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A Novel Small Molecule Met Inhibitor Induces Apoptosis in Cells Transformed by the Oncogenic TPR-MET Tyrosine Kinase[1]

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ABSTRACT

The Met receptor tyrosine kinase has been shown to be overexpressed or mutated in a variety of solid tumors and has, therefore, been identified as a good candidate for molecularly targeted therapy. Activation of the Met tyrosine kinase by the *TPR* gene was originally described *in vitro* through carcinogen-induced rearrangement. The TPR-MET fusion protein contains constitutively elevated Met tyrosine kinase activity and constitutes an ideal model to study the transforming activity of the Met kinase. We found, when introduced into an interleukin 3-dependent cell line, TPR-MET induces factor independence and constitutive tyrosine phosphorylation of several cellular proteins. One major tyrosine phosphorylated protein was identified as the TPR-MET oncoprotein itself. Inhibition of the Met kinase activity by the novel small molecule drug SU11274 [(3Z)-*N*-(3-chlorophenyl)-3-({3,5-dimethyl-4-[(4-methylpiperazin-1-yl)carbonyl]-1H-pyrrol-2-yl}methylene)-*N*-methyl-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide] led to time- and dose-dependent reduced cell growth. The inhibitor did not affect other tyrosine kinase oncoproteins, including BCR-ABL, TEL-JAK2, TEL-PDGF*β*R, or TEL-ABL. The Met inhibitor induced G_1 cell cycle arrest and apoptosis with increased Annexin V staining and caspase 3 activity. The autophosphorylation of the Met kinase was reduced on sites that have been shown previously to be important for activation of pathways involved in cell growth and survival, especially the phosphatidylinositol-3′-kinase and the Ras pathway. In particular, we found that the inhibitor blocked phosphorylation of AKT, GSK-3*β*, and the pro-apoptotic transcription factor FKHR. The characterization of SU11274 as an effective inhibitor of Met tyrosine kinase activity illustrates the potential of targeting for Met therapeutic use in cancers associated with activated forms of this kinase.

INTRODUCTION

The *TPR-MET* oncogene was originally identified as the transforming counterpart of the Met receptor tyrosine kinase after treatment of a human osteogenic sarcoma cell line with the chemical carcinogen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (1–3). *TPR-MET* is generated by a chromosomal translocation, placing the *TPR*[3] locus on chromosome 1 upstream of a portion of the *Met* gene on chromosome 7 encoding for a cytoplasmic region (2, 3). TPR-MET is not only detected in experimental cancer, but it may potentially also be involved in human cancer, such as gastric carcinomas (4, 5). Dimerization of the M_r 65,000 TPR-MET oncoprotein through a leucine zipper motif encoded by *TPR* leads to constitutive activation of the Met kinase, which is crucial for transformation (6, 7). In many aspects, TPR-MET behaves like the activated Met receptor tyrosine kinase and can activate crucial growth pathways, including the Ras pathway (8–11) and the PI3K pathway (12, 13). However, there are also distinct pathways that may not be used directly, *e.g.*, TPR-MET lacks the CBL binding site present in Met and is mainly cytoplasmic in contrast to the membrane-bound receptor tyrosine kinase (1–3, 14, 15).

Met serves as the high-affinity receptor for HGF. HGF was identified as a growth factor for hepatocytes and as a fibroblast-derived cell motility factor, or scatter factor. HGF is a disulfide-linked heterodimeric molecule produced predominantly by mesenchymal cells and acting in an endocrine or paracrine fashion. Ligation of the Met receptor by HGF has been shown to regulate cell growth and motility, as well as embryological development, wound healing, tissue regeneration, angiogenesis, growth, invasion, and morphogenic differentiation. Therefore, deregulation of these processes by activated forms of Met in human cancer is of special interest. Met mutations have been described in hereditary and sporadic human papillary renal carcinomas and have been reported in ovarian cancer, childhood hepatocellular carcinoma, metastatic head and neck squamous cell carcinomas, and gastric cancer. There is also overexpression of Met in both non-small cell lung cancer and small cell lung cancer cells (for review, see Ref. 16).

Because Met and transforming forms of Met are thought to play an important role in oncogenesis of a variety of tumors (16), we sought to target the Met tyrosine kinase for drug development. c-Met would be an attractive therapeutic target for inhibition in diseases with mutated Met or overexpression of Met, such as small cell lung cancer. However, unlike Gleevec for CML (targeting BCR-ABL) and gastrointestinal stromal tumor (targeting c-Kit), no targeted small molecule inhibitors against c-Met have been developed yet. Nonetheless, Morotti *et al.* (17) have recently reported inhibitory activities of K252a, a member of a group of natural alkaloids, against the oncogenic properties of c-Met at a concentration 100 nM or less. K252a acts as a kinase inhibitor by competing with the binding of ATP to the catalytic domain and was originally described as a serine/threonine kinase inhibitor, which later was also found to be a potent inhibitor of Trk family members and a partial inhibitor of PDGF receptor. Interestingly, K252a exhibited more potent inhibition for the strongly activating M1268T-Met mutation, than for wild-type Met. However, it remains an open question whether broad-spectrum or highly specific kinase inhibitors would exhibit more effective therapeutic value with reasonable safety profiles.

Using the *TPR-MET* oncogene with constitutively activated Met tyrosine kinase activity, we developed a cell line model system suitable to identify small molecule drugs as inhibitors of Met kinase activity. We have developed a small molecule drug specifically inhibiting the Met tyrosine kinase *in vitro* and in cell lines. Inhibition of the Met kinase activity by the novel small molecule drug SU11274 [(3Z)-*N*-(3-chlorophenyl)-3-({3,5-dimethyl-4-[(4-methylpiperazin-1-yl)carbonyl]-1H-pyrrol-2-yl}methylene)-*N*-methyl-2-oxo-2,3-dihy-

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[3] The abbreviations used are: TPR, translocated promoter region; HGF, hepatocyte growth factor; CML, chronic myelogenous leukemia; EGFR, epidermal growth factor receptor; PDGF, platelet-derived growth factor; FGFR, fibroblast growth factor receptor; JAK, Janus-activated kinase; PI3K, phosphatidylinositol-3′-kinase.

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dro-1H-indole-5-sulfonamide] led to time- and dose-dependent reduced cell growth and did not inhibit BCR-ABL, TEL-JAK2, TEL-PDGFβR, or TEL-ABL tyrosine kinases. SU11274 inhibition of TPR-MET kinase activity induced G_1 cell cycle arrest and apoptosis with increased Annexin V staining and caspase 3 activity. Also, SU11274 inhibited phosphorylation of key regulators of the PI3K pathway, including AKT, FKHR, or GSK3β. Lung cancer cell lines overexpressing c-Met, H69 and H345, also had growth inhibition with SU11274, with biochemical evidence of inhibition of phosphorylated c-Met. The characterization of SU11274 as an effective inhibitor of Met tyrosine kinase activity illustrates the potential of targeting Met in cancers associated with activated forms of the Met receptor tyrosine kinase.

MATERIALS AND METHODS

Cells. The murine pre-B cell line BaF3 was grown in RPMI 1640 containing 10% FCS and 10% WEHI-conditioned medium as a source of murine interleukin 3. BaF3 cell lines transfected with a BCR-ABL, TEL-ABL, TEL-JAK2, or TEL-PDGFβR cDNA were grown in the absence of growth factors. A TPR-MET-expressing BaF3 cell line was generated by transfection of an expression vector containing the TPR-MET cDNA (kindly provided by Dr. George F. Vande Woude, Van Andel Research Institute, Grand Rapids, MI) and subsequent selection in G418 (Mediatech, Herndon, VA) and outgrowth of the cell population in the presence of interleukin 3. The number of viable cells after treatment with DMSO or SU11274 was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (In Vitro Toxicology Assay Kit; Sigma Chemical Co., St. Louis, MO) or trypan blue exclusion.

Drugs. SU11274 (SUGEN, Inc.) and Gleevec (STI-571, imatinib mesylate; Novartis Pharmaceuticals) were dissolved in DMSO and used at the concentrations described.

***In Vitro* Kinase Assays.** The IC_{50} (50% inhibitory concentration) values of SU11274 for the inhibition of various kinases was determined as described previously (18). Human c-Src was a full-length purified recombinant protein, and Met, FGFR1, EGFR, Flk-1, and Tie-2 were generated as glutathione *S*-transferase fusion proteins. IC_{50} measurements of PDGFβR autophosphorylation were determined on immunoprecipitated PDGFβR. The phosphorylation of histone in the presence of ^{33}P-ATP was used for IC_{50} determination of cyclin-dependent kinase 2 activity. IC_{50} measurements with all other kinases were made using poly-Glu-Tyr (4:1) as a peptide substrate. The divalent cation in the reaction was 20 mM $MgCl_2$ (Src) or 10 mM $MnCl_2$ (EGFR, Met, FGFR1, Flk-1, Tie2). The linear range (*i.e.*, the time period over which the rate remained equivalent to the initial rate) was determined for each kinase. All kinetic measurements and IC_{50} determinations were performed within this range. K_m values were calculated using the Eadie-Hofstee method, and the final ATP concentrations were within two to three times the K_m value.

Transwell Migration Assay. The lower chamber of a transwell plate (8-μm pore size polycarbonate membrane; Corning Costar Corp., Cambridge, MA) was filled with 600 μl starvation media [0.5% (w/v) BSA in RPMI 1640]. Cells were counted using a Coulter particle counter (Coulter Counter Z2; Beckman Coulter, Fullerton, CA) and resuspended at 2×10^6 cells/ml in starvation media. One hundred microliters of this cell suspension was transferred to the upper chamber. The medium contained either SU11274 (3 μM) or DMSO in the control samples. After 4 h, cells in the lower compartment were resuspended and counted using a Coulter particle counter. The spontaneous transwell migration of cells was expressed as a "migration index" (number of migrating cells treated with SU11274 divided by the number of migrating cells left untreated). The SE was calculated from the migration indices of independently performed experiments. The statistical significance of the data was analyzed using the Student's *t* test.

Immunoblotting. Proteins were extracted from whole cells by lysing them in a Tris buffer (50 mM, pH 8.0) containing NaCl (150 mM), NP40 (1% v/v), deoxycholic acid (0.5% w/v), SDS (0.1% w/v), NaF (1 mM), Na_3VO_4 (1 mM), and glycerol (10% v/v; Sigma Chemical Co.) supplemented with a protease inhibitor mixture (complete; Roche, Indianapolis, IN). Polyclonal rabbit antibodies against tyrosine phosphorylated Met (Biosource International, Camarillo, CA), PI3K (Upstate Biotechnology, Lake Placid, NY), or tyrosine phosphorylated GSK3β, FKHR, or AKT (Cell Signaling, Beverly, MA) and mouse monoclonal antibodies against Abl (Oncogene, San Diego, CA) or phospho-tyrosine (Upstate Biotechnology) were used for immunoblotting or immunoprecipitation.

Apoptosis Assays. The activity of caspase-3 was measured in cell lysates (CaspACE Assay System; Promega), and Annexin V-positive staining was determined by fluorescence-activated cell sorter analysis (Annexin-V-Fluos Staining Kit; Roche Diagnostics), according to the manufacturer's directions, in cells that were either treated with SU11274 or the solvent DMSO.

Cell Cycle Analysis. Fixed cells were stained with propidium iodide, and cell cycle parameters were analyzed by fluorescence-activated cell sorter analysis.

RESULTS

SU11274 Is a Small Molecule Drug That Selectively Inhibits Met Tyrosine Kinase Activity. SU11274 was identified as a prototype ATP-competitive small molecule inhibitor of the catalytic activity of Met (Fig. 1, *top*). SU11274 exhibited activity against Met enzyme *in vitro* with an average IC_{50} of 0.02 μM. In addition, SU11274 exhibited greater than 50-fold selectivity for Met enzyme *versus* a panel of other tyrosine kinases (Table 1). For example, at the IC_{50} of SU11274 for Met inhibition, there was no inhibition of PDGFβR kinase activity and only about 2% inhibition of FGFR-1 kinase activity (Fig. 1, *bottom*). The IC_{50} of SU11274 for PDGFβR inhibition was more than 1000-fold higher, and that for FGFR-1 inhibition about 500 times higher than the IC_{50} of SU11274 for inhibition of Met. The cellular activity and good selectivity against other kinases suggests that SU11274 should have use as a prototype inhibitor for the investigation of Met biology.

The Small Molecule Drug SU11274 Specifically Regulates Cell Growth in TPR-MET-transformed BaF3 Cells. We initially sought to determine whether SU11274 could inhibit cell growth in TPR-MET-transformed BaF3 cells (Fig. 2*A*). Treatment of BaF3.TPR-MET cells with SU11274 was found to inhibit cell growth in a dose-dependent manner with an IC_{50} <3 μM. In the presence of

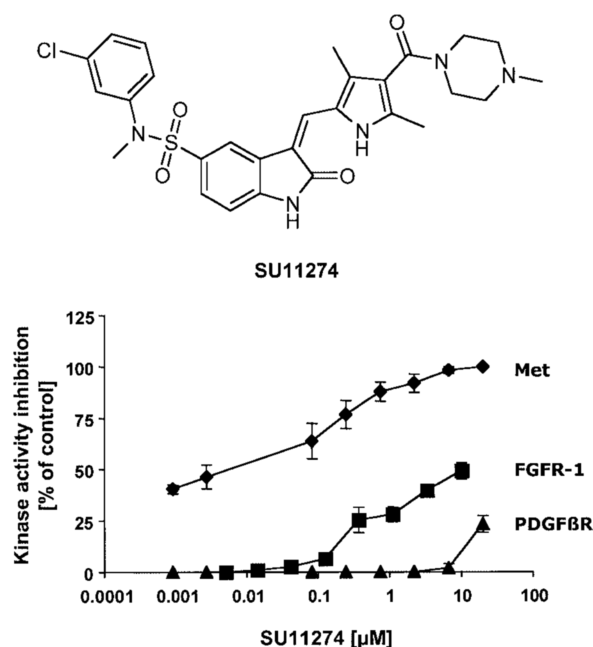

Fig. 1. The Met kinase inhibitor SU11274. Chemical structure of the Met kinase inhibitor SU11274 (*top*). The IC_{50} values of SU11274 for the inhibition of Met, FGFR-1, and PDGFβR kinases were determined at the indicated concentrations in the presence of ^{33}P-ATP using poly-Glu-Tyr (4:1) as a peptide substrate (*bottom*).

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Table 1 *IC_{50} values for SU11274 against isolated kinases*

Tyrosine kinase	IC_{50} [μM]
Met	0.02
Flk	1.3
EGFR	>100
PDGFβR	>20
Tie2	>100
c-src	>10
cdk2	>10
FGFR-1	9.7

interleukin 3, the kinase inhibitor also inhibited cell growth at concentrations larger than 1 μM. Inhibition of cell growth at higher concentrations was not a result of general toxicity of the drug, because there was no growth inhibition in BCR-ABL-transformed BaF3 cells (Fig. 2A). This difference is likely because of the regulation of a pathway that is required for interleukin 3 signaling by the TPR-MET kinase. SU11274 (1 μM, 18 h) also did not inhibit cell growth of BaF3 cells transformed by other oncogenic tyrosine kinases, including BCR-ABL, TEL-JAK2, TEL-ABL, and TEL-PDGFβR (Fig. 2B). To further determine whether the growth inhibitory effect of SU11274 on BaF3.TPR-MET cells accumulates over time, the cell growth was determined over a 3-day culture. In the presence of interleukin 3, SU11274 did not reduce cell growth of TPR-MET-transformed cells or BCR-ABL-transformed cells in a control experiment (Fig. 2*C*, *top*). In contrast, SU11274 completely blocked cell growth in the absence of interleukin 3 in BaF3.TPR-MET ($P < 0.0001$) but not in BaF3.BCR-ABL (Fig. 2*C*, *bottom*). This suggests that interleukin 3 completely rescues the BaF3.TPR-MET cells from SU11274-depend-

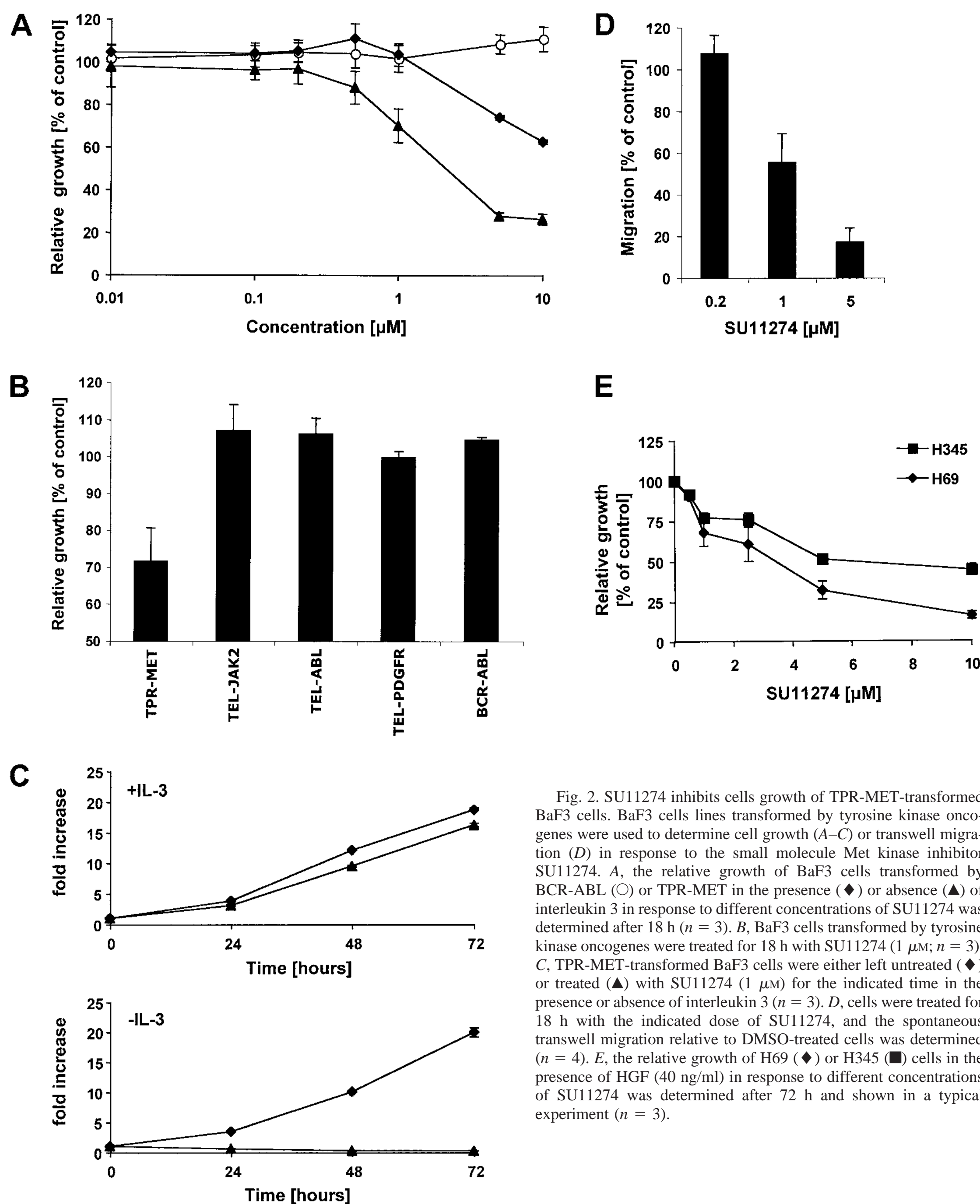

Fig. 2. SU11274 inhibits cells growth of TPR-MET-transformed BaF3 cells. BaF3 cells lines transformed by tyrosine kinase oncogenes were used to determine cell growth (*A–C*) or transwell migration (*D*) in response to the small molecule Met kinase inhibitor SU11274. *A*, the relative growth of BaF3 cells transformed by BCR-ABL (○) or TPR-MET in the presence (♦) or absence (▲) of interleukin 3 in response to different concentrations of SU11274 was determined after 18 h ($n = 3$). *B*, BaF3 cells transformed by tyrosine kinase oncogenes were treated for 18 h with SU11274 (1 μM; $n = 3$). *C*, TPR-MET-transformed BaF3 cells were either left untreated (♦) or treated (▲) with SU11274 (1 μM) for the indicated time in the presence or absence of interleukin 3 ($n = 3$). *D*, cells were treated for 18 h with the indicated dose of SU11274, and the spontaneous transwell migration relative to DMSO-treated cells was determined ($n = 4$). *E*, the relative growth of H69 (♦) or H345 (■) cells in the presence of HGF (40 ng/ml) in response to different concentrations of SU11274 was determined after 72 h and shown in a typical experiment ($n = 3$).

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ent growth inhibition. We did not observe a significant growth inhibitory effect of SU11274 at 1 μM in interleukin 3-stimulated parental BaF3 cells in a 3-day culture (data not shown). TPR-MET is, therefore, implicated in the deregulation of pathways normally used by the activated interleukin 3 receptor, similarly to the relation between the Abl inhibitor STI-571 and the BCR-ABL oncoprotein.

Untransformed BaF3 cells do not migrate through a transwell membrane. However, when transformed by TPR-MET, the cells display spontaneous transwell migration. In addition to cell growth, SU11274 was also found to inhibit this aspect of transformation in a dose-dependent manner (Fig. 2*D*). Migration of BaF3.TPR-MET cells was inhibited with 1 μM SU11274 (44.8 $\pm$ 13.8% inhibition of the cell migration; $P < 0.01$) and with 5 μM SU11274 (about 80% inhibition of the cell migration; $P < 0.0001$), compared with DMSO-treated cells. This demonstrates that the TPR-MET kinase activity regulates cell growth and migration of BaF3 cells.

In cellular assays, SU11274 demonstrated inhibition of HGF-dependent phosphorylation of Met as well as HGF-dependent cell proliferation and motility with a mean IC_{50} of 1–1.5 μM (data not shown). Cytotoxicity was not observed in non-Met-expressing cells at concentrations up to 25 μM. In addition to TPR-MET-dependent cell growth, we also determined the effect of SU11274 on proliferation in the H69 and H345 lung cancer cell lines. H69 and H345 cells express the functional Met receptor but do not require exogenous HGF for cell growth and survival. Cell growth in the presence of HGF (40 ng/ml) was found to be inhibited in a dose-dependent manner (Fig. 2*E*). The IC_{50} values of SU11274 for the inhibition of cell growth in H69 cells (3.4 μM) and H345 cells (6.5 μM) were comparable with the IC_{50} in BaF3.TPR-MET cells.

SU11274 Inhibits Tyrosine Phosphorylation of Cellular Proteins in TPR-MET-transformed BaF3 Cells. To determine the biochemical consequences of Met kinase inhibition by SU11274 in BaF3 cells, changes in tyrosine phosphorylation of cellular proteins were evaluated. In whole cell lysates of BaF3.TPR-MET cells, compared with BaF3.BCR-ABL, tyrosine phosphorylation of a set of unique and overlapping tyrosine phoshorylated proteins were observed, with prominent tyrosine phosphorylation of either oncogenic tyrosine kinase (Fig. 3*A*, *right*). In particular, the M_r 65,000 TPR-MET kinase induced significant tyrosine phosphorylation of a M_r 140,000–145,000 protein band that appeared only weakly tyrosine phosphorylated in BCR-ABL-transformed cells. Similarly, TPR-MET was found to be tyrosine phosphorylated itself in Met immunoprecipitations and coprecipitated with a prominent M_r 140,000–145,000 band. There were additional prominently tyrosine phosphorylated proteins with apparent molecular masses of M_r 50,000–60,000 and larger than M_r 300,000 found in a complex with TPR-MET (Fig. 3*A*, *left*). Comparing TPR-MET and BCR-ABL immunoprecipitations also indicated that both oncogenes formed different signaling complex with tyrosine phosphorylated proteins in BaF3 cells. Treatment of BaF3.TPR-MET cells with SU11274 reduced tyrosine phosphorylation within 2 h but was not maximal until 18 h of treatment (Fig. 3*B*). In addition, the Abl kinase inhibitor STI-571 did not alter tyrosine phosphorylation of cellular proteins in BaF3.TPR-MET cells (Fig. 3*C*). These data suggest that SU11274 specifically inhibits TPR-MET-induced tyrosine phosphorylation relative to BCR-ABL.

Using phospho-specific antibodies against tyrosine phosphorylation sites in Met, we found that SU11274 inhibits autophosphorylation at Tyr361/365/366 (autophosphorylation site), Tyr480 (Grb2 binding site), and Tyr496 (important in cell morphogenesis; Fig. 4*A*, *top*). To determine whether SU11274 also inhibits the activation of the c-Met receptor, we used HGF-stimulated and -unstimulated H69 cells. After ligand activation, the Met receptor becomes inducibly phosphorylated at multiple sites, including the major autophosphorylation sites Tyr1230/1234/1235 (homologous to Tyr361/365/366 in TPR-MET). Phosphorylation at these sites correlates with activation of the Met kinase. Using phospho-specific antibodies to Tyr1230/1234/1235, we found that SU11274 completely blocked the increase in phosphorylation (Fig. 4*A*, *bottom*). In addition, there was no detectable tyrosine phosphorylation of Met in anti-Met immunoprecipitations of HGF-stimulated H69 cells after SU11274 treatment (data not shown). This is consistent with the above data demonstrating an inhibition of HGF-induced proliferation in H69 cells at micromolar concentrations.

It has previously been suggested that these tyrosine phosphorylation sites in TPR-MET have similar functions as in Met and are crucial for the transforming activity of TPR-MET. A crucial pathway regulated by TPR-MET includes activation of PI3K. Therefore, we sought to determine whether inhibition of TPR-MET would reduce the phosphorylation and alter the activation status of downstream signaling targets in the PI3K pathway. We found that the dose-dependent reduction in tyrosine phosphorylation of cellular proteins after SU11274 treatment correlated with reduced serine phosphorylation of AKT (Ser308, Ser473) as well as its targets GSK-3β (Ser21/9) and the proapoptotic transcription factor FKHR (Ser256; Fig. 4*B*). This would suggest that inhibition of Met kinase activity leads to reduced activation of the PI3K pathway in these cells.

Inhibition of Met Kinase Activity by SU11274 Induces Apoptosis and Cell Cycle Arrest in TPR-MET-transformed BaF3 Cells. Apoptosis is a complex cellular function that is regulated in part through the PI3K pathway. Down-regulation of PI3K activity through inhibition of Met tyrosine kinase activity in TPR-MET-transformed cells is, therefore, expected to induce an increase in apoptosis. We measured the change in Annexin V-positive staining of

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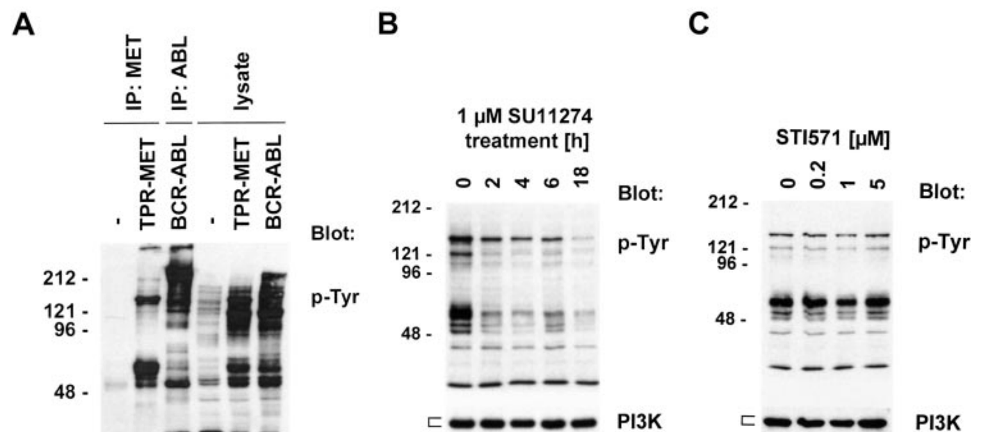

Fig. 3. SU11274 inhibits tyrosine phosphorylation of cellular proteins by TPR-MET. Tyrosine phosphorylation of cellular proteins was determined by immunoblotting in Met and Abl immunoprecipitations (*A*) as well as in whole cell lysate (*A–C*). BaF3 cells (-) as well as TPR-MET-transformed (*A* and *B*) and BCR-ABL-transformed (*A* and *C*) BaF3 cell were either left untreated (*A*) or treated with the indicated amount of SU11274 (*B*) or STI-571 (*C*). Blots were stripped and reprobed for equal loading with antibodies against p85 PI3K.

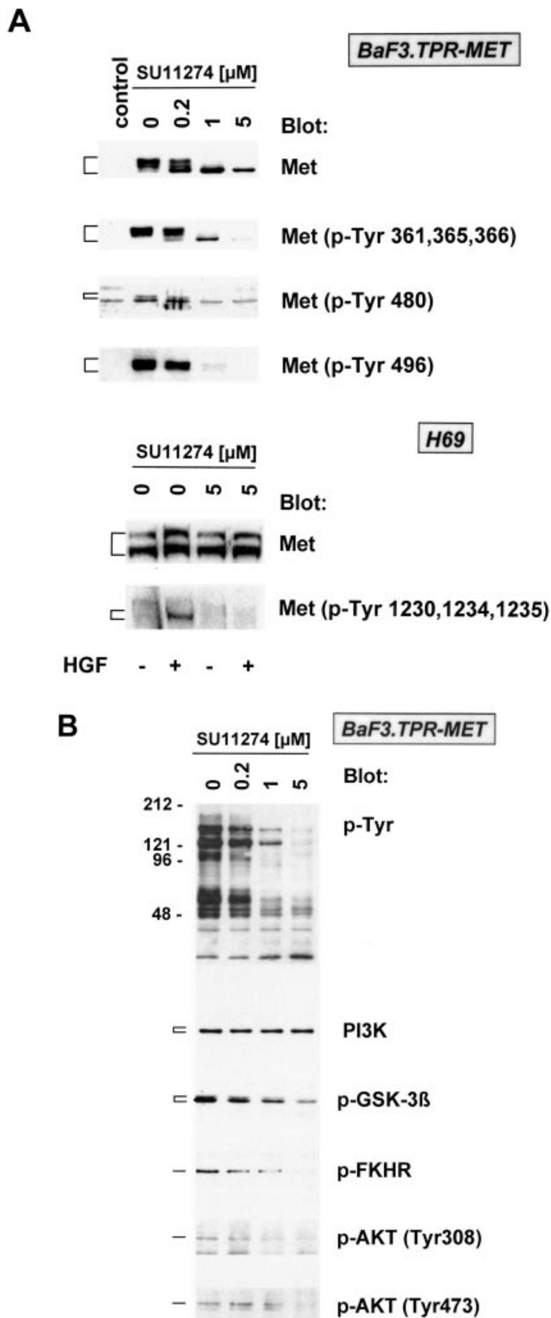

Fig. 4. SU11274 inhibits tyrosine phosphorylation of regulatory phospho-tyrosines in TPR-MET and the PI3K pathway. Tyrosine phosphorylation of cellular proteins using lysates from untransformed BaF3 cells (control), TPR-MET-transformed BaF3 cells, or H69 cells treated with the indicated amounts of SU11274 (*A* and *B*) were used to determine site-specific tyrosine phosphorylation. *A*, TPR-MET (*MET*) expression or phosphorylation of specific tyrosine residues in TPR-MET (*top*) or Met (*bottom*) was detected as indicated. H69 cells were either left untreated or treated with 40 ng/ml HGF. *B*, p85 PI3K expression, phosphorylation of protein tyrosine phosphorylation (*p-Tyr*), or phosphorylation on specific tyrosine residues in GSK-3β, AKT, and FKHR was detected as indicated.

cells, an indication for increased exposure of phosphatidylserine to the outer cell membrane during apoptosis. Using TPR-MET-transformed BaF3 cells, we found that treatment with SU11274 (18 h, 1 μM) led to an increase in Annexin V-positive cells compared with DMSO-treated cells (Fig. 5*A*, *top left*). In the control cells, 5% of the total population showed signs of apoptosis, however, the number of apoptotic cells increased to 24% after SU11274 treatment. On average, 9.0 ± 2.5% of the cells were in early apoptosis (Annexin V positive), and 14.8 ± 4.9% of the cells were in late apoptosis (Annexin V plus propidium iodide positive). We next measured the activation status of caspase-3, a downstream effector of the proapoptotic caspase-9. Similar to the previous data, we observed a consistent increase in caspase-3 activity (2.5 ± 0.6-fold increase; $n = 3$; $P < 0.01$) compared with DMSO-treated cells (Fig. 5*B*).

We also determined whether inhibition of the TPR-MET tyrosine kinase would induce cell cycle arrest. Cells were treated with DMSO or different amounts of the Met kinase inhibitor, and the different phases of cell cycle distribution were determined (Fig. 5*C*). The percentage of cells in G_1 phase increased from 42.4% to 70.6% in SU11274 (5 μM)-treated cells, whereas the percentage of cells in S phase (reduced from 45.4% to 21.1%) and G_2-M phase (reduced from 12.2% to 8.3%) decreased. This suggests that inhibition of TPR-MET kinase activity leads to G_1 cell cycle arrest in the transformed cells. In addition, there was an increase of cells in sub-G_1 phase, which was consistent with apoptotic cells. These data demonstrate that SU11274 induces cell cycle arrest as well as apoptosis, and both events in combination are likely to contribute to the reduced cell growth of SU11274-treated TPR-MET-transformed cells.

DISCUSSION

The Met receptor tyrosine kinase pathway can be regulated through mutation, overexpression, and stimulation via its ligand HGF. In a considerable number of solid malignancies, Met has been shown to have effects on tumorigenesis and metastases (16). Here, we report the effects of the novel Met inhibitor SU11274 on a model system with TPR-MET, an oncogenic form of Met with constitutively activated tyrosine kinase. In this study, it was shown that there was specific *in vitro* inhibition of Met (IC_{50} = 0.02 μM) as compared with other kinases. We had shown previously that the BaF3 cell line system can serve as an elegant model to study a variety of transforming tyrosine kinase oncogenes. Using TPR-MET stably transfected BaF3 cells, we found that the oncogene induced growth factor independence and an increase in tyrosine phosphorylation of cellular proteins, including TPR-MET itself. This is consistent with previous experiments using the oncogenic BCR-ABL, TEL-ABL, TEL-PDGFβR, or TEL-JAK2 tyrosine kinases in this cell system (19–22). We further showed that growth factor independence was abrogated by SU11274 with an IC_{50} of <3 μM. This could be rescued with interleukin 3, demonstrating that SU11274 did not inhibit growth pathways regulated through the activated interleukin 3 receptor. The specificity of SU11274 was further shown by its inability to inhibit growth mediated by transforming forms of ABL, JAK2, or the PDGFβ receptor.

The Met pathway itself shares homology with semaphorins and semaphorin receptors (plexins), which are also involved in cell scattering (23, 24). There is also evidence that semaphorins may use Met for regulation of cell scattering (25). Here, we show that TPR-MET can lead to enhanced migration of BaF3 cells, and this can be readily inhibited with SU11274. This is likely mediated through downstream inhibition of the PI3K pathway and cytoskeletal proteins. Because HGF/Met signaling has been implicated in processes involving cellular motility and invasive growth, it has been closely associated with integrins. In the MDA-MB-231 carcinoma cell line, HGF/Met signaling sustained cell adhesion on laminin-1, laminin-5, fibronectin, and vitronectin via a PI3K-dependent mechanism (26). This was followed by increased invasiveness through reconstituted basement membranes. HGF stimulation of Met has also been shown to induce phosphorylation of cytoskeletal proteins paxillin, p125FAK, and Pyk2, and potential activation of cdc42 and Rac, thus forming filopodia and lamellopodia (26). With the increased migration of cells, activated Met can then ultimately lead to enhanced metastasis.

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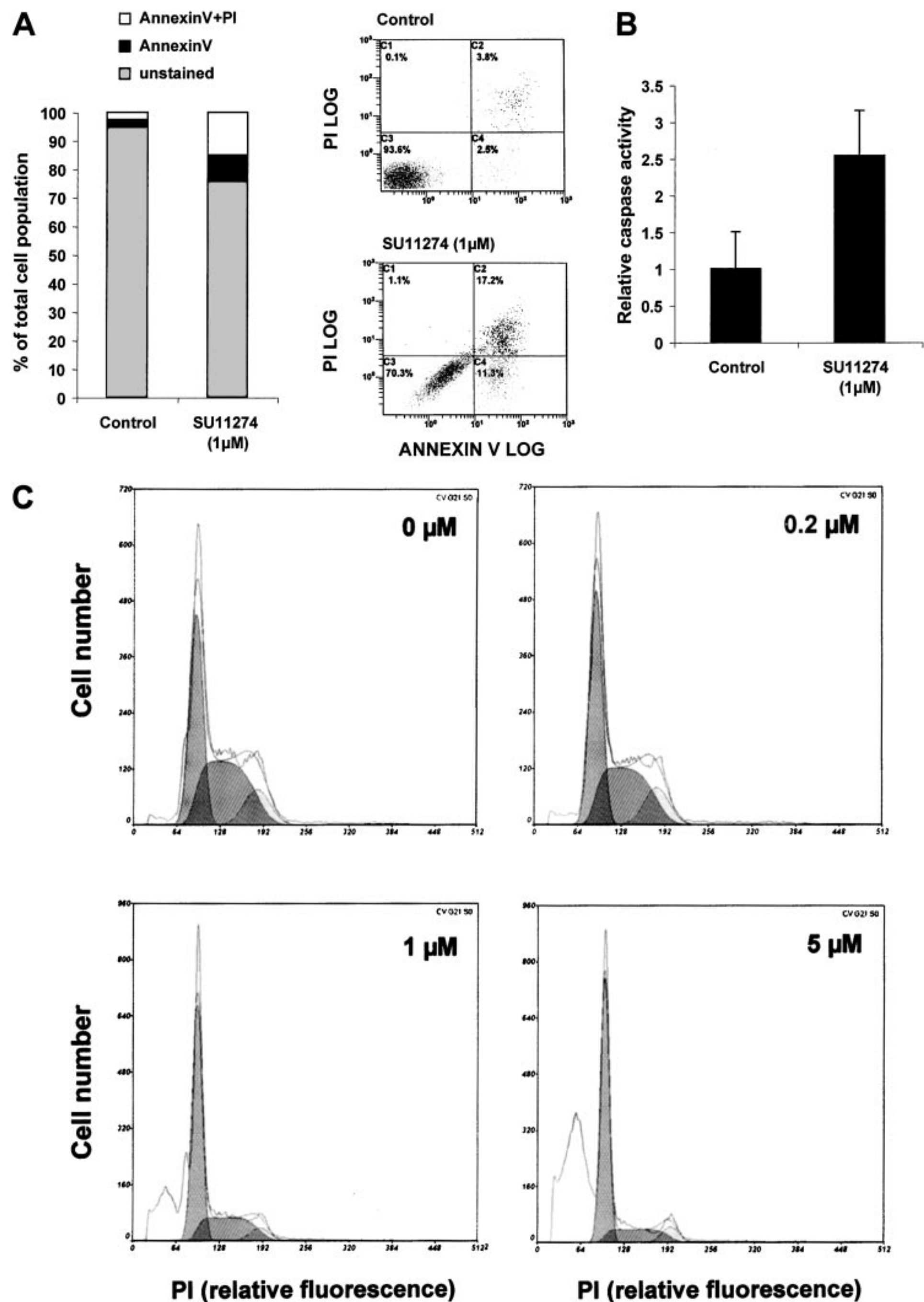

Met inhibitor SU11274 [μM]	% of cells in phase: G1	S	G2/M
0	42.4	45.4	12.2
0.2	46.6	40	13.4
1	64.2	29.8	6
5	70.6	21.1	8.3

Fig. 5. SU11274 induces apoptosis and cell cycle arrest in TPR-MET-transformed BaF3 cells. TPR-MET-transformed BaF3 cells were treated for 18 h with either DMSO or the indicated amount of SU11274 (n = 3). *A*, annexin V and propidium iodide staining was determined by flow cytometry. *B*, activity of caspase-3 was determined in cell lysate (n = 3). *C*, the percentage of cells in different cell cycle phases was determined by flow cytometry after propidium iodide staining (n = 3).

It is, therefore, proposed that inhibition of Met function would block tumor invasion, angiogenesis, growth, and proliferation and multiple other biological effects associated with Met activation. As pointed out before, small molecule inhibitors specific against c-Met have not yet been reported. However, in addition to targeting the c-Met tyrosine kinase, there are several additional pathways that are currently being explored. Bardelli *et al.* (27) have reported using peptides against tyrosines of c-Met located in the kinase activation loop with N-terminal sequence from the Antennapedia protein for internalization, as well as located in the COOH-tail of c-Met. Peptides derived from the c-Met receptor tail, and not from the kinase domain, bind the receptor, inhibit kinase activity, and inhibit HGF-mediated invasive growth on A549 cells by approximately 50% (invasion, cell migration, and branched morphogenesis). HGF also leads to plasmin activation. Webb *et al.* (28) have reported that geldanamycins, anisamycin antibiotics, lead to decreased plasmin activation at femtomolar concentrations. Geldanamycins, at nanomolar concentrations, have been shown to result in down-regulation of c-Met, inhibition of

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HGF-mediated cell motility and invasion in MDCK-2 cells, and phenotypic reversion of Met-transformed NIH3T3 cells. We have recently shown that geldanamycins can modulate the c-Met pathway in small cell lung cancer (29). However, geldanamycins may not be specific only for c-Met. Another inhibitor of the HGF/Met pathway is NK2, a naturally occurring alternatively spliced form of HGF. NK2 possesses the N-domain and the first two kringle domains and can act as either an antagonist or partial agonist, depending on the target cells and culture conditions. NK2 has been reported to inhibit growth but facilitates metastasis of transplanted Met-containing melanoma cells in NK2-HGF bitransgenic mice (30). In addition, another antagonist of HGF, NK4, was reported to be generated by proteolytic digestion of HGF (31). NK4 is a truncated HGF composed of the NH2-terminal hairpin domain and four kringle domains in the α chain of HGF. It retains c-Met receptor binding properties without mediating biological responses. NK4 antagonizes HGF-induced tyrosine phosphorylation of c-Met, resulting in inhibition of HGF-induced motility and invasion of HT115 human colorectal cancer cells, as well as angiogenesis (32). Also, when administered to pancreatic tumor-bearing mice, NK4 inhibited growth, invasion, and disseminating metastasis of pancreatic cancer cells and prolonged the life span of these mice (33). Also, a soluble chimeric form of c-Met was shown to retain full capacity to bind HGF and, therefore, neutralize HGF activity (34). NK4, pro-HGF (uncleavable HGF), and the decoy c-Met receptor have been shown to inhibit mutant c-Met-induced transformation of NIH3T3 cells (35). Attempts at inhibiting c-Met signaling through ribozyme-mediated down-regulation have been reported in breast cancer and glioblastoma with interesting and positive results (36, 37). Examples include decreased tumorigenic growth and reduced induction of HGF-dependent gene expression in glioblastoma cells (36). Interestingly, the reduction in c-Met protein levels leads to a decrease in the activation of survival pathway and, therefore, to increased apoptosis (38, 39). It still remains unknown as to how much c-Met inhibition is required to achieve clinically beneficial antitumor and antimetastatic results. The answer may potentially be different depending on whether the "tumorigenic culprit" lies with c-Met expression or mutations of the receptor tyrosine kinase. Another intriguing area of research, when specific c-Met inhibitors are available, would be to test the drug efficacies against different mutant forms of the Met oncoprotein. It is plausible that some mutations of c-Met may cause resistance, or conversely a greater susceptibility, to the inhibitors. This certainly translates into important clinical decisions regarding therapeutic choices. Obviously, much more work needs to be done in dissecting the potential correlation between c-Met mutations and its specific inhibitors to arrive at better designed anticancer therapies. Efforts in resolving the crystal structure of the c-Met receptor tyrosine kinase would likely be helpful to allow a starting point in rational design of specific c-Met kinase inhibitors in the future.

As done in CML (with activated Abl in BCR-ABL; Ref. 40), gastrointestinal stromal tumor (with activated c-Kit; Ref. 41) and non-small cell lung cancer (with EGFR overexpression; Ref. 42), small molecule inhibitors are rationally designed to the ATP pocket of the tyrosine kinase. Our model of TPR-MET has considerable homologies with the BCR-ABL model. Similar to BCR-ABL, TPR-MET constitutively activates signaling pathways that are thought to be crucial for transformation, such as pathways regulating the function of Ras (43, 44) or PI3K (45). In addition, at least in our cell line model system, TPR-MET, like BCR-ABL, seems to be sufficient for transformation and does not require an apparent second mutation. Thus, designing inhibitors against the tyrosine kinase domain of Met would be very useful, similar to Gleevec in CML with BCR-ABL.

In studying signal transduction pathways downstream of activated Met, we have recently shown that PI3K is an important pathway. PI3K is responsible for cellular regulation, including events such as proliferation, reduced apoptosis, anchorage independence, and intracellular vesicle trafficking/secretion (46, 47). Phosphorylation of AKT (48, 49) and FKHR (50, 51), downstream of PI3K, leads to cell survival, whereas herein we show SU11274 treatment of TPR-MET cells leads to decreased phosphorylation of FKHR and AKT, thereby leading to apoptosis. HGF/Met activation has been shown to protect against cell death in human glioblastoma cells treated with cytotoxic agents. Treating cells with inhibitors of PI3K, such as wortmannin and LY249002, can block this promotion of cell survival by HGF (39). It would now be useful to determine whether there is any synergism between SU11274 and PI3K inhibition.

In summary, these studies have characterized the dramatic effects of a prototype Met inhibitor, SU11274, on Met tyrosine kinase activity and function in a novel model system. Collectively, these results illustrate the potential of targeting Met in cancers associated with activated forms of the Met receptor tyrosine kinase. In the future, it would be useful to extend these results to evaluate Met inhibitors in mouse tumor models in support of potential clinical studies.

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