (26). However, response to this regimen is short lived and overall survival is still on the order of months. Thus, there is intense focus on discovering new agents or combinations of therapies that are efficacious. A novel therapeutic approach in SCLC, particularly in refractory or relapsed disease, involves the use of Top1 inhibitors (27). These agents, primarily consisting of camptothecin analogues, irinotecan and topotecan, stabilize the Top1/DNA complex during the
process of DNA replication. In doing so, the Top1-induced DNA single-strand breaks cannot be re-sealed and DNA synthesis is halted thereby initiating apoptotic cell death (28). In extensive stage disease, irinotecan combined with platinum-based agents increases overall survival compared to the combination of platinum and etoposide (27, 29). Irinotecan is a water-soluble compound that is metabolized into 7-ethyl-10-hydroxycamptothecin (SN-38) by the liver. SN-38 has a 100-fold greater anti-tumor effect over irinotecan (30).

In this investigation we determined the relationship between MET and Top1 in SCLC. MET and TOP1 gene copy numbers as well as protein expression were determined in 29 SCLC patient tumor tissues - 11 with limited disease and 18 with extensive disease. MET gene copy numbers and nuclear expression of Top1 were increased in extensive disease. Interestingly, there was a correlation between MET gene copy number and nuclear Top1 expression. In vitro cell culture modeling revealed modulation of Top1 activity via activation of the HGF/MET axis. We next evaluated the therapeutic benefits of the novel combination of the Top1 inhibitor, SN-38, and the small molecule MET inhibitor, SU11274. HGF stimulation induced nuclear co-localization of p-MET and Top1. Moreover, HGF stimulation enhanced Top1 activity, which was abrogated by MET inhibition using SU11274. Combined MET and Top1 inhibition decreased cell viability in two SCLC cell lines, H69 and H345. Collectively, these findings highlight the potential for co-targeting MET and Top1 as a novel therapeutic modality for SCLC.

MATERIALS AND METHODS

Obtaining Patient Samples and Clinical Information

The Institutional Review Board at The University of Chicago (Chicago, USA) approved all of the human subjects research performed in this study. Written informed consent was obtained from the patients prior to the harvest of tumor and paired adjacent normal lung tissues. The tissues used in this study were obtained from patients who were treated for SCLC at The University of Chicago Medical Center.

Gene copy number analysis by qPCR

Genomic DNA was isolated from formalin fixed paraffin embedded (FFPE) tumor tissues using a protocol that was standardized in our laboratory (31); and cell line genomic DNA was isolated using the Qiagen kit (Qiagen, Valencia, CA). In brief, 30 to 50ng of genomic DNA was PCR amplified using FastSYBR green 2X MasterMix (Applied Biosystems, CA) and ABI StepOnePlus (Applied Biosystems, CA). MET and TOP1 gene copy numbers were determined using the primers listed in Supplementary Table 1 and the following reaction conditions: 1 cycle - 95°C for 20s; 40 cycles - 95°C for 3s, 55°C for 10s, 68°C for 10s; and 1 cycle - 95°C for 3s, 55°C for 10s, 95°C for 3s. Samples were assayed in triplicate for the gene of interest (GOI) being analyzed as well as LINE1 as an internal control for normalization of the quantity values. Control genomic DNA that was not amplified for the GOI was used to normalize and calculate GOI amplification using the following equations:

\[
\text{quantity(Sample-Normalized)} = \frac{\text{quantity(Sample-GOI)}}{\text{quantity(Sample-LINE1)}} \\
\text{quantity(Control -Normalized)} = \frac{\text{quantity(Control-GOI)}}{\text{quantity(Control-LINE1)}} \\
\text{Gene copy number} = \frac{\text{quantity (Sample-Normalized)}}{\text{quantity (Control -Normalized)}}
\]
**Immunohistochemistry**
Paraffin-embedded, formalin-fixed tissues from 29 patients with SCLC diagnosed at The University of Chicago Medical Center were utilized. Immunohistochemical (IHC) staining was performed using standard techniques previously described (25) with antibodies against the following proteins: MET (Invitrogen, Carlsbad, CA), phospho-MET (BioSource International), and Top1 (Abcam). In brief, tissue cores from normal and tumor tissues from each patient were obtained and arranged into a tumor tissue microarray using the ATA-27 Arrayer (Beecher Instruments, Sun Prairie, WI). Slides were reviewed and scored by two independent pathologists on a scale of 0 to 3: with 0=no staining, no expression; 1=weak staining, low expression; 2=moderate staining, moderate expression; and 3=strong staining, high expression.

**Cell Lines**
H69 and H82 SCLC cell lines were obtained from the American Type Culture Collection (Rockville, MD), and maintained in RPMI 1640 media supplemented with 10% (v/v) heat inactivated fetal calf serum, 1% (v/v) penicillin-streptomycin and 2 mM L-glutamine. Cells were incubated at 37°C, 5% CO2 in air atmosphere with humidity, and harvested during the log phase of growth. The cell lines were characterized by ATCC based on cytogenic analysis, DNA profiling, and growth properties, and no additional authentication was performed by the authors.

**Small molecule inhibitors**
SU11274 (Selleck Chemicals, Houston, TX) and SN-38 (Tocris, Ellisville, MO) were dissolved in DMSO to a concentration of 10mM and stored in small aliquots at -20°C.

**Immunoblotting**
Whole cell lysates were prepared in M-PER buffer (Thermo Fisher Scientific, Rockford, IL) containing HALT protease and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentrations were determined using theNanoDrop 2000 (Thermo Fisher Scientific), and then 100 µg of proteins were separated by 10% SDS-PAGE under reducing conditions and transferred electrophoretically to a PVDF membrane (Millipore, Bedford, MA). The membranes were probed with antibodies specific for MET (Invitrogen), Top1 (BD Pharmingen, San Diego, CA) and β-actin (Sigma, St. Louis, MO). Proteins bands were detected using the Immun-Star WesternCChemiluminescent Detection Kit.

**Selective reaction monitoring**
Cells (10^7) were washed, fixed with 10%-buffered formalin, and solubilized to tryptic peptides by Liquid Tissue® processing. The soluble mixtures were analyzed by selective reaction monitoring (SRM) on a Thermo Vantage triple quadruple mass spectrometer. The quantification of MET expression was achieved by spiking in a heavy isotope labeled MET peptide into the samples, for the comparison of the ratio of AUC to the endogenous MET analyte.

**Immunofluorescence and confocal microscopy**
H82 cells were deprived of growth factors by incubation in serum-free medium containing 0.5% BSA (Sigma, St. Louis, MO) for 16h. The cells were stimulated with HGF (Calbiochem, La Jolla, CA) at 40 ng/ml for 10 min. The cells were plated on poly-L-lysine coated glass cover slips, then
fixed using 4% Paraformaldehyde (Boston Scientific, Natick, MA), and permeabilized with 0.1% TritonX-100 (Sigma) in PBS. Fixed cells were washed with ice cold PBS plus 0.1% Tween-20 (PBST) and nonspecific binding was inhibited using 2.5% BSA in PBS, then incubated with the appropriate primary antibodies: anti-MET (Abcam, Cambridge, MA), anti-pY1349/Y1350-MET (Cell Signaling, British Colombia, Canada), and anti-Top1 (Abcam) in PBS with 1% BSA. Secondary antibodies conjugated with fluorescein (CY3 and CY5) were utilized to detect the binding of primary antibodies. The cover slips were mounted and images were captured using a laser scanning confocal microscope. Cell nuclei were visualized by DAPI staining.

*Topoisomerase I activity assays of nuclear extracts*
Nuclear extracts were prepared as described by Deffie et al. (32). In brief, H82 cells were collected by centrifugation and washed once with ice-cold TEMP (10mM Tris-HCl pH 7.5, 1mM EDTA, 4mM MgCl2, 0.5mM PMSF). Cells were subsequently suspended in 1 ml of cold TEMP for 10 min and then centrifuged at 1500xg for 10 min. The nuclear pellet was resuspended in 20 µL of TEP (same as TEMP but lacking MgCl₂) plus 20 µL of 1M NaCl and placed on ice for at least 30 min and then centrifuged at 15,000xg for 15 min. The supernatant was collected as the nuclear extract. Top1 enzymatic activity in the nuclear extracts was measured using a DNA relaxation assay as per manufacturer’s instructions (TopoGen, Port Orange, FL). Supercoiled plasmid DNA in a reaction mixture (20µl) containing 10 mM Tris-HCl, pH 7.9, 1mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine and 5% glycerol was incubated at 37°C for 30 min with neat and serially diluted (1:4) nuclear extracts, purified recombinant human Top1 (positive control), or assay diluent (negative control). The reactions were terminated by addition of 5 µl of 5X loading buffer (5% SDS, 0.3% bromophenol blue). Samples were resolved on a 1% agarose gel and imaged using the BioRad GelDoc (BioRad, Hercules, CA). The conditions assayed were as follows: (1) unstimulated cells (Media) – cells that were cultured in media alone. (2) HGF stimulated cells (HGF) - cells were stimulated for 15 mins with 50 ng/ml of HGF then harvested. (3) SU11274 treated cells (SU11274) – cells were cultured for 4 hours with 5 µM of SU11274 then harvested. (4) HGF stimulation and SU11274 treatment (HGF/SU11274) – were cultured for 4 hours with 5 µM of SU11274 then stimulated for 15 mins with 50 ng/ml of HGF prior to harvesting.

*Cell Viability Assay*
H69 and H82 cells (1 x 10⁴ cells/well in 96-well plate) were cultured overnight in RPMI-1640 supplemented with 1% FBS. The next day the cells were treated with SU11274 alone, SN-38 alone or SU11274 and SN-38 in combination for 72 h. Cell viability was estimated using Alamar blue (final concentration of 10% v/v), a non-radioactive, non-toxic compound that is reduced by viable cell such that the amount of reduced Alamar blue is proportional to the metabolic activity of the cells. Plates were incubated at 37°C for 4 to 5 h and fluorescence was measured using a plate reader (530nm/590nm for excitation/emission). Cell viability represents the percentage of cells affected by drug treatment following normalization to cells cultured in media alone.

*Statistical Analysis*
A Wilcoxon Signed Ranks test was performed to compare differences in the gene copy number between MET and Top1 in cell lines and patient samples. Mann-Whitney testing was performed to compare protein expression by stage. Correlational analysis was performed using a Pearson correlation. All statistical analyses were conducted using SPSS 17.0 (SPSS Inc., Chicago, IL), with statistical significance set at p< 0.05.

RESULTS

MET and TOP1 gene copy number and protein expression in SCLC tumors

Tumor samples were obtained from 29 patients treated for SCLC at The University of Chicago (Supplementary Table 2). There were 11 patients with limited stage disease and 18 patients with extensive stage disease. Gene copy numbers for MET and TOP1 were determined using genomic DNA isolated from patient tumor samples (Fig. 1A). MET gene copy number was increased (> 6 copies) in 9/29 patient samples. In 21 of the 29 patients, there was a statistically significant greater MET gene copy number compared to TOP1 gene copy number (p=0.005). When patients were grouped by disease stage (limited or extensive), there was a statistically significant difference between the mean MET gene copy number for limited disease (2.7) and extensive disease (7.9) (p=0.015). No difference was observed for TOP1 gene copy number (Fig. 1B).

The expression and distribution of MET and Top1 was determined by IHC in 29 patient tumor samples. Figure 2A shows representative IHC images of MET and Top1 staining in limited and extensive disease stage tumors. Nuclear Top1 expression was significantly higher in extensive stage disease than in limited stage disease (p= 0.04); cytoplasmic Top1 was not differentially expressed based on disease stage (Fig. 2B). Moreover, there was no difference in the expression or distribution of MET and p-MET based on disease stage.

We next sought to determine whether MET or TOP1 gene copy number correlated with protein expression/distribution of MET, p-MET, and Top1 (Table 1). Having shown that MET gene copy number and nuclear Top1 were increased in extensive disease compared to limited disease it was interesting to determine that MET gene copy number was correlated with increased nuclear Top-1 expression (r= 0.5) (Fig. 2C).

MET and TOP1 gene copy number and protein expression in SCLC cell lines

MET and TOP1 gene copy number was determined in 20 SCLC cell lines (Fig. 3A). MET gene copy number was increased (> 6 copies) in 4/20 cell lines and TOP1 was increased (> 6 copies) in 2/20 cell lines. In 18 of the 20 cell lines, MET gene copy number was greater than the TOP1 gene copy number. In fact, the mean MET gene copy number (4.6) was significantly higher than the mean TOP1 gene copy number (2.2) (p=0.004).

We next determined MET and Top1 protein expression in a panel of eight SCLC cell lines by immunoblotting and SRM. Figure 3B shows high levels of MET expression in H69 and H345, while moderate expression was detected in H82, H249, and H526 cells. In contrast, MET expression was low or undetectable in H184, H446, and H2171 cells. The expression pattern of MET in the cell lines was largely recapitulated using SRM technology (Fig. 3C). Highest MET
expression was detected in H69 (255.09±8.5 amol/mg) and H345 (244.03±11.59 amol/mg) cells. Moderate levels were detected in H82 (158.63±10.27 amol/mg), H249 (115.66±8.77 amol/mg), and H526 (134.69±18.49 amol/mg), and low to undetectable levels were detected in H184 (0 amol/mg) and H446 (88.53±8.24 amol/mg). The only exception was the detection of moderate levels of MET expression in H2171 (148.25±12.65 amol/mg) by SRM compared to relatively low expression by immunoblotting.

**Modulation of Topoisomerase I activity via the HGF/MET axis**

The modulation of Top1 expression by the HGF/MET axis was investigated in H82 cells. Figure 4A shows low basal expression of p-MET and Top1 in H82 cells. HGF stimulation increased the expression of pMET as well as Top1. Moreover, p-MET and Top1 co-localized to the nucleus of HGF stimulated cells.

DNA relaxation assays were performed to determine the induction of Top1 activity in response to HGF stimulation (Fig. 4B). Increasing amounts of nuclear extracts from H82 cells stimulated with HGF in the absence or presence of SU11274 were incubated with supercoiled plasmid DNA. SU11274 decreased Top1 activity as evidenced by the presence of strong supercoiled bands in the SU11274 treated cells compared to HGF treated and control cells. The addition of SU11274 to HGF-stimulated cells reduced Top1 activity compared to cells treated with HGF alone, suggesting that activation of the HGF/MET axis mediated Top1 activity.

**SU11274 and SN-38 synergize to decrease cell viability**

We investigated the potential synergism between SU11274 and SN-38 to decrease cell viability in H69 and H82 cells. The cell lines were treated with SU11274 and SN-38 alone or in combination and cell viability was determined using the standard Alamar blue assay. H69 cells (R988C MET mutation) were highly sensitive to SU11274 alone and SN-38 alone across the concentrations investigated (Fig. 5A). Moreover, SU11274 and SN-38 combined to significantly decrease cell viability compared to either SU11274 alone or SN-38 alone. In contrast, H82 cells (wild-type MET) were less sensitive to SU11274 and SN-38 inhibition. However, SU1127 and SN-38 worked in combination at the lower doses of SU11274 and SN-38 to significantly decrease cell viability (Fig. 5B).

**DISCUSSION**

Small cell lung cancer is an aggressive malignancy for which novel therapies are desperately needed. Herein, we show that MET can be specifically inhibited with a novel small molecule inhibitor SU11274 in SCLC cell lines, and that SN-38, a Top1 inhibitor, can synergize with SU11274. This synergism was most evident in the H69 SCLC cell line that expresses the R988C mutation in the MET JM domain, which renders MET constitutively active (16). Further support of the MET/HGF axis modulation of Top1 activity is evidenced by the association of Top1 with p-MET and the nuclear co-localization of MET and Top1 in response to HGF stimulation. Moreover, MET inhibition by SU11274 decreased the activity of Top1 leading less relaxed DNA in the Top1 activity assay. Taken together, these data show a link between the activation of the HGF/MET axis and Top1 activity in the nucleus.
We have previously shown that MET is expressed in a variety of SCLC cell lines, with abundant expression in H69 and moderate expression in H82 cells (16). Activity along the HGF/MET axis has been shown to be critical for embryogenesis and wound healing (33, 34). These processes require appropriate cell proliferation, motility, and angiogenesis, key factors also necessary for tumorigenesis and metastasis. Studies using the H69 cell line have demonstrated that HGF stimulation leads to increased SCLC cell motility through the induction of focal adhesion proteins paxillin, focal adhesion kinase, and PYK2 (35). HGF stimulation also led to downstream phosphorylation at sites associated with transcriptional control, G1/S cell cycle checkpoint, cell survival, and cell proliferation. The missense R988C MET mutation in the H69 cell line results in constitutive activation, cellular proliferation and cell motility, decreased cellular adhesion, which may collectively contribute to enhanced tumorigenicity (16). Though not specifically studied, our data supports the notion that increased cellular activity would require increased DNA replication, likely achieved through overexpressed/activated MET enhancing Top1 activity. In fact, activation of the HGF/MET axis induced MET and Top1 nuclear co-localization. Moreover, HGF stimulation increased Top1 activity, which was abrogated by the addition of SU11274. This suggests that inhibiting MET activity likely downregulates Top1 activity, either directly or indirectly.

*MET* gene amplification has been more commonly associated with metastatic disease (23). While we detected increased *MET* gene copy number in primary tumor samples in this study, we cannot rule out similar increases in metastatic tissues. However, we found an association between increased *MET* gene copy number and advanced stage disease. It is likely that increased *MET* gene copy number and activation of MET in SCLC may lead to enhanced Top1 activity and disease progression. Specifically inhibiting the HGF/MET pathway through the use of targeted molecular therapies holds significant promise, particularly for patients with *MET* overexpression or activating mutations.

SU11274 was utilized for this study given its higher selectively (50-fold) for MET compared to other tyrosine kinases such as EGFR, PDGFR, and FGFR-1 (36). Through such targeted inhibition, SU11274 has been shown to abrogate HGF induced tyrosine phosphorylation of the MET autophosphorylation sites at amino acid residues Y1230/1234/1235, as well as downstream phosphorylation of AKT and ERK 1/2, in a dose dependent manner in SCLC (22). However, work with this compound stimulated the development of other compounds based on an indolinone core, such as PF2341066 (Crizotinib, Pfizer). It is to be noted, crizotinib has both anti-MET activity and anti-EML4-ALK activity in lung cancer. Other small molecule inhibitors such as ARQ197 and MET mAb (onartuzumab) are also in clinical testing (22). The proliferation of anti-MET therapeutics for the treatment of lung cancer, and their success in the treatment of SCLC, validates MET as a key player in SCLC tumorigenesis.

To date Topoisomerase inhibition is one of the cytotoxic therapies for SCLC. As shown initially by the Japanese Cooperative Oncology Group (29) and subsequently by Hermes and colleagues (27), the Top1 inhibitor irinotecan in combination with a platinum-based agent improved median survival and the one-year survival rate, without causing additional toxicity in patients...
with extensive SCLC. In the Hermes phase III study, 220 patients were randomized to receive irinotecan plus carboplatin (IC) or etoposide plus carboplatin (EC). The median survival was 8.5 months in the IC arm compared with 7.1 months in the EC arm. Although the study size was small, this study suggested that Top1 inhibition was more effective than previously considered treatments of metastatic SCLC.

It is appreciated from the imatinib studies in SCLC that a single novel therapy may not be sufficient to eradicate SCLC. Keeping this view in mind, we utilized SN-38, which is the active metabolite of irinotecan in this study to determine whether there was synergism with SU11274. Our results showed that SU11274 does synergize with SN-38 to inhibit the growth of SCLC cells in vitro. This appears to be a potential unique pathway that can be targeted in SCLC expressing MET. We observed synergism between SU11274 and SN-38 in two SCLC cell lines, however, the extent of synergism varied between H69 cells to H82 cells. The drugs synergized at much lower doses in H69 cells compared to H82 cells, with H82 cells having a strong trend toward antagonism at higher doses. The postulated reason for this difference is that the H69 cell line harbors a mutation, R988C in the exon encoding the JM domain of MET, while H82 expresses wild-type MET. The fact the R988C mutations makes MET constitutively active renders cells expressing this mutation much more susceptible to targeted MET inhibition, and by extension co-targeted therapies.

In summary, the studies described here have established the in vitro effects of MET inhibition, on HGF-induced signal transduction, as well as demonstrated the benefits of combined therapy with the clinically efficacious Top1 inhibitor. This is a novel treatment modality since it not only targets the growth factor addiction that is characteristic of many tumors via the inhibition of the HGF/MET axis, it also targets a putative downstream effector molecule, Top1. These results also highlight the impact of mutations on the efficacy of molecularly-targeted drugs, as well as emphasize the potential synergistic effects of combining therapeutics to treat SCLC. It would now be useful to investigate this phenomenon in other cancers such as NSCLC, breast carcinoma, head and neck malignancies, in which MET has been shown to play an integral role in tumorigenesis. Furthermore, it would also be beneficial to study other MET mutations in SCLC, which may influence the efficacy of this and other drug combinations.
REFERENCES


Table 1. Correlation of gene copy number with protein expression.

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FIGURE LEGENDS

Figure 1. MET and TOP1 gene copy number in patient samples. (A) Gene copy numbers for MET and TOP1 were determined by real-time qPCR using genomic DNA isolated from 29 SCLC patient tumor samples. (B) Patient samples were grouped in limited (n=11) versus extensive (n=18) disease for comparison of gene copy number.

Figure 2. MET and Top1 protein expression based on disease stage. Patient samples were subjected to IHC staining for the detection of MET, p-MET, and Top1 expression. (A) Representative images of IHC staining in limited disease versus extensive disease. (B) Summary IHC scores (mean ± SD) based on disease stage for limited (n=11) versus extensive (n=18). (C) IHC scores for nuclear Top1 were correlated with MET gene copy number. There was a significant positive correlation between MET gene copy number and nuclear Top1 expression (r=0.5).

Figure 3. Gene copy number and protein expression of MET and Top1 in SCLC cell lines. (A) Genomic DNA was isolated from 20 SCLC cell lines for the determination of MET and TOP1 gene copy numbers by real-time qPCR. (B) Whole cell lysates were immunoblotted with anti-MET and anti-Top1 specific antibodies. β-actin served as the loading control. (C) Quantification of MET expression by SRM. Data shown is the mean±SD of three independent mass spectrophotometry runs.

Figure 4. HGF/MET activation modulates Top1 activity in SCLC. (A) H82 cells were stimulated with HGF (40 ng/ml) for 10 mins, then fixed and permeabilized for immunofluorescence analysis to determine the cellular localization of p-MET and Top1. The nucleus was counterstained with DAPI (blue), p-MET is shown in green, and Top1 is shown in red. (B) H82 cells were cultured overnight in serum-free medium in the absence or presence of 5µM SU11274, and then stimulated for 15 mins with 50 ng/ml HGF. Nuclei were harvested and Top1 activity was assayed in the neat nuclear lysates and across a range of 1:4 serial dilutions. Reaction mixture plus purified recombinant human Top1 served as the positive (+) control and reaction mixture plus Top1 assay dilution buffer served as the negative (-) control. The reaction mixtures were resolved on a 1% agarose with EtBr and then imaged under a UV lamp. Relaxed DNA, R and supercoiled DNA, S.

Figure 5. SU11274 and SN-38 combined to decrease the cell viability of SCLC in vitro. (A) H69 and (B) H82 cells were cultured for 72 h in media, SU11274 alone, SN-38 alone, or SU11274 and SN-38 in combination. Cell viability was determined using the Alamar blue assay. Data shown are the mean ± SEM of 5 independent experiments.
Figure 1

(A) Gene copy number of MET and TOP1 in patient samples (n=29).

(B) Comparison of gene copy number between limited (n=11) and extensive (n=18) cases. *p=0.015.
Figure 2
Figure 5.
Molecular Cancer Therapeutics

Combination MET inhibition and Topoisomerase I inhibition block cell growth of Small Cell Lung Cancer

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