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HER kinase activation confers resistance to MET tyrosine kinase inhibition in MET oncogene-addicted gastric cancer cells

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Abstract

Tumor cells with genomic amplification of MET display constitutive activation of the MET tyrosine kinase, which renders them highly sensitive to MET inhibition. Several MET inhibitors have recently entered clinical trials; however, as with other molecularly targeted agents, resistance is likely to develop. Therefore, elucidating possible mechanisms of resistance is of clinical interest. We hypothesized that collateral growth factor receptor pathway activation can overcome the effects of MET inhibition in MET-amplified cancer cells by reactivating key survival pathways. Treatment of MET-amplified GTL-16 and MKN-45 gastric cancer cells with the highly selective MET inhibitor PHA-665752 abrogated MEK/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT signaling, resulting in cyclin D1 loss and G_1 arrest. PHA-665752 also inhibited baseline phosphorylation of epidermal growth factor receptor (EGFR) and HER-3, which are transactivated via MET-driven receptor cross-talk in these cells. However, MET-independent HER kinase activation using EGF (which binds to and activates EGFR) or heregulin-β1 (which binds to and activates HER-3) was able to overcome the growth-inhibitory effects of MET inhibition by restimulating MEK/MAPK and/or PI3K/AKT signaling, suggesting a possible escape mechanism. Importantly, dual inhibition of MET and HER kinase signaling using PHA-665752 in combination with the EGFR inhibitor gefitinib or in combination with inhibitors of MEK and AKT prevented the above rescue effects. Our results illustrate that highly targeted MET tyrosine kinase inhibition leaves MET oncogene-''addicted'' cancer cells vulnerable to HER kinase-mediated reactivation of the MEK/MAPK and PI3K/AKT pathways, providing a rationale for combined inhibition of MET and HER kinase signaling in MET-amplified tumors that coexpress EGFR and/or HER-3. [Mol Cancer Ther 2008;7(11):3499–508]

Introduction

The MET proto-oncogene encodes a heterodimeric receptor tyrosine kinase that consists of an extracellular α-chain and a membrane-spanning β-chain (1, 2). Hepatocyte growth factor binds to MET with high affinity and induces receptor dimerization and conformational changes that trigger MET tyrosine kinase activity (3, 4). MET activates several downstream signaling pathways, including the phosphoinositide 3-kinase (PI3K)/AKT, Ras/Raf/MEK/mitogen-activated protein kinase (MAPK), and phospholipase Cγ pathways (3).

In tumors where MET is overexpressed due to genomic amplification, the MET tyrosine kinase becomes constitutively active, rendering the malignant cells highly dependent on MET signaling for proliferation and survival (''MET oncogene addiction''; refs. 5, 6). MET amplification occurs most frequently in gastric cancer (10-15%) but is also found in lung (4%) and esophageal cancers (4%; refs. 7–12). Several small-molecule MET inhibitors have been developed and have shown powerful antitumor efficacy *in vitro* as well as in mouse models, which has led to first clinical trials in humans (13–15).

Recently, two studies were able to show that MET transactivates epidermal growth factor receptor (EGFR) and/or HER-3 via receptor cross-talk in MET-amplified non-small cell lung cancer cells (5, 16). This receptor cross-talk has attracted wide interest because one of the studies was able to identify secondary amplification of MET as a new mechanism of acquired resistance to the EGFR tyrosine kinase inhibitor gefitinib: by exposing EGFR-mutant non-small cell lung cancer cells to increasing doses of gefitinib, the authors found that the occurrence of secondary MET gene amplification and activation led to cross-activation of HER-3 and persistent PI3K/AKT signaling, which was no longer sensitive to gefitinib treatment as in the parental cells (16, 17).

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The above study illustrates that collateral growth factor receptor pathway activation is a mechanism of resistance to receptor tyrosine kinase inhibition. Because MET, EGFR, and HER-3 share common downstream signaling pathways such as MEK/MAPK and PI3K/AKT, we hypothesized that HER kinase activation could similarly overcome the growth-inhibitory effects of a MET kinase inhibitor in MET-amplified cancer cells. To test our hypothesis, we selected gastric cancer cells as a model system, because (*a*) MET amplification is most prevalent in this tumor entity and (*b*) gastric cancers are frequently HER receptor positive, with HER receptor overexpression being associated with an unfavorable prognosis (18–20).

We treated MET-amplified GTL-16 and MKN-45 gastric cancer cells that coexpress EGFR and HER-3 with the highly selective small-molecule MET tyrosine kinase inhibitor PHA-665752 with or without addition of EGF or heregulin-β1 (HRG). We show that PHA-665752 potently inhibits MET, MET-driven receptor cross-talk with EGFR and HER-3, as well as MEK/MAPK and PI3K/AKT signaling, thereby inducing complete growth arrest of the cells. However, MET-independent HER kinase activation using EGF (which binds to and activates EGFR) or HRG (which binds to and activates HER-3) can rescue the cells from the effects of PHA-665752, suggesting a possible mechanism of tumor cell escape. The cellular rescue from MET inhibition is mediated either through reactivation of the MEK/MAPK pathway (EGF) or both MEK/MAPK and PI3K/AKT pathways (HRG). Importantly, inhibition of HER kinase signaling using either gefitinib or combined inhibition of MEK and AKT prevents the above rescue effects. Our data provide a strong rationale for combined inhibition of MET and HER kinase signaling in patients with MET-amplified tumors that coexpress EGFR and/or HER-3.

Materials and Methods

Cell Lines

GTL-16 cells were a gift from Dr. Silvia Giordano (Institute for Cancer Research and Treatment, Torino School of Medicine). MKN-45 cells were obtained from the RIKEN BRC Cell Bank (RIKEN BioResource Center). SNU-16 cells were purchased from the American Type Culture Collection. GTL-16 cells were grown in DMEM, MKN-45 cells were grown in RPMI 1640, and SNU-16 cells were grown in RPMI 1640 + 10 mmol/L HEPES + 2 mmol/L L-glutamine + 1 mmol/L sodium pyruvate + 1.5 g/L L-sodium bicarbonate, and 4.5 g/L L-glucose. All media were supplemented with 10% FCS and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Chemicals

PHA-665752 and PD0325901 were provided by Pfizer Global Research & Development. Human EGF and HRG were purchased from R&D Systems. Gefitinib (ZD-1839; Iressa) was obtained from AstraZeneca Pharmaceuticals. AKT/protein kinase B signaling inhibitor-2 (API-2) was purchased from Calbiochem.

Quantitative PCR for Analysis of MET Genomic Amplification

Primers and probe for MET and the single-copy reference gene RNase P were obtained from Applied Biosystems. Primer and probe sequences for MET were (5′-3′): F-GGAGCCAAAGTCCTTTCATCTGTAA, R-GCAATGGATGATCTGGGAAATAAGAAGAAT, and FAM-CCGGTTCATCAACTTC. Reactions were done in triplicate under standard thermocycling conditions using 10 ng genomic DNA, primers at 900 nmol/L, and probes at 250 nmol/L.

Fluorescence *In situ* Fluorescence Analysis

DNA probes for MET (bacterial artificial chromosome clone RPC11-163C9; Invitrogen Life Technologies) and the centromere of chromosome 7 were directly labeled via nick translation with SpectrumRed (MET) and SpectrumGreen (centromere of chromosome 7) fluorophores, respectively. Metaphase slides were prepared using standard cytogenetic techniques. The slides were denatured in 70% formamide/2× SSC for 5 min at 72°C and dehydrated in 70%, 85%, and 100% ethanol. The slides were then hybridized in 50% formamide, 2× SSC, Cot-1 DNA, and 50 ng of each probe at 37°C in a humid chamber overnight. After washing in 2× SSC/0.3% NP-40 at 72°C for 2 min, the slides were air-dried, counterstained with 0.2 μmol/L 4′,6-diamidino-2-phenylindole, and coverslipped. The signals were visualized with a Nikon Eclipse florescence microscope containing SpectrumRed, SpectrumGreen, and 4′,6-diamidino-2-phenylindole filters (Nikon Instruments). Images were captured using the Metasystem software (Digital Scientific).

Real-time-PCR

TaqMan Gene Expression Assays for MET, EGFR, and 18S rRNA were purchased from Applied Biosystems. Gene expression was measured using the ABI Prism 7900HT Sequence Detection System from Applied Biosystems. Real-time PCR of cDNA specimens was conducted as described previously (21).

Western Blot

After removal of growth medium, tissue culture flasks were placed on ice and washed twice with ice-cold TBS, scraped off the culture flasks, centrifuged, and placed in ice-cold Cell Lysis Buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktails; Pierce). After shaking for 15 min at 4°C, the lysates were centrifuged at 20,000 × *g* for 15 min and stored at -70°C until further use. For Western blotting, equal amounts of protein (50 μg) were boiled in Laemmli buffer for 5 min, resolved by 10% SDS-PAGE (Invitrogen Life Technologies), and electrophoretically transferred onto a polyvinylidene difluoride membrane (Bio-Rad). After blocking nonspecific binding sites with 5% nonfat dry milk in TBS + 0.05% Tween 20, the membrane was incubated with the respective primary antibodies. After three washes with TBS + 0.05% Tween 20, the membrane was incubated for 1 h at room temperature with a horseradish peroxidase-linked secondary antibody followed by several washes with TBS + 0.05% Tween 20.

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The immunocomplexes were visualized using the Enhanced Chemiluminescence Plus detection system (GE Healthcare).

Antibodies

Antibodies against MET (C-12), EGFR (1005), phospho-EGFR (Tyr^{1173}), and cyclin D1 (M-20) as well as secondary bovine anti-goat IgG horseradish peroxidase and secondary goat anti-mouse IgG horseradish peroxidase antibodies were from Santa Cruz Biotechnology. Antibodies against p44/42 MAPK, phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}), AKT, phospho-AKT (Ser^{473}), Gab-1, phospho-Gab-1 (Tyr^{627}), phospho-HER-2 (Tyr^{1221}/Tyr^{1222}), and phospho-HER-3 (Tyr^{1289}) were from Cell Signaling Technology. Anti-phospho-MET (Tyr^{1230}/Tyr^{1234}/Tyr^{1235}) was from Biosource, secondary donkey anti-rabbit horseradish peroxidase antibody was from GE Healthcare, and anti-HER-3 Ab-2 was from Lab Vision.

Cell Proliferation Assays

Cellular proliferation was measured using a commercially available 5-bromo-2-deoxyuridine cell proliferation assay kit (Roche). Briefly, the cells were seeded in triplicate in flat-bottomed 96-well plates at 5,000 per well and allowed to adhere for 48 h. Thereafter, the cells were treated for 24 h as indicated. After incubation with 5-bromo-2-deoxyuridine labeling reagent for 2 h, the cells were fixed and 5-bromo-2-deoxyuridine incorporation into newly synthesized DNA was assessed by incubation with an anti-5-bromo-2-deoxyuridine peroxidase-conjugated antibody for 90 min followed by addition of substrate solution and colorimetric detection at 450 and 690 nm, respectively.

Cell Cycle Analysis

Cells were seeded in 100 mm dishes at a density of 5×10^5 per dish. Forty-eight hours later, the cells were treated with drug or vehicle (DMSO) for 24h. Both adherent and floating cells were harvested and stained with ethidium bromide (22). Quantitation of the cell cycle distribution was done by flow cytometric analysis.

Transfection Experiments

For transient transfections, 2×10^4 GTL-16 cells per well were seeded into 96-well plates and transfected with 0.2 μg plasmid DNA [EGFRΔL747-S752 exon 19 deletion mutant cloned into the pcDNA3.1(-) expression vector (23, 24); kindly provided by Dr. William Pao] using Lipofectamine 2000 transfection reagent (Invitrogen). Following transfection, the cells were grown in DMEM supplemented with 10% FCS for 48 h. Thereafter, the cells were treated with 0.4 μmol/L PHA-665752 for 24 h or left untreated followed by a 5-bromo-2-deoxyuridine cell proliferation assay. For control, GTL-16 cells transfected with empty pcDNA3.1(-) expression vector were used.

Immunohistochemistry for MET, EGFR, and HER-3 Expression in Human Gastric Cancer Tissue

Standard ABC peroxidase techniques were used for immunohistochemistry done on 4 μm paraffin sections of formalin-fixed and paraffin-embedded human gastric cancer tissue. The following antibodies were used: anti-MET

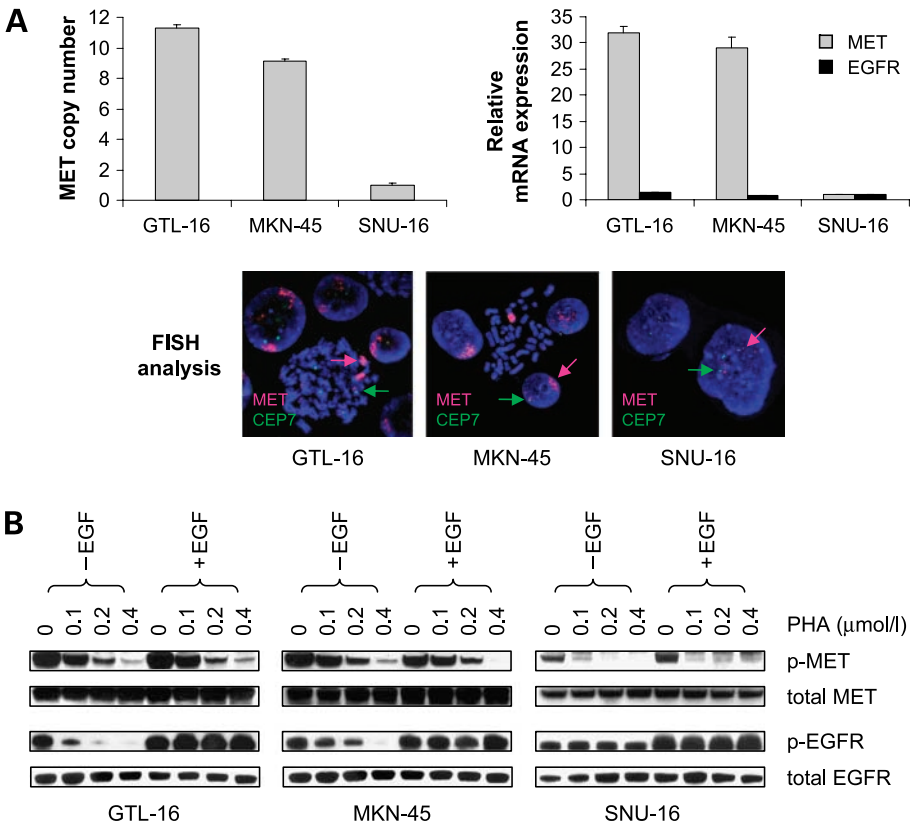

Figure 1. MET is amplified and overexpressed in GTL-16 and MKN-45 but not SNU-16 gastric cancer cells. **A,** *top left,* DNA from the indicated cell lines was subjected to quantitative PCR analysis as described in Materials and Methods. Mean MET copy number relative to RNase P copy number, setting the value for SNU-16 cells arbitrarily as 1. *Top right,* expression of MET and EGFR mRNA relative to 18S rRNA in gastric cancer cell lines as determined by real-time PCR. *Bottom,* representative fluorescence *in situ* fluorescence analysis, showing amplification of MET (*red*) in GTL-16 and MKN-45 but not SNU-16 cells. *Green,* control probe against the centromere of chromosome 7. **B,** cells were treated for 4 h with increasing concentrations (0.1-0.4 μmol/L) of PHA-665752 with or without addition of EGF (100 ng/mL) for the final 15 min.

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(C-12) from Santa Cruz Biotechnology, anti-EGFR from Zymed Laboratories, and anti-HER-3 from Lab Vision. For all antibodies, antigen retrieval in heated citrate buffer (pH 6.0) was applied. Appropriate positive and negative control tissues were stained in parallel with the study cases. MET, EGFR, and HER-3 expression was quantified using the following scoring system. First, a proportion score was assigned, representing the proportion of positive staining tumor cells (0, 0-24%; 1, 25-49%; 2, 50-74%; 3, 75-100%). Next, an intensity score was assigned, representing the average intensity of positive tumor cells (0, none; 1, weak; 2, intermediate; 3, strong). The proportion and intensity scores were then multiplied to obtain a total score that ranged from 0 to 9.

Results

MET-Amplified GTL-16 and MKN-45 Gastric Cancer Cells Display MET-Dependent EGFR Phosphorylation, Which Can Be Inhibited by the Selective MET Inhibitor PHA-665752

The gastric cancer cells GTL-16 and MKN-45 display MET gene amplification and overexpression as determined by quantitative PCR for MET gene copy number, fluorescence *in situ* fluorescence analysis, and real-time PCR for MET mRNA (Fig. 1A). MET is constitutively active and can be inhibited in a dose-dependent manner by the highly selective MET tyrosine kinase inhibitor PHA-665752 (Fig. 1B). GTL-16 and MKN-45 cells also display high levels of baseline EGFR phosphorylation despite the absence of EGFR amplification or mutation (data not shown). Baseline EGFR phosphorylation is MET dependent, because inhibition of MET is paralleled by dephosphorylation of EGFR (Fig. 1B). Exogenous EGF restores EGFR phosphorylation despite the presence of PHA-665752, suggesting that the MET inhibitor does not have a direct EGFR-inhibitory effect. Additional evidence against unspecific EGFR inhibition includes the fact that PHA-665752 induces complete dephosphorylation of EGFR at a 10-fold lower concentration (0.4 μmol/L) than its reported IC_{50} (3.8 μmol/L) against the recombinant EGFR enzyme (13). Furthermore, EGFR phosphorylation remains unaffected by PHA-665752 in non-MET-amplified SNU-16 gastric cancer cells, which display baseline EGFR phosphorylation that is independent of MET (Fig. 1B).

PHA-665752 Also Inhibits MET-Dependent HER-3 Phosphorylation as Well as Downstream Gab-1, MAPK, and AKT Activation

Consistent with previous findings in MET-amplified lung cancer cells (16), we also observed high levels of baseline HER-3 phosphorylation in MET-amplified GTL-16 and MKN-45 gastric cancer cells, whereas HER-2 phosphorylation was minimal (Fig. 2A). As observed with EGFR, HER-3 phosphorylation was dependent on MET and could be inhibited by PHA-665752. Downstream effects of PHA-665752 included dephosphorylation of the docking protein Gab-1 as well as MAPK and AKT (Fig. 2A).

PHA-665752-Mediated Dephosphorylation of EGFR and the MEK/MAPK Pathway Can Be Overcome by Addition of Exogenous EGF

When exogenous EGF was added to PHA-665752-treated cells, it restimulated EGFR, HER-2, and the MEK/MAPK

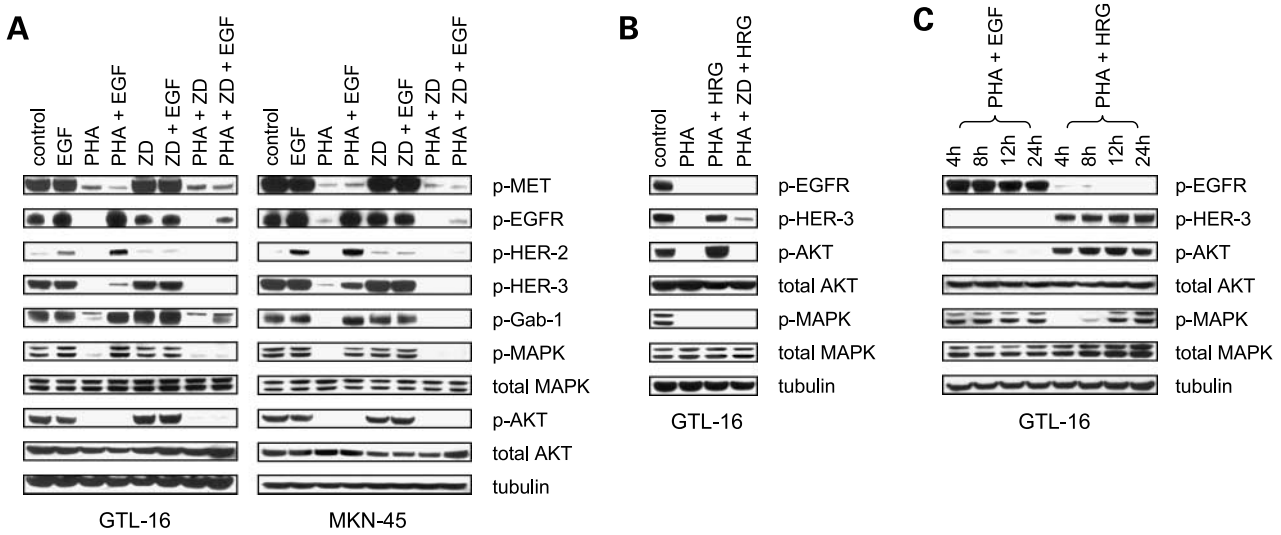

Figure 2. Effects of HER kinase activation on receptor tyrosine kinase phosphorylation and downstream signaling in PHA-665752-treated GTL-16 and MKN-45 cells. **A,** GTL-16 and MKN-45 cells were treated for 4 h with 0.4 μmol/L PHA-665752 ± 1 μmol/L gefitinib with or without addition of 100 ng/mL EGF for the final 15 min. **B,** GTL-16 cells were treated for 4 h with 0.4 μmol/L PHA-665752 ± 1 μmol/L gefitinib followed by addition of 20 ng/mL HRG for the final 15 min. HRG alone did not have an effect on the phosphorylation status of the cells (data not shown). **C,** GTL-16 cells were treated for up to 24 h with 0.4 μmol/L PHA-665752 + 100 ng/mL EGF or 20 ng/mL HRG, with EGF or HRG being present for the entire culture period. For control, the cells were treated with PHA-665752 alone, which persistently inhibited phosphorylation of EGFR, HER-3, AKT, and MAPK (data not shown). Identical results in **B** and **C** were obtained using cell lysates derived from MKN-45 cells. *ZD,* gefitinib.

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pathway, whereas it had no effect on AKT phosphorylation even after 24 h of treatment (Fig. 2A and C). Interestingly, the EGFR-specific tyrosine kinase inhibitor gefitinib did not affect baseline EGFR phosphorylation, further substantiating the fact that EGFR is transactivated by MET in MET-amplified tumor cells. However, when gefitinib was added to cells treated with PHA-665752 and EGF, it could block EGF-mediated restimulation of EGFR, HER-2, Gab-1, and the MEK/MAPK pathway (Fig. 2A).

Addition of the HER-3 Ligand HRG to PHA-665752-Treated Cells Restimulates HER-3 and Both MEK/MAPK and PI3K/AKT

Next, we assessed the effects of HER-3 restimulation in GTL-16 cells treated with PHA-665752 using the HER-3 ligand HRG. As shown in Fig. 2B and C, HRG restimulated HER-3 and both PI3K/AKT and MEK/MAPK pathways, with PI3K/AKT pathway activation preceding MEK/MAPK pathway activation. As with EGF, the signaling effects of HRG on PHA-665752-treated cells could be blocked using combined treatment with gefitinib.

EGF- or HRG-Mediated Restimulation of the MEK/MAPK and/or PI3K/AKT Pathways Rescues MET-Amplified Tumor Cells from PHA-665752-Mediated Growth Inhibition

We then evaluated whether EGF- or HRG-mediated restimulation of the MEK/MAPK and/or PI3K/AKT pathways can rescue MET-amplified tumor cells from PHA-665752-mediated growth inhibition. As shown in Fig. 3A, MET-amplified GTL-16 and MKN-45 cells were highly sensitive to PHA-665752, whereas non-MET-amplified SNU-16 cells were resistant. None of the cell lines were sensitive to gefitinib alone. Also, treatment of the cells with either EGF or HRG alone did not affect proliferation of the cells (data not shown). However, as shown in Fig. 3B, EGF and HRG were able to rescue GTL-16 and MKN-45 cells from PHA-665752-mediated growth inhibition. In accordance with the above signaling data, gefitinib was able to abrogate the rescue effects of both agents in PHA-665752-treated cells.

Transfection of GTL-16 Cells with Mutant EGFR Harboring an Exon 19 Deletion (EGFRΔL747-S752) Confers Resistance to PHA-665752 Treatment

To assess whether expression of mutant EGFR renders GTL-16 cells resistant to PHA-665752-mediated growth inhibition, we transiently transfected the cells with an EGFR exon 19 deletion mutant (EGFRΔL747-S752), which leads to constitutive activation of the EGFR pathway (24). As shown in Fig. 3C, GTL-16 cells transfected with EGFRΔL747-S752 became resistant to PHA-665752-mediated growth inhibition, whereas cells containing the empty expression vector remained sensitive to PHA-665752.

PHA-665752 Induces G_1 Arrest of the Cells, Which Can Be Reversed Using EGF or HRG

Figure 4A illustrates the cell cycle distribution of GTL-16 cells following treatment: at 24 h, 96.6% of PHA-665752-treated cells accumulated in G_1, whereas the percentage of cells in S phase decreased from 36.5% (control) to 1.5% (PHA-665752). Neither EGF nor HRG had an effect on the cell cycle distribution in otherwise untreated cells (data not shown). However, both EGF and HRG were able to counteract the effects of PHA-665752, increasing the proportion of cells in S phase from 1.5% to 29.2% and 24.4%, respectively. Again, gefitinib abrogated the effects of both EGF and HRG when combined with PHA-665752, reducing the proportion of cells in S phase from 29.2% to 1.2% and from 24.4% to 3.1%, respectively.

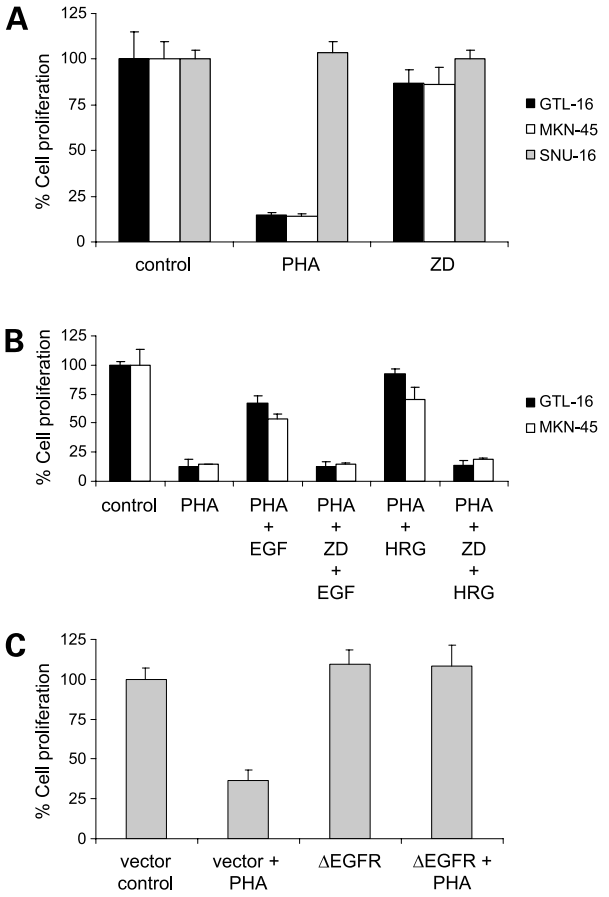

Figure 3. HER kinase activation rescues MET-amplified gastric cancer cells from PHA-665752-mediated growth inhibition. **A,** GTL-16, MKN-45, and SNU-16 cells were treated for 24 h with 0.4 μmol/L PHA-665752 or 1 μmol/L gefitinib followed by determination of cell proliferation as described in Materials and Methods. In cells treated with PHA-665752 and gefitinib, no combined effect was observed (data not shown). **B,** GTL-16 and MKN-45 cells were treated for 24 h with 0.4 μmol/L PHA-665752 ± 1 μmol/L gefitinib with or without addition of 100 ng/mL EGF (or 20 ng/mL HRG). In cultures treated with EGF or HRG, the respective ligands were present for the entire experiment. **C,** GTL-16 cells were transiently transfected with a constitutively active EGFR exon 19 deletion mutant (EGFRΔL747-S752) followed by treatment with PHA-665752 for 24 h. For control, cells transfected with the empty pcDNA3.1(-) expression vector were used. *ΔEGFR,* EGFRΔL747-S752 exon 19 deletion mutant.

PHA-665752 Induces Loss of Cyclin D1 Expression, Which Can Be Prevented by Combined EGF or HRG Treatment

Figure 4B shows that PHA-665752-mediated G_1 arrest was paralleled by loss of expression of cyclin D1, a key cell

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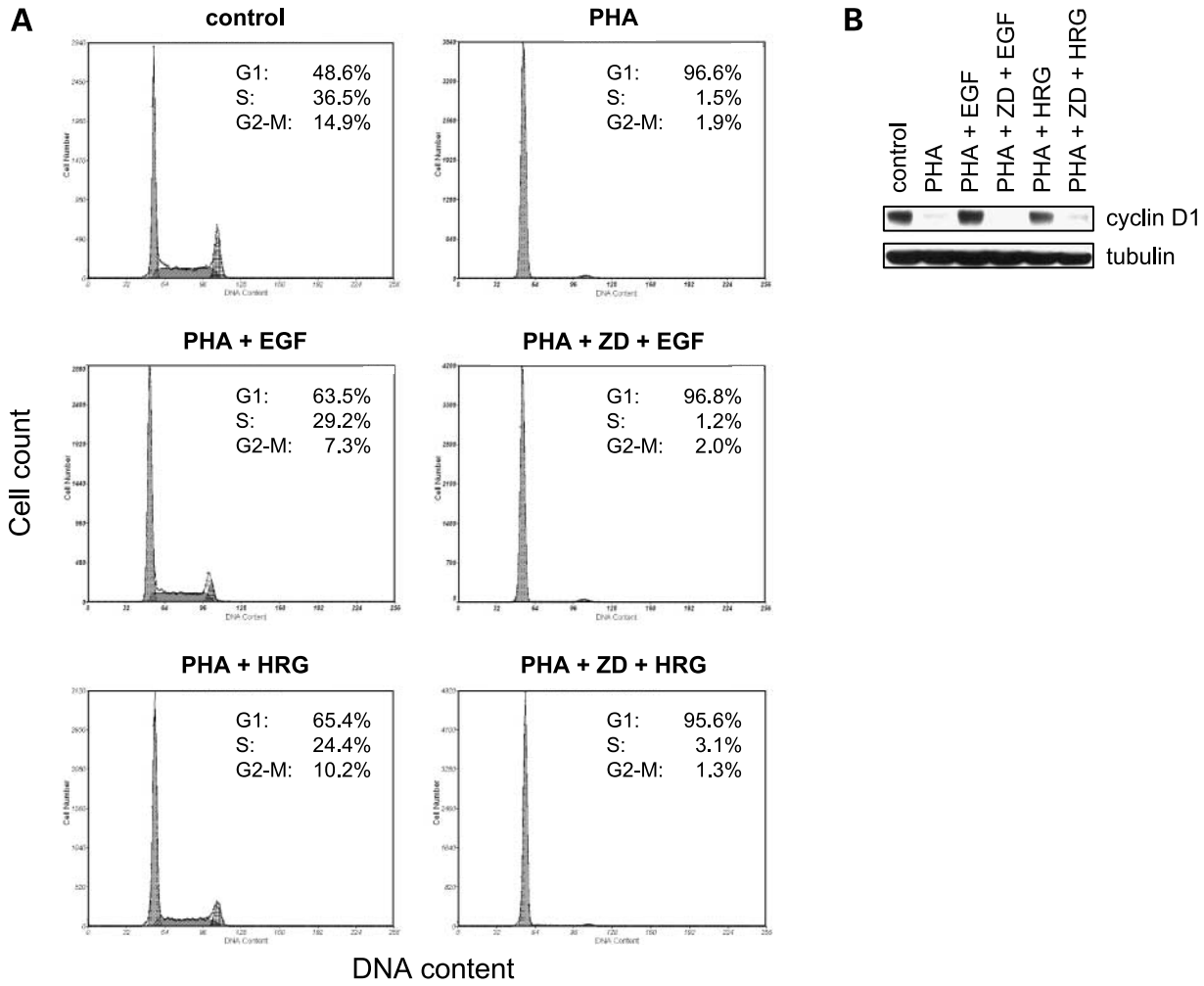

Figure 4. Cell cycle analysis and Western blot for cyclin D1 expression. **A,** GTL-16 and MKN-45 cells were treated for 24 h with 0.4 μmol/L PHA-665752 ± 1 μmol/L gefitinib with or without addition of 100 ng/mL EGF (or 20 ng/mL HRG) followed by flow cytometric determination of the cell cycle distribution as described in Materials and Methods. Gefitinib alone did not have an effect on the cell cycle distribution of the cells (data not shown). **B,** immunoblot for cyclin D1 in cell lysates derived from the same experiment as in **A**.

cycle regulator that allows for cell cycle progression from G_1 to S phase. EGF and HRG prevented PHA-665752-mediated cyclin D1 loss and promoted progression of cells into S phase. Concordant with the cell cycle distribution data, the addition of gefitinib abrogated the effects of EGF and HRG in PHA-665752-treated cells, again promoting cyclin D1 loss and G_1 arrest.

MEK/MAPK Inhibition Is Able to Abrogate the EGF Rescue Effect in PHA-665752-Treated Cells, Whereas Only Combined Inhibition of MEK/MAPK and PI3K/AKT Is Able to Abrogate the HRG Rescue Effect

Having established a mechanism by which MET-amplified gastric cancer cells could escape the effects of a specific MET kinase inhibitor, we further investigated to which extent the MEK/MAPK and PI3K/AKT pathways contribute to the rescue effects of EGF and HRG. To inhibit activation of MAPK, we used PD0325901, a highly selective MEK inhibitor (25). To inhibit activation of AKT, we used API-2, a highly selective small-molecule inhibitor of cellular phosphorylation of AKT (26). Despite completely abrogating either MAPK or AKT activation, neither PD0325901 (0.1 μmol/L) nor API-2 (2 μmol/L) alone inhibited proliferation of our cells (data not shown). However, as shown in Fig. 5A, PD0325901 abrogated the rescue effect of EGF in PHA-665752-treated cells, indicating that EGF confers MET inhibitor resistance through stimulation of the MEK/MAPK pathway. Conversely, only combined treatment with PD0325901 and API-2 was able to completely abrogate the rescue effect of HRG on PHA-665752-treated cells ($P < 0.001$ compared with either PD0325901 or API-2 inhibition alone), indicating that HRG mediates its effects through stimulation of both MEK/MAPK and PI3K/AKT pathways. In accordance with the cell proliferation data, cyclin D1 expression in cells treated with PHA-665752 + EGF could be completely abolished using PD0325901, whereas cyclin D1 expression

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in cells treated with PHA-665752 + HRG could only be completely abolished using combined treatment with PD0325901 and API-2 (Fig. 5B).

Clinical Gastric Cancer Samples Display Coexpression of MET, EGFR, and/or HER-3

Our data indicate that MET-independent HER kinase activation is a possible mechanism of MET tyrosine kinase inhibitor resistance in MET-amplified tumor cells that coexpress EGFR and/or HER-3. We therefore investigated human gastric cancer specimens for coexpression of MET, EGFR, and HER-3 (Fig. 6A and B): Positive MET expression was detected in 9 of 10 (90%) tumors, with 4 (40%) of the tumors displaying MET overexpression (immunohistochemistry score $\geq$ 6). Noticeably, all tumors with MET overexpression coexpressed either EGFR, HER-3, or both, suggesting that the above rescue mechanisms could be clinically relevant.

Discussion

Constitutive activation of the MET tyrosine kinase is a hallmark of tumor cells with MET gene amplification (5, 6). Proliferation and survival of these cells are driven by MET (''MET oncogene addiction''), which renders them highly sensitive to MET inhibition (5, 6). Approximately 15% of gastric, 4% of lung, and 4% of esophageal cancers harbor MET amplification and are therefore attractive candidates for MET kinase inhibitor therapy (7–12). Several MET inhibitors have been developed and have recently entered early stage clinical trials (13, 15). Here, we show that MET inhibition induces complete growth arrest of MET-amplified gastric cancer cells but leaves the malignant cells vulnerable toward collateral growth factor receptor-mediated MEK/MAPK and/or PI3K/AKT activation, suggesting a potential escape mechanism from MET inhibition.

Collateral growth factor receptor pathway activation has recently been characterized as a novel mechanism of resistance to tyrosine kinase inhibition: Engelman et al. found that chronic exposure of EGFR-mutant non-small-cell lung cancer cells to increasing concentrations of the EGFR tyrosine kinase inhibitor gefitinib led to the emergence of several gefitinib-resistant subclones with acquired amplification of the MET oncogene. In the resistant cells, the constitutively active MET tyrosine kinase triggered persistent activation of PI3K/AKT signaling, which was no longer sensitive to gefitinib treatment as in the parental cells. The underlying mechanism of PI3K/AKT activation was identified as MET-dependent HER-3 receptor cross-talk, which could be abrogated by PHA-665752, a highly specific MET kinase inhibitor (16).

We show that, in MET-amplified GTL-16 and MKN-45 gastric cancer cells, PHA-665752 abolishes constitutive activation of MET, MET-dependent HER kinase receptor cross-talk, as well as downstream MEK/MAPK and PI3K/AKT signaling, inducing complete growth arrest of the cells. However, we also show that MET-independent reactivation of the MEK/MAPK and/or PI3K/AKT pathways using EGF (which binds to and activates EGFR) or HRG (which binds to and activates HER-3) can overcome the growth-inhibitory effects of MET kinase inhibition. Furthermore, we show that transfection of MET-amplified cancer cells with a constitutively active EGFR exon 19 deletion mutant (EGFRΔL747-S752) similarly confers resistance to MET inhibition. Our findings illustrate the potential limitations of monotherapy with a highly selective receptor tyrosine kinase inhibitor in ''kinase-addicted'' cancer cells: despite their extreme sensitivity to inhibition of the target kinase, the cells remain responsive to collateral growth receptor activation, which suggests that combined pathway inhibition may be required.

Noticeably, EGF- or HRG-mediated rescue from MET inhibition was mediated through distinct downstream signaling events: although both ligands led to reactivation of the MEK/MAPK pathway in PHA-665752-treated cells,

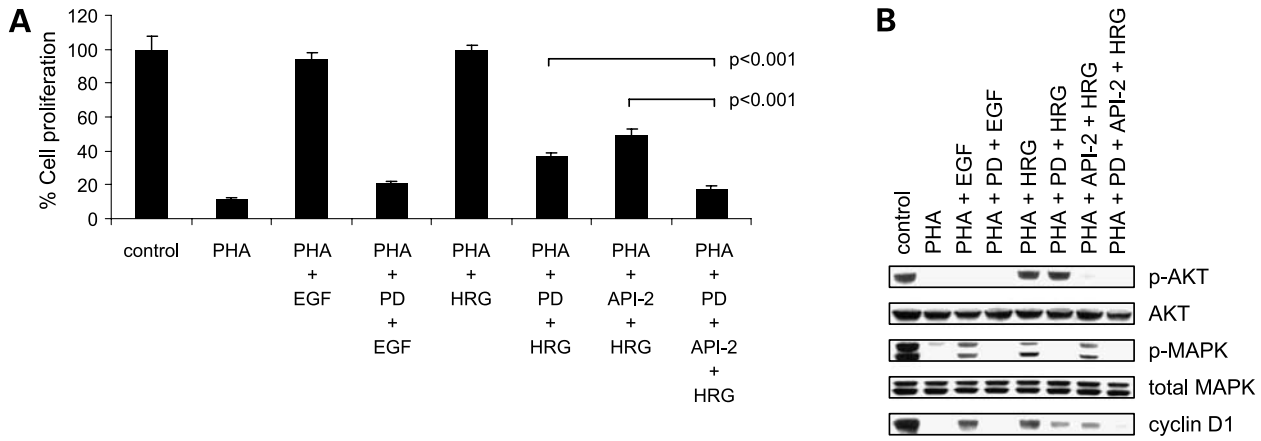

Figure 5. Effects of selective MEK and/or AKT inhibition on HER kinase-mediated activation of downstream signaling pathways, cyclin D1 expression, and cell proliferation in PHA-665752-treated cells. **A,** GTL-16 cells were treated for 24 h with 0.4 μmol/L PHA-665752 ± 0.1 μmol/L PD0325901 ± 2 μmol/L API-2 with or without addition of 100 ng/mL EGF (or 20 ng/mL HRG). Statistical analysis was done using paired, two-sided Student's *t* test. **B,** immunoblot analysis for phosphorylated AKT, phosphorylated MAPK, and cyclin D1 expression in cell lysates derived from the same experiment as in **A**.

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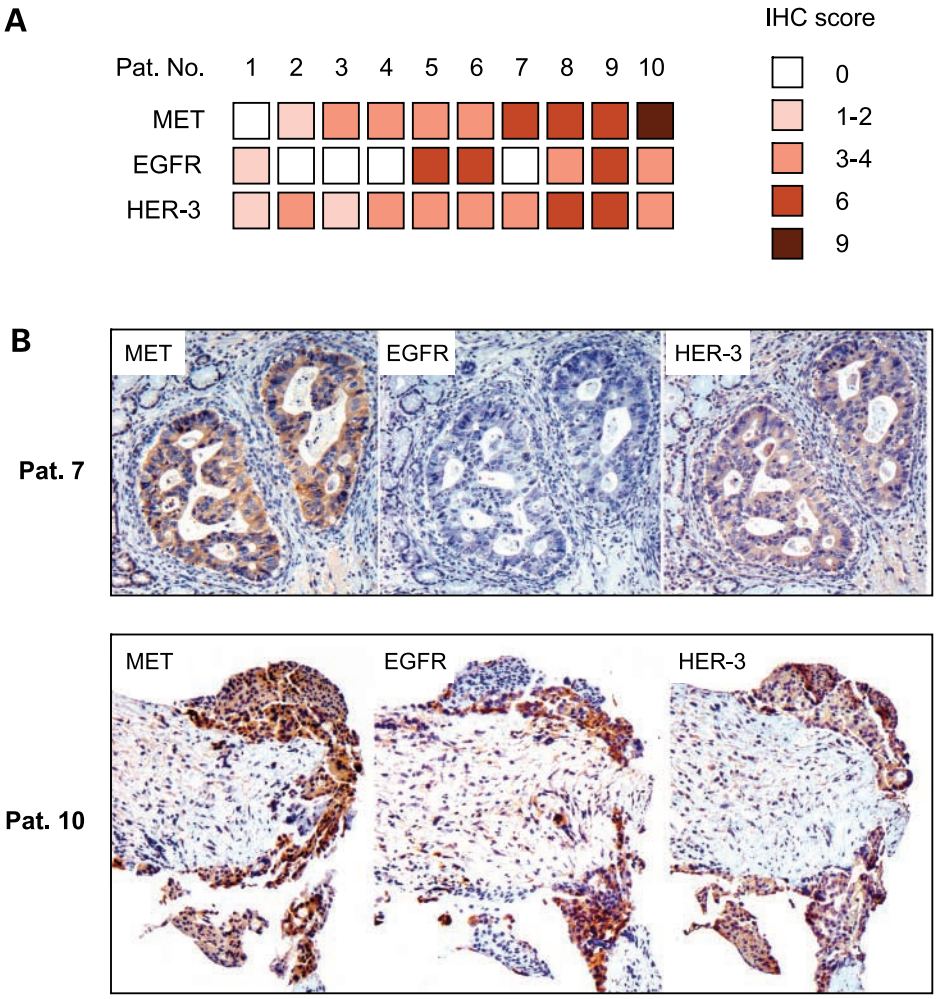

Figure 6. Coexpression of EGFR and/or HER-3 in gastric cancer tissue with MET overexpression. **A,** formalin-fixed, paraffin-embedded tissue specimens from 10 cases with gastric cancer were analyzed for MET, EGFR, and HER-3 expression using immunohistochemistry. MET, EGFR, and HER-3 expression was scored as described in Materials and Methods. MET overexpression, defined as a MET immunohistochemistry score ≥ 6, was observed in 4 of 10 tumors. Importantly, all of these tumors were found to coexpress either EGFR, HER-3, or both. **B,** representative immunohistochemical analysis of two patients with MET overexpression.

only HRG induced reactivation of the PI3K pathway. It is well known that HER-3 induces potent activation of PI3K/AKT signaling through several Tyr-Xaa-Xaa-Met sequence motifs, which bind to the p85 regulatory subunit of PI3K, whereas EGFR, which lacks similar binding sites, is only a weak activator of this pathway (27–29). As a result, EGFR relies on the docking protein Gab-1 or HER-3 as a heterodimerization partner to mediate PI3K activation (28, 30). Our data indicate that the latter mechanisms are of minor importance in MET-amplified GTL-16 and MKN-45 gastric cancer cells. (*a*) Although Gab-1 is phosphorylated after addition of EGF, no activation of PI3K is observed. (*b*) EGF-mediated activation of EGFR induces only weak transphosphorylation of HER-3 in MET-amplified cancer cells, suggesting that only few EGFR/HER-3 heterodimers are formed in response to EGF stimulation.

Both EGF and HRG rescued cells from PHA-665752-induced growth arrest by preventing loss of cyclin D1, a key cell cycle regulator (31). Because the induction of cyclin D1 expression has been linked to both MEK/MAPK and PI3K/AKT signaling (32, 33), we selectively inhibited each pathway and assessed the effects on EGF- and HRG-mediated cyclin D1 expression. Consistent with previous observations (32, 33), activation of either pathway was sufficient to maintain cyclin D1 expression in our cells. However, because EGF induced MEK/MAPK activation only, MEK inhibition alone abrogated EGF-induced cyclin D1 expression and the EGF rescue effect in PHA-665752-treated cells. In contrast, only combined inhibition of MEK and AKT was able to abrogate HRG-induced cyclin D1 expression and the HRG rescue effect.

The EGFR inhibitor gefitinib potently inhibited both EGF- and HRG-induced cellular rescue from MET inhibition

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despite the fact that HRG signaling is EGFR independent. Previous studies have shown that HRG activates HER-3 through heterodimer formation between HER-3 and HER-2, which together constitute a high-affinity HRG coreceptor complex (29). On ligand binding, HER-2 transactivates HER-3 because the latter lacks intrinsic tyrosine kinase activity (34, 35). Despite its relative selectivity for EGFR, gefitinib also inhibits the formation of active phosphorylated HER-2/HER-3 heterodimers and the association of HER-3 with the p85 regulatory subunit of PI3K, which explains the abrogation of the HRG rescue effect by gefitinib in our cells (36, 37).

Immunohistochemical analysis revealed that human gastric cancers frequently express EGFR and/or HER-3, suggesting that they are responsive to EGF and/or HRG *in vivo*. The expression of EGFR and HER-3 is particularly prominent in tumors with MET overexpression, which substantiates the clinical relevance of the above rescue mechanisms. Importantly, cells from the tumor microenvironment may serve as a source of HER ligands such as EGF and HRG: for example, it could be shown that gastric fibroblasts produce HRG and are able to stimulate HER-3 phosphorylation in MKN-28 gastric cancer cells (38). Tumor-associated macrophages secrete a wide variety of growth factors and are the most significant source of EGF in tumors (39–41). It has also been shown that prostaglandin E_2 derived from inflammatory cells can transactivate EGFR and trigger MEK/MAPK signaling in malignant cells (42). Taken together, the available data therefore provide a strong rationale for combining a MET kinase inhibitor with gefitinib in MET-amplified gastric cancers that coexpress EGFR and/or HER-3.

It may be argued that the combination of a MET kinase inhibitor with gefitinib does not account for potential HER kinase-independent MEK/MAPK and/or PI3K/AKT activation and rescue. Clearly, our data indicate that a possible alternative strategy taking into account both HER kinase-dependent and HER kinase-independent mechanisms of rescue would be to combine a MET kinase inhibitor with inhibitors of MEK and AKT. Several compounds have been developed and warrant further investigation in this context. In conclusion, the present study adds to the growing evidence that complementary pathway inhibition using rational combinations of molecularly targeted agents is advantageous over kinase inhibitor monotherapy and therefore should become the future mainstay of molecularly targeted treatment in cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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