PF-03732010: A Fully Human Monoclonal Antibody against P-Cadherin with Antitumor and Antimetastatic Activity

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Abstract

Purpose: P-cadherin is a membrane glycoprotein that functionally mediates tumor cell adhesion, proliferation, and invasiveness. We characterized the biological properties of PF-03732010, a human monoclonal antibody against P-cadherin, in cell-based assays and tumor models.

Experimental Design: The affinity, selectivity, and cellular inhibitory activity of PF-03732010 were tested in vitro. Multiple orthotopic and metastatic tumor models were used for assessing the antitumor and antimetastatic activities of PF-03732010. Treatment-associated pharmacodynamic changes were also investigated.

Results: PF-03732010 selectively inhibits P-cadherin–mediated cell adhesion and aggregation in vitro. In the P-cadherin–overexpressing tumor models, including MDA-MB-231-CDH3, 4T1-CDH3, MDA-MB-435HAL-CDH3, HCT116, H1650, PC3M-CDH3, and DU145, PF-03732010 inhibited the growth of primary tumors and metastatic progression, as determined by bioluminescence imaging. Computed tomography imaging, H&E stain, and quantitative PCR analysis confirmed the antimetastatic activity of PF-03732010. In contrast, PF-03732010 did not show antitumor and antimetastatic efficacy in the counterpart tumor models exhibiting low P-cadherin expression. Mechanistic studies via immunofluorescence, immunohistochemical analyses, and 3′-[18F]fluoro-3′-deoxythymidine–positron emission tomography imaging revealed that PF-03732010 suppressed P-cadherin levels, caused degradation of membrane β-catenin, and concurrently suppressed cytoplasmic vimentin, resulting in diminished metastatic capacity. Changes in the levels of Ki67, caspase-3, and 3′-[18F]fluoro-3′-deoxythymidine tracer uptake also indicated antiproliferative activity and increased apoptosis in the tested xenografts.

Conclusions: These findings suggest that interrupting the P-cadherin signaling pathway may be a novel therapeutic approach for cancer therapy. PF-03732010 is presently undergoing evaluation in Phase 1 clinical trials. Clin Cancer Res; 16(21); 5177–88. ©2010 AACR.

The classic cadherins constitute a superfamily of molecules that mediate calcium-dependent cell-cell adhesions. The intracellular domains of cadherins directly bind to β-catenin and link with cytoskeletal components, providing the molecular basis for stable cell-cell adhesion (1). The cadherin/catenin signaling pathway also represents a major regulatory mechanism for oncogenic signaling pathways (2, 3) that guide cell fate decisions through the regulation of cell growth, differentiation, motility, and survival (4). Several cadherins, such as E-cadherin, N-cadherin, VE-cadherin, P-cadherin, and catenin 11, have been implicated in cancer disease progression (5).

Among the cadherin families, E-cadherin and N-cadherin are the most highly characterized subgroup of adhesion proteins. E-cadherin is ubiquitously expressed throughout most epithelial tissues and serves as a negative regulator to functionally block the β-catenin signaling pathway and suppress tumor cell growth (6, 7) and invasion (8, 9). Numerous preclinical and clinical studies have shown that the loss of E-cadherin occurs concurrently with the upregulation of N-cadherin (10) or other cadherin family members implicated in invasive growth (5). This process, known as cadherin switching, has been reported to promote epithelial-mesenchymal transition and leads to tumor cell invasion and metastasis. N-cadherin overexpression via cadherin switching was observed in various invasive cancer
Translational Relevance

P-cadherin–mediated adherens junctions and the associated signaling pathway play diverse roles in the regulation of tumor cell proliferation, invasiveness, and metastatic potential. Upregulation of P-cadherin was frequently observed in various malignancies, including breast, colon, lung, and pancreatic tumors, and P-cadherin increase correlated with poor survival of breast cancer patients. PF-03732010, a novel, highly selective human monoclonal antibody against P-cadherin, showed antitumor and antitumor metastatic activity in a diverse panel of P-cadherin–overexpressing tumor models without introducing any adverse effects. Mechanistic studies revealed that PF-03732010 disrupted the P-cadherin signaling pathway, suppressed β-catenin transcriptional targets, and resulted in an antitumor metastatic and antiproliferative activity and the induction of apoptosis. Thus, antagonizing P-cadherin with PF-03732010 represents a novel approach for anticancer therapy targeting tumors with high P-cadherin expression.

In vitro selectivity over E-cadherin

E-cadherin antibody–binding assessment was tested via fluorescence-activated cell sorting analysis. A549 cells were blocked with serum and incubated with either 50 μg/mL PF-03732010 or an E-cadherin–specific antibody (R&D Systems) at 5 μg/mL for 1 hour. Cells were subsequently processed according to the manufacturer’s instructions and analyzed using a FACSCalibur system (BD Biosciences).

Transfection of tumor cell lines

DU145, H1650, and HCT116 cell lines, which express high levels of endogenous P-cadherin, were transfected with luciferase expression vector as previously reported (24). The paired isogenic cell lines, MDA-MB-231-CDH3 (P-cadherin)/MDA-MB-231-pCL (vector), PC3M-CDH3/PC3M-pCL (from PC3M-luc-C6, Xenogen-Caliper Company), and MDA-MB-435HAL-CDH3/MDA-MB-435HAL-pCL (25, 26), were derived so that they differ only in P-cadherin expression. Full-length human or mouse P-cadherin cDNAs were cloned into the retroviral transfer plasmid pCLNCX (Imgenex), and retroviral vectors were prepared in the PT67 packaging cell line (Clontech), according to the manufacturer’s protocols. 4T1-CDH3 cells were transfected to express high levels of P-cadherin. Target cell lines were transduced with retroviral vectors, and drug-resistant pools of cells were selected with geneticin at 0.5 mg/mL. DU145, H1650, and HCT116, along with the MDA-MB-435HAL and PC3M isogenic pairs, stably expressed luciferase to allow disease progression to be monitored by BLI.

Cell lines and tumors and, therefore, is emerging as a potential therapeutic target. ADH1-1, a peptide antagonist that disrupts N-cadherin–mediated adhesion, has been shown to inhibit cell growth and motility in vitro, as well as tumor growth and invasion in vivo (11, 12); ADH1 is currently undergoing phase 1 clinical trials.

P-cadherin (CDH3), which shares moderate homology with N-cadherin (41%) and E-cadherin (46%) in the extracellular domain (13), was often reported to correlate with increased tumor cell motility and invasiveness when overexpressed (14–16). P-cadherin–associated tumor cell growth, motility, and invasiveness is likely or at least partially mediated by the relocalization of membrane β-catenin to the cytoplasm and subsequently into the nucleus where it partners with the T-cell factor and activates transcription of the target genes (14, 17), including vimentin (18), cyclin D1, survivin, and BCL-2 (19). The upregulation of P-cadherin was frequently observed in various malignant tumors, including breast, colon, lung, and pancreatic tumors, and correlated with poor survival of breast cancer patients (15, 20, 21). In contrast, significantly low levels of the P-cadherin gene expression were detected in a diverse panel of normal tissues (22). Thus, disruption of P-cadherin signaling represents an intriguing opportunity for the development of novel targeted therapeutic agents.

PF-03732010, a humanized monoclonal antibody, was discovered to antagonize P-cadherin–regulated cell-cell adhesion and the associated signaling pathway. Because P-cadherin is implicated in tumor cell invasion, proliferation, and metastatic progression, in this report, we used spontaneous and experimental metastasis models to evaluate the antitumor and antimetastatic activity of PF-03732010 as well as the underlying mechanism. Noninvasive bioluminescence imaging (BLI) technology was used to longitudinally monitor disease progression. We also used 3′-[(18F)fluoro-3′-deoxythymidine ([18F]FLT)–positron emission tomography imaging (PET) to assess the antiproliferative activity of PF-03732010 and determine the potential clinical utility of this imaging modality as a surrogate biomarker for proof of mechanism (23). These findings provide insights into the P-cadherin signaling pathway and warrant the clinical investigation of PF-03732010 as a novel agent for anticancer therapy.

Materials and Methods

The PF-03732010 humanized monoclonal antibody was generated by affinity purification at Pfizer St. Louis Protein Therapeutics group (Pfizer, Inc.). D-Luciferin was purchased from Caliper Life Sciences. The antibodies used include anti-β-catenin (BioSource International), anti-Ki67 (Lab Vision), and anti-vimentin (Dako). Anti-P-cadherin, anti-E-cadherin, anti-N-cadherin, anticyclin D1, anti-caspase-3, anti-survivin, and anti-Bcl-2 were purchased from Cell Signaling. Unless otherwise noted, all cell lines and fine chemicals were purchased from American Type Culture Collection and Sigma-Aldrich, respectively.
Cell aggregation and spheroid disruption assays
To perform the aggregation assay, cells were detached and resuspended as single cells at a concentration of 1 × 10^6 cells/mL. Cells were plated into 96-well poly-HEMA–coated plates and incubated with and without the test agent(s) at 37°C on a horizontal shaker for 16 hours to facilitate aggregate formation. For the spheroid disruption experiments, cell aggregates were formed before being treated, and activity was expressed as fold increase in disrupted cells compared with untreated controls.

Cells or aggregates were fixed with 4% formaldehyde, and the degree of aggregation was scanned and calculated using a Cellomics ArrayScan (Thermo Fisher Scientific) equipped with Target Acquisition software. Maximal inhibition was defined as the value when EGTA was present, during which the maximum number of single nonaggregated cells was produced, whereas the negative control was the value when the smallest number of cell-cell aggregates was formed in complete medium. EC_{50} values were determined using Graphpad Prism software.

In vivo studies and drug administration
All animal experimental procedures complied with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 1996) and were approved by the Pfizer Global Research and Development Institutional Animal Care and Use Committee. For pharmacokinetic analysis, serum samples were collected until ready for ELISA analysis. PF-03732010 exhibited a half-life of 5.7 to 6.6 days in mice via s.c. or i.v. dosing (Supplementary Fig. S1). The once-weekly s.c. dosing schedule was also used in the subsequent in vivo experiments for efficacy and pharmacodynamic end point evaluations. For the efficacy assessments, mice (Charles River) were randomly assigned to groups (12 mice per group) before treatment, such that the mean tumor size was equal for all groups. In studies assessing efficacy, PF-03732010 was administered s.c. once per week for 6 to 9 weeks. For all studies in this report, no adverse effects were observed in mice treated with PF-03732010.

Tumor models
Severe combined immunodeficient-beige mice were used for all studies in this report, except when using 4T1-CD1H3 cells, for which normal BALB/c mice were used. For the mammary orthotopic implant, 2 × 10^6 cells were combined with Matrigel and injected into the mice under the mammary fat pad. Details for the prostate orthotopic and subrenal capsule (SRC) tumor models were described previously (27, 28). For the H1650 orthotopic or lung metastasis models, 2 × 10^8 cells were i.v. injected through the tail vein of anesthetized mice. Tumors in the lungs were identified via bioluminescence within 2 weeks after tumor inoculation. For the HCT116-luc liver metastasis model, mice were intrasplenically injected with 2 × 10^5 cells, and liver metastases were visualized within a week after tumor cell implant.

Prostate orthotopic surgical implants were done by injecting 5 × 10^5 tumor cells into male severe combined immunodeficient mice. In the SRC model, lung metastases were visualized at 21 to 28 days after tumor implant. At later stages of the SRC and experimental metastasis disease models, mice exhibited physiologic signs consistent with advanced metastatic disease, moribund mice were euthanized, and the survival times were recorded for Kaplan-Meier survival analyses.

BLI
In vivo BLI was conducted using the IVIS100 system with Living Image acquisition and analysis software (Caliper Life Sciences). Anesthetized mice were i.p. injected with 75 mg/kg b-luciferin and imaged 10 minutes after luciferin injection.

Quantitative PCR and Western blot analysis
The circulating tumor cells (CTC) in mouse whole blood were evaluated by real-time quantitative PCR (qPCR) analysis of human Alu DNA sequences, which has been previously validated by laser scanning cytometry (29). Western blot analysis was done according to the antibody manufacturer's instructions. Tumors were snap-frozen and pulverized in a liquid nitrogen-cooled mortar before analysis.

Immunofluorescence and immunohistochemical staining
For in vitro immunofluorescence staining, cells were plated on coverslips in triplicate before treatment. When ready, cells were fixed in 4% formaldehyde and permeabilized in 100% methanol before staining. Tumor samples were collected after 4 to 5 weeks of treatment and either frozen in OCT medium (VWR) or prepared into formalin-fixed, paraffin-embedded tissue blocks. The staining procedure was done according to the manufacturer's instructions. Fluorescence images were captured using a Nikon Eclipse TE2000 fluorescent microscope with Q-Capture software, and image analysis was done using Image Pro Plus 5.1 (Media Cybernetics). Quantitative analysis of immunohistochemical staining was done using the Chromovision automated cell imaging system.

[^F]FLT-PET imaging
[^F]FLT-PET and computed tomography (CT) imaging was done with a microPET Focus F220 scanner (Siemens Medical Solutions) and a GE explore microCT scanner (GE Healthcare), respectively, as described previously (30). The standardized uptake value (SUV) was calculated using the following formula:

\[
\text{SUV} = \frac{C_{\text{PET}}(T)}{(\text{ID}/W)},
\]

where \(C_{\text{PET}}\) is the measured activity in the volume of interest, ID is the injected dose (\(\mu\)Ci), and W is the mouse body weight.
Data analysis

Tumor growth inhibition (TGI) was calculated as $100 \times (1 - \Delta T/\Delta C)$. The $\Delta C$ ($\Delta T$) was obtained by subtracting the mean tumor volume in the vehicle (treated) group on the first day of treatment from the mean tumor volume at the day of assessment. Statistical analyses were conducted using GraphPad Prism with one-way ANOVA analysis, followed by Dunnett’s $t$ test to compare the tumor sizes between the vehicle- and drug-treated groups.

Results

Affinity and specificity of PF-03732010 against P-cadherin

The single-chain Fc versions of PF-03732010 bind to both human and mouse P-cadherin/Fc proteins, with dissociation constant values ($K_d$) of 2.5 and 1.8 nmol/L, respectively, suggesting similar affinities toward both proteins. Because the extracellular domain of human and mouse mature P-cadherin proteins share 87% homology (31), PF-03732010 showed comparable cross reactivity with human and mouse P-cadherins. The selectivity of PF-03732010 was tested on immobilized P-cadherin versus other related cadherins via an ELISA format (Supplementary Fig. S2), and PF-03732010 did not exhibit significant binding to E-cadherin, N-cadherin, or VE-cadherin recombinant proteins across a broad range of concentrations.

To confirm that PF-03732010 does not bind to E-cadherin, cell-based flow cytometric analysis was done using A549 cells, which exhibit high E-cadherin expression but no detectable P-cadherin expression. Figure 1A shows that binding of PF-03732010 to A549 cells was not detected.

In vitro functional assay of PF-03732010

P-cadherin expression in the cell lines used in this report was tested by immunoblot analysis (Supplementary Fig. S3). High levels of P-cadherin were observed in HCT116, DU145, and H1650, as well as the cell lines engineered to express P-cadherin (CDH3). In contrast, vector-transfected (pCL) lines showed minimal expression of P-cadherin.

In cell-based functional assays, PF-03732010 significantly disrupted the MDA-MB-435HAL-CDH3 cell aggregates with an $IC_{50}$ of 5 nmol/L (Fig. 1B and C). PF-03732010 also disrupted existing P-cadherin-mediated adhesion. When the preestablished DU145 spheroids were treated for 24 hours, PF-03732010 (>5 nmol/L) exhibited an 11-fold to 15-fold increase in disrupted spheroids when compared with vehicle-treated cells (Fig. 1C, bottom). DU145 cells treated with PF-03732010 (>5 nmol/L) for 24 hours showed a reduction in membrane-localized $\beta$-catenin (Fig. 1D).

Together, PF-03732010 showed a similar degree of inhibition of cellular aggregation, disruption of spheroids, and suppression of membrane $\beta$-catenin levels across a panel

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**Fig. 1.** In vitro characteristics of PF-03732010. A, PF-03732010 does not show nonspecific binding to E-cadherin. B and C, PF-03732010 inhibits MDA-MB-435HAL-CDH3 cell aggregate formation. C, PF-03732010 disrupts DU145 spheroids. D, PF-03732010 modulates membrane $\beta$-catenin level in DU145 cells. Images are at 40× magnification.
of P-cadherin–expressing cell lines, including HCT116, DU145, MDA-MB-231-CDH3, PC3M-CDH3, and MDA-MB-435HAL-CDH3.

The antitumor and antimetastatic properties of PF-03732010 against multiple tumor models, including mammary, non–small cell lung, colon, and prostate cancer models

The primary tumor sizes in the 4T1-CDH3, MDA-MB-231-CDH3, and MDA-MB-231-pCL mammary orthotopic models were assessed by caliper measurement. For other tumor models, luciferase-expressing DU145, PC3M-pCL, PC3M-CDH3, H1650, and HCT116 lines were used to enable BLI assessment of the primary tumor and metastasis.

In all tested models, treatment of PF-03732010 (s.c. injection) was done once per week until the study ended. The representative BLI images of selected tumor models (Fig. 2A) and the quantitative measurements of the tumor burdens in all tested tumor models (Fig. 2B) showed the antitumor and antimetastatic efficacy of PF-03732010 (20 mg/kg) in a panel of orthotopic and metastasis models. In HCT116 liver metastasis and DU145 lung metastasis models, PF-03732010 displayed significant ($P < 0.05$) antimetastatic activity. PF-03732010 also exhibited significant ($P < 0.05$) antitumor efficacy against tumor growth in 4T1-CDH3, MDA-MB-231-CDH3, DU145, PC3M-CDH3, and H1650 orthotopic models. Dose-dependent efficacy was shown in the PC3M-CDH3 orthotopic model.
PF-03732010 at 5 and 20 mg/kg (data not shown) induced 49% and 73% TGI \( (P < 0.05) \), respectively. In contrast, in the MDA-MB-231-pCL and PC3M-pCL orthotopic models, which expressed low levels of P-cadherin, PF-03732010 (20 mg/kg) did not exhibit antitumor activity. Because 4T1-CDH3, MDA-MB-231-CDH3, DU145, PC3-CDH3, H11650, and HCT116 cell lines express high levels of P-cadherin, these results suggest that the efficacy of PF-03732010 was dependent on the blockade of P-cadherin signaling.

In the PC3M-CDH3 tumor-bearing mice, qPCR analysis of the human Alu gene in mouse whole blood (at day 34) showed that PF-03732010 at 5 and 20 mg/kg significantly reduced the number of human CTCs compared with vehicle treatment (data not shown).

The antitumor and antimetastatic properties of PF-03732010 in MDA-MB-435HAL-CDH3 SRC and experimental metastasis tumor models

We next tested the antitumor and antimetastatic properties of PF-03732010 in SRC tumor models using antigen-expressing MDA-MB-435HAL-CDH3 cells, which form spontaneous metastases in lungs. Treatment of PF-03732010 was initiated on day 9 after tumor cell implantation, and representative BLI images of the primary and secondary tumor burdens on day 50 (Fig. 3A) showed the antitumor and antimetastatic properties of PF-03732010. H&E staining of lungs from a separate cohort of five mice in each group (Fig. 3B) indicated a marked reduction of the tumor burden in PF-03732010-treated groups compared with vehicle controls, in agreement with...
the BLI images. In addition, the reduction in the human Alu gene, detected by qPCR analysis, suggested a reduced level of CTCs in mice treated with PF-03732010 (Fig. 3C).

Significant inhibition ($P < 0.05$) of the primary tumor burden was observed at each dose level (Fig. 3D) evaluated at or beyond day 36 after tumor implantation. On day 50, PF-03732010 at 10, 20, and 40 mg/kg showed TGI values of 52%, 66%, and 68%, respectively, against the primary tumor growth. In addition, PF-03732010 showed significant dose-dependent inhibition of tumor growth at secondary organ sites starting on day 28, with TGI values on day 50 of 54%, 73%, and 82% for the 10, 20, and 40 mg/kg–treated groups, respectively (Fig. 3E). The median survival time for mice treated with PF-03732010 at 10, 20, and 40 mg/kg significantly ($P < 0.05$) increased to 92, 101, and 96 days, respectively, compared with 71 days for the vehicle-treated mice.

To further understand how the kinetics of metastasis was mitigated, we tested PF-03732010 in the MDA-MB-435HAL-CDH3 experimental lung metastasis model by directly injecting cells into the vasculature, which omits the first steps of the metastatic cascade and enables the assessment of tumor cell colonization in host organs. PF-03732010 was administered either 24 hours before tumor cell injection using the prophylactic model or after tumors were established in lungs (at day 14) in the established tumor model.

Figure 4A depicts the effect of PF-03732010 on tumor burden in the prophylactic setting. PF-03732010 at the 10 and 20 mg/kg dose levels significantly ($P < 0.01$) inhibited tumor cell colonization (80% and 90% inhibition, respectively) compared with the vehicle-treated group, and the median survival time significantly ($P < 0.01$) improved to 87 and 93 days, respectively, over 54 days for the vehicle-treated mice (Fig. 4B). In the therapeutic setting, PF-03732010 significantly inhibited tumor growth ($P < 0.01$) in lungs at both 10 and 20 mg/kg dose levels with TGI values of 77.2% and 86.8%, respectively (Fig. 4C). Both evaluated dose levels also improved ($P < 0.05$) the median survival time to 74 days, compared with 55 days for vehicle-treated mice.

The overall results in the experimental metastasis model show that PF-03732010 not only inhibits tumor cell colonization in lungs but also inhibits tumor growth after being established in the host organ.

**PF-03732010 negatively modulates P-cadherin and β-catenin levels, results in antiproliferation and antimetastatic activities, as well as increased apoptosis**

P-cadherin mediates adherens junctions through direct binding of β-catenin with cadherin cytoplasmic domains. In addition to serving a functional role through P-cadherin to stabilize cell-cell adhesion, β-catenin acts as a signaling molecule to mediate cell invasion, migration, proliferation and survival through the transcription of target genes, including vimentin, cyclin D1, survivin, and BCL-2.

**Fig. 4.** PF-03732010 inhibits tumor cell colonization and lung tumor burden growth in the MDA-MB-435HAL-CDH3 experimental metastasis model. Values represent the mean ± SEM from 12 mice. In the prophylactic model, the representative BLI images (A) and line plots (B) depict the disease progression and the treatment effect. The IgG was also tested in the therapeutic setting (C). The difference in BLI measurements of the control groups between B and C was due to interexperimental variability.

At the efficacious doses (10, 20, and 40 mg/kg), PF-03732010 suppressed the levels of P-cadherin, β-catenin, vimentin, and Ki67 and increased caspase-3 activation, as shown in representative immunofluorescence or...
immunohistochemical images (Fig. 5A) and semiquantitative analysis (Fig. 5B and C) of the biomarkers in the MDA-MB-435HAL-CDH3 SRC tumor model. These data show that the disruption of the P-cadherin/β-catenin signaling pathway corresponds with diminished tumor cell invasiveness, proliferation, and increased apoptosis. E-cadherin levels were below the level of detection in this model; therefore, we were unable to determine whether E-cadherin plays a role in this signaling cascade.

In the PC3M-CDH3 prostate orthotopic model (Fig. 5D), Western blot and immunohistochemical analyses showed that the degradation of β-catenin induced by PF-03732010 (20 mg/kg) was accompanied by a decrease in cyclin D1 levels and the increase in caspase-3 activation, indicating that tumor cell antiproliferation and apoptosis were associated with the blockade of β-catenin signaling. Suppression of vimentin levels was also observed after treatment (not shown). PF-03732010 concurrently modulated the levels of survivin and BCL2, suggesting that apoptosis may result from suppression of these antiapoptotic proteins. Diminished P-cadherin levels were also observed after the treatment with PF-03732010, whereas E-cadherin levels were below the level of detection. These findings support the hypothesis that the disruption of the P-cadherin/β-catenin signaling pathway is critical for the antiproliferative and proapoptotic effects of PF-03732010 in these tumor models.

**Fig. 5.** PF-03732010 suppresses P-cadherin/β-catenin signaling and reduces levels of β-catenin signaling-mediated target proteins, including vimentin, cyclin D1, survivin, and Bcl2. Values represent the mean ± SEM from six mice. The representative images of tumors treated with vehicle or 20 mg/kg PF-03732010 (A) and semiquantitative analyses (B, C) of P-cadherin, β-catenin, vimentin, Ki67, and caspase-3 staining at all doses in the MDA-MB-435HAL-CDH3 SRC tumors. Western blot analysis of P-cadherin, cyclin D1, survivin, and Bcl2 levels and the representative immunohistochemical images of β-catenin, E-cadherin, and caspase-3 staining (D) in PC3M-CDH3 tumors. Images of P-cadherin (A), β-catenin (A, D), P-cadherin/β-catenin (A), vimentin (A), and E-cadherin (D) are at 40× magnification. The remaining immunohistochemical images (A, D) are at 10× magnification. All sample analyses were done after mice were treated with vehicle or PF-03732010 for 5 wk.
expression remained unchanged. PF-03732010 caused similar results in the DU145 (Supplementary Fig. S4) and H1650 orthotopic models.

**PF-03732010 shows antiproliferative activity via [18F] FLT-PET imaging in the MDA-MB-435HAL-CDH3 SRC tumor model**

In MDA-MB-435HAL-CDH3 SRC tumors, PF-03732010 at 10 and 20 mg/kg significantly (P < 0.01) reduced the uptake of [18F]FLT by 26.8% and 23.4%, respectively, in primary tumors on day 42 (Fig. 6A and B). The observed reduction in [18F]FLT uptake also occurred in the lung tumor burden (Fig. 6C and D). PF-03732010 at 10 and 20 mg/kg suppressed [18F]FLT uptake in the lung tumor burden by 31.7% and 37.8% (P < 0.01), respectively, compared with the vehicle treatment, showing the antiproliferative activity of PF-03732010 as a downstream effect of blocking P-cadherin signaling. Similar results were obtained on day 55.

**Discussion**

Here, we report the preclinical characteristics of PF-03732010, a human monoclonal antibody against P-cadherin. PF-03732010 efficiently blocks P-cadherin-mediated cell-cell adhesion by antagonizing the β-catenin signaling pathway, resulting in significant antiproliferative and antimetastatic activity. Because the IgG shows no noticeable signs of adverse effects at the efficacious doses, antagonizing P-cadherin with PF-03732010 represents a novel approach to anticancer therapy.
PF-03732010 showed high specificity and selectivity for P-cadherin targeting. The IgG failed to bind to the most closely target-related family members, including E-cadherin, N-cadherin, and VE-cadherin, as assessed by ELISA using recombinant proteins. In PC3M-pCL and MDA-MB-435HAL-pCL models, which express high level of N-cadherin but lack P-cadherin, treatment with PF-03732010 showed no therapeutic benefit. These data suggest that the IgG-associated efficacy was not due to the off-target activity by antagonizing N-cadherin. Specifically, PF-03732010 also lacked the ability to bind to endogenous E-cadherin in cell-based fluorescence-activated cell sorting analyses, confirming the high selectivity of the antibody to P-cadherin over E-cadherin. Because E-cadherin shares some homology with P-cadherin and E-cadherin functionally suppresses tumorigenesis or metastasis (32), the high specificity of PF-03732010 for P-cadherin over E-cadherin would allow a better therapeutic window to be achieved.

PF-03732010 impairs P-cadherin-mediated cell function, and at nanomolar concentrations, the antibody also blocked P-cadherin-mediated cell-cell adhesion and disrupted preestablished spheroids.

In tumor models, PF-03732010 induced significant TGI, although this antiproliferative activity was not observed when cells were treated with PF-03732010 in vitro. This discrepancy suggests that fully functioning P-cadherin signaling may require the cell-cell and cell-stroma crosstalk in an intact tumor architecture during tumorigenesis and metastasis, a process that may not be recapitulated under in vitro conditions. In the experimental system, we used P-cadherin-deficient MDA-MB-231, 4T1, PC3M, and MDA-MB-435HAL cells to construct cell lines that stably overexpress P-cadherin. PF-03732010 showed significant antitumor activity in the 4T1-CDH3 orthotopic, MDA-MB-231-CDH3 orthotopic, PC3M-CDH3 orthotopic, and the MDA-MB-435HAL-CDH3 SRC models, as well as in H1650 and DU145 orthotopic models, in which tumor cells endogenously expressed high levels of P-cadherin. By comparison, tumor models with minimal P-cadherin expression, including the MDA-MB-231-CDH3 orthotopic, PC3M-pCL orthotopic, MDA-MB-435HAL-pCL SRC (Supplementary Fig. S5A and B), and the MDA-MB-435HAL-pCL experimental metastasis (Supplementary Fig. S5C and D) models, showed no antitumor response to PF-03732010; these results suggest that PF-03732010 only inflicts efficacy in tumors with high P-cadherin expression. P-cadherin is frequently upregulated in a diverse panel of malignancies (15, 33, 34) and often associated with high histologic grade, invasiveness, and poor survival rate (14, 35). The broad spectrum activity of PF-03732010 provides rationale and opportunity in the clinical development of the antibody for treating patients with tumors with high P-cadherin expression.

Increasing evidence indicates that high P-cadherin expression in human cancer cells correlates with increased cell motility (15) and invasiveness (20), which led us to investigate whether PF-03732010 slows down metastasis by blocking the P-cadherin signaling pathway. During the metastatic cascade, malignant cells break away from the primary tumor, shed into the bloodstream or migrate through the lymphatic system, and eventually colonize at the preferred organ microenvironment, as proposed in Paget’s “seed and soil” theory (36). PF-03732010 reduced lymph node metastases via BLI in the PC3M-CDH3 and DU145 prostate orthotopic models and lowered CTC levels in mouse whole blood of PC3M-CDH3 tumor-bearing mice. The antimetastatic property of the antibody was further confirmed in the MDA-MB-435HAL-CDH3 SRC model; via BLI and CT imaging, as well as H&E staining of lungs, PF-03732010 significantly inhibited tumor cell infiltration into the lungs at all doses tested (10, 20, and 40 mg/kg). Also using this model, PF-03732010 reduced CTC levels in whole blood. Supported by the in vitro assay results (data not shown), the ability of PF-03732010 to suppress CTCs in mouse whole blood could be partially caused by antibody-dependent cell-mediated cytotoxicity.

Among the millions of cells that primary tumors release into the circulation, only a small minority of them will adhere at a distant organ and eventually establish metastases (37). We therefore assessed whether the antimetastatic characteristic of PF-03732010 was entirely due to the reduction of CTCs in whole blood. In the MDA-MB-435HAL-CDH3 prophylactic metastasis model, mice were pretreated with PF-03732010 to ensure that plasma levels of the IgG were maintained above the effective biological concentration when the tumor cells were i.v. injected (24 hours posttreatment). PF-03732010 significantly inhibited tumor cell adhesion in the lungs, and similar results were observed in the HCT-116 and DU145 experimental metastasis models. Notably, under the same settings, MDA-MB-435HAL-pCL cells did not respond to PF-03732010 in the prophylactic metastasis model, suggesting that the blockade of MDA-MB-435HAL-CH3 colonization in the lungs was due to impaired P-cadherin signaling. Overall, the antimetastatic activity of PF-03732010 is attributed to its ability to reduce tumor cells in the circulatory system and mitigate tumor cell colonization in the host organ.

P-cadherin knockdown in cells resulted in reduced tumor cell invasiveness regardless of E-cadherin expression levels (17). Indeed, loss of P-cadherin expression after PF-03732010 treatment resulted in decreased metastasis without any effect on the E-cadherin level, presumably, in part, due to the reduced tumor cell adhesiveness. More importantly, the antimetastatic activity was derived from the diminished level of β-catenin, which not only plays a functional role in stabilizing P-cadherin-mediated cell-cell adhesion but also regulates molecular signaling pathways to promote tumor growth, invasiveness, and metastasis (17, 19). It has been shown that P-cadherin failed to induce invasion when the catenin-binding domain was mutated (38). When released from the plasma membrane, β-catenin is stabilized by Wnt signaling and subsequently translocates into the nucleus, where it acts as a coactivator with T-cell factor and induces transcriptional activation of target genes (32, 39). This pathway activation is critical for epithelial-mesenchymal transition, a mechanism that
contributes to metastasis (40). During epithelial-mesenchymal transition, one of the activated targets of β-catenin signaling is vimentin (18), which has been implicated in tumor cell invasiveness and migratory potential. In several tested xenografts, including MDA-MB-435HAL-CDH3 SRC, PC3M-CDH3, and DU145 orthotopic models, efficacious doses of PF-03732010 concurrently suppressed membrane β-catenin and vimentin levels and resulted in antimetastatic activity, further confirming that the IgG-induced disruption of β-catenin signaling contributes to antibody efficacy. In addition, as Bcl-2 overexpression leads to enhanced metastatic potential in human cancers (41, 42), the suppression of Bcl-2 could account for the PF-03732010-induced antimetastatic property. The cause of the IgG-induced suppression of β-catenin levels is yet to be fully understood; one possibility is that once β-catenin is released into the cytoplasm, it is stabilized by Wnt signaling in a coordinated fashion. The blockade of P-cadherin/β-catenin signaling disrupts this process and results in the subsequent degradation of β-catenin by nonspecific enzymes (43).

β-catenin is well known for controlling a wide array of cellular functions. High levels of nuclear β-catenin have been frequently observed in cells with β-catenin or APC gene mutations (44). We were unable to detect nuclear β-catenin levels in the cell lines used in this report, including MDA-MB-435-CDH3, PC3-CDH3, and DU145, possibly due to their functional (wild-type) β-catenin and APC proteins. One noteworthy observation is that, although the cellular distribution of β-catenin was primarily detected on the membrane, nuclear β-catenin remains transcriptionally active, which is consistent with previous findings (45). The supporting data are that the decrease in membrane β-catenin by PF-03732010 not only resulted in diminished levels of vimentin (Fig. 5A) but also the reduction in other β-catenin transcription-mediated target proteins (Fig. 5D), including cyclin D1, survivin, and Bcl-2 (46, 47). Cyclin D1 mediates tumor cell proliferation and survival, and Bcl-2 prevents tumor cells from undergoing apoptosis. These observations provide evidence that, in addition to the reduced metastatic potential, suppressing β-catenin levels by PF-03732010 also resulted in antiproliferative activity and apoptosis in tumor cells. The concurrent decrease in β-catenin and Ki67 levels, as well as the increase of caspase-3 staining, further confirmed the biological relevance of a β-catenin signaling blockade with the PF-03732010–induced antitumor activity.

The antiproliferative activity of PF-03732010 was further assessed by using the hybrid [18F]FLT-PET/CT imaging in the MDA-MB-435HAL-CDH3 SRC model. In agreement with the results from Ki67 immunohistochemical staining, PF-03732010 significantly (P < 0.05 versus vehicle group) suppressed [18F]FLT uptake in both primary and secondary tumors, indicative of antiproliferative activity as a downstream effect of blocking P-cadherin/β-catenin signaling. The results also suggest that [18F]FLT-PET imaging may be used in early clinical development of PF-03732010 as a supplementary surrogate biomarker. Comparing to the conventional biopsy approach, the imaging technology in some cases presents advantages due to the noninvasive nature and less heterogeneity. In the clinical setting, showing the sufficient reduction in [18F]FLT uptake in patients on treatment of PF-03732010 at biologically effective doses would constitute proof of mechanism (48).

Taken together, these data highlight the critical role of P-cadherin/β-catenin signaling in regulating tumor cell metastasis, proliferation, and apoptosis. PF-03732010 antagonizes P-cadherin signaling and leads to the antitumor and antimetastatic efficacy in target-associated tumor models without introducing noticeable adverse effect. This work provides the rationale and guidance for the clinical development of PF-03732010, in which tumors with high P-cadherin expression will be essential criteria for patient selection. Future work is warranted to seek a refined therapeutic strategy for patient stratification and combination therapies, aiming to reach the full potential for clinical development of the IgG. PF-03732010 is currently under Phase 1 trial development.

Disclosure of Potential Conflicts of Interest

All authors are current or former employees and shareholders of Pfizer, Inc.

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