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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. 06-112.530

Solicitud de patente de invención titulada: "DERIVADOS DE 5-(4-AMINO-5-(TRIFLUOROMETIL)PIRIMIDIN-2-ILAMINO)INDOLIN-2-ONA, ACTIVOS CONTRA EL CRECIMIENTO CELULAR ANORMAL"

Solicitante: PFIZER PRODUCTS INC

Nuestra Ref.: P2006/000220

RESPUESTA AL OFICIO No.13725 NOTIFICADO POR FIJACION EN LISTA No. 328
DE FECHA 02 DE SEPTIEMBRE DE 2011
(TERCER EXAMEN DE FONDO)

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad solicitante, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del plazo legal previsto en el artículo 45 de la Decisión 486, en los siguientes términos:

1. En primer lugar, doy por entendido que mediante el Oficio No. 13725 se revoca en su totalidad el Oficio 5123 notificado por fijación de lista de fecha 18 de marzo, por incluir como anterioridad para el estudio de la novedad una solicitud PCT que fue retirada del trámite en Colombia; por lo tanto, el presente memorial corresponde a mi respuesta al nuevo tercer examen de fondo.
2. Ahora bien, con el ánimo de brindar mayor claridad a la presente solicitud, me permito adjuntar un **Capítulo Reivindicatorio Modificado**, que reemplaza en su totalidad al Capítulo Reivindicatorio presentado en respuesta al segundo examen de fondo, de fecha 21 de febrero de 2011.

Así las cosas, el Capítulo Reivindicatorio Modificado adjunto contiene los siguientes cambios:

- se han eliminado las Reivindicaciones 1 a 12;
- las nuevas Reivindicaciones 1 y 2 corresponden a las antiguas Reivindicaciones 13 y 14, que reclaman específicamente el compuesto N-metil-N-(3-{[2-(2-oxo-2,3-dihidro-1H-indol-5-ilamino)-5-trifluorometil-pirimidin-4-ilamino]-metil}-piridin-2-il)-metanosulfonamida, sales farmacéuticamente aceptables del mismos, y composiciones farmacéuticas que los contienen.

Valga anotar que las modificaciones efectuadas al Capítulo Reivindicatorio cumplen a cabalidad con lo estipulado en el Artículo 34 de la Decisión 486, ya que éstas de ninguna manera implican una ampliación de la protección correspondiente a la divulgación contenida en la solicitud inicial, por el contrario, éstas corresponden a una drástica limitación de la materia reivindicada.

3. Reitero en este memorial el cambio de título realizado de acuerdo con la sugerencia del Examinador en su Oficio 5123 (revocado), de tal forma que el título de la solicitud ha sido modificado a:

“DERIVADOS DE 5-(4-AMINO-5-(TRIFLUOROMETIL)PIRIMIDIN-2-ILAMINO)INDOLIN-2-ONA, ACTIVOS CONTRA EL CRECIMIENTO CELULAR ANORMAL”.

4. a- El Examinador insiste en que las reivindicaciones no son claras, en tanto que contienen términos muy amplios tales como “heterociclilo”, “O-heterociclilo”, “heteroarilo”, “(CO)heteroarilo”, “(CO)heterociclilo”, “(CO)O-heterociclilo”, “(CO)O-heteroarilo”, entre otros, que contienen la expresión “hetero”. El Examinador señala que dichas expresiones no se encuentran definidas, puesto que no se especifica el tipo o número de heteroátomos en el anillo, y podían comprenderse compuestos que no se encuentran divulgados en el Capítulo Descriptivo.

b- Llamo respetuosamente la atención del Examinador sobre las modificaciones presentadas en el Capítulo Reivindicatorio adjunto, de acuerdo con lo explicado en el punto 2 de este memorial, en las que la presente invención ahora reclama específicamente el compuesto N-metil-N-(3-{[2-(2-oxo-2,3-dihidro-1H-indol-5-ilamino)-5-trifluorometil-pirimidin-4-ilamino]-metil}-piridin-2-il)-metanosulfonamida, sales farmacéuticamente aceptables del mismos, y composiciones farmacéuticas que los contienen. Teniendo en cuenta lo anterior, considero superada la anterior objeción.

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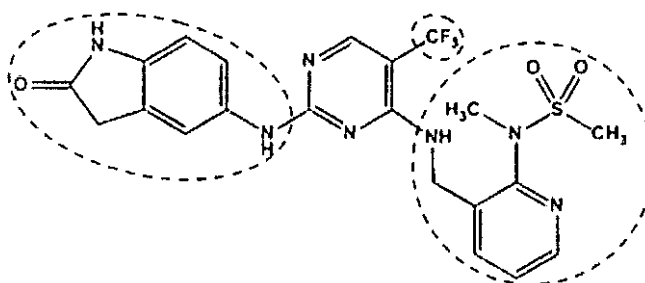
5. a- El Examinador insiste en que la patente WO03030909 (D1) y el documento "Inhibidores de proteína quinasa como agentes anticáncer"¹ (D4), afectan el nivel inventivo de la presente invención.

El Examinador señala que D1 divulga derivados de pirimidina, activos como agentes neoplásicos, que se diferencian de la presente invención en el radical amino-2-oxo-2,3-dihidro-indol que no se enseña en D1.

Sin embargo, el Examinador señala que en D4, un documento que hace un resumen de compuestos patentados en el campo de los inhibidores de proteína quinasa, se enseñan algunos compuestos que contienen el anillo 2-oxo-2,3-dihidroindol, demostrando que dicho anillo es una característica esencial en compuestos anticancerígenos.

El Examinador concluye que una persona medianamente versada en la materia habría tomado los compuestos divulgados en D1, incluyendo el anillo 2-oxo-2,3-dihidroindol divulgado en los compuestos de D4, llegando de manera obvia a los compuestos de la presente invención. Adicionalmente, el Examinador señala que no se evidencia un efecto mejorado en los compuestos aquí reclamados.

b- i) En primer lugar, me permito llamar nuevamente la atención del Examinador respecto a las modificaciones realizadas en el Capítulo Reivindicatorio Modificado adjunto, en las que se eliminaron las Reivindicaciones 1 a 14, y ahora se reclama específicamente el compuesto N-metil-N-(3-{[2-(2-oxo-2,3-dihidro-1H-indol-5-ilamino)-5-trifluorometil-pirimidin-4-ilamino]-metil}-piridin-2-il)-metanosulfonamida, ilustrado a continuación:



sus sales farmacéuticamente aceptables y composiciones farmacéuticas que lo contienen.

¹ Supuran C. & Scozzafava A. "Review: Protein tyrosine kinase inhibitors as anticancer agents" Expert Opinion on Therapeutic Patents. Ashley Publications. Vol. 14. Pages 35-53. 2004.

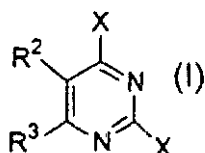
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ii) Ahora bien, es importante resaltar que el compuesto de la presente invención incluye una combinación de características estructurales esenciales, que incluyen un núcleo pirimidina trisustituido en las posiciones 2, 4 y 5, de la siguiente manera:

- a) un grupo 5-amino-2-oxo-2,3-dihidro-indol en posición 2 unido a través del grupo 5-amino;
- b) un grupo trifluorometilo en posición 5; y
- c) una fracción aminometil-3-piridilo en la posición 4, en donde el anillo piridina se encuentra sustituido adicionalmente por una fracción N-metil-N-metanosulfonilo.

Por el contrario, y como lo señalé en respuesta al primer examen de fondo (Oficio 08812, notificado por fijación en lista No. 242 del 09 de julio de 2010), D1 divulga una gran familia de derivados de 2- y 4-aminopirimidinas, sustituidas en el nitrógeno por un anillo bicíclico, como inhibidores de serin y tirosin quinasas, particularmente CDK. Sin embargo, D1 no divulga o sugiere específicamente que tales compuestos presenten actividad como inhibidores FAK. En particular, los compuestos de D1 se encuentran sustituidos por un radical X en posición 2 y 4 del anillo pirimidina, que comprende los grupos NR^1R^6 , NR^4R^5 y R^4 ; dichos sustituyentes abarcan una gran cantidad de grupos químicos diversos, dentro de los cuales se encuentran particularmente ilustrados en los 375 ejemplos, los grupos quinolina y 2,3-dihidro-1H indazol solos o sustituidos por uno o varios grupos metilo.



Ahora bien, D1 proporciona datos sobre actividad *in vitro* para 8 compuestos de la Tabla 8 (ver de la página 88, línea 30 a la página 89, línea 1), en donde el compuesto divulgado de mayor actividad es el Ejemplo 56, que presenta un IC_{50} de 360nM en ese ensayo². Adicionalmente, en D1 se enseña que el Ejemplo 59 presenta actividad en un ensayo de inhibición de crecimiento tumoral *in vivo*, sin embargo, no se proporciona ningún tipo de datos experimentales (ver página 90, líneas 32 y 33).

Así entonces, todos los compuestos 2,4-diaminopirimidinas para los cuales se proporcionan datos experimentales se encuentran sustituidos en la posición 4 por un grupo aminoquinolina o aminoindazol; todos ellos, con excepción del Ejemplo 10 (que presenta una fracción 2-

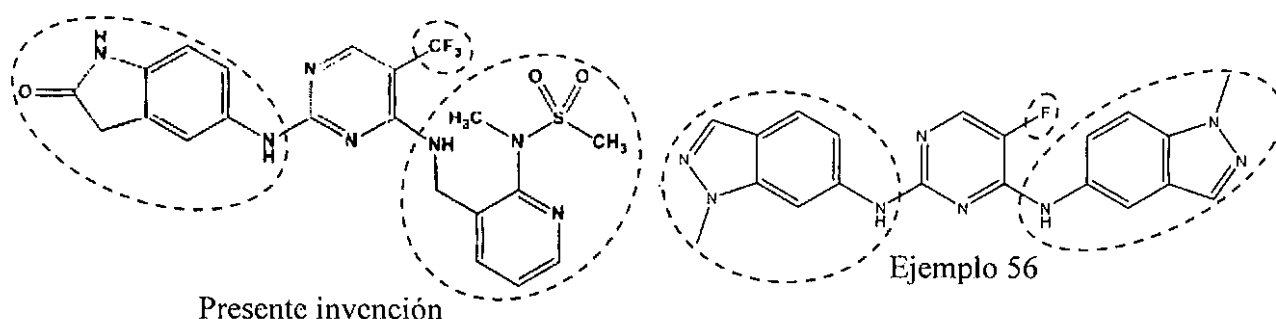
² Es importante señalar que el Compuesto 390 presenta una mejor actividad con un IC_{50} de 240nM, sin embargo, tal ejemplo no existe en D1.

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hidrazinilo), se encuentran sustituidos en la posición 2 por un grupo aminoindazol, anilina o aminoquinolina. De lo anterior es posible concluir que todos los derivados del núcleo 2,4-diaminopirimidina de D1 que se identifican como compuestos activos, contienen dos grupos heteroarilo unidos directamente al grupo anillo pirimidina a través de un átomo de nitrógeno.

Por el contrario, el compuesto reivindicado en la presente solicitud difiere del compuesto más activo en D1 (Ejemplo 56) en los grupos que sustituyen las posiciones 2, 4 y 5 el anillo pirimidina central, como se evidencia a continuación:

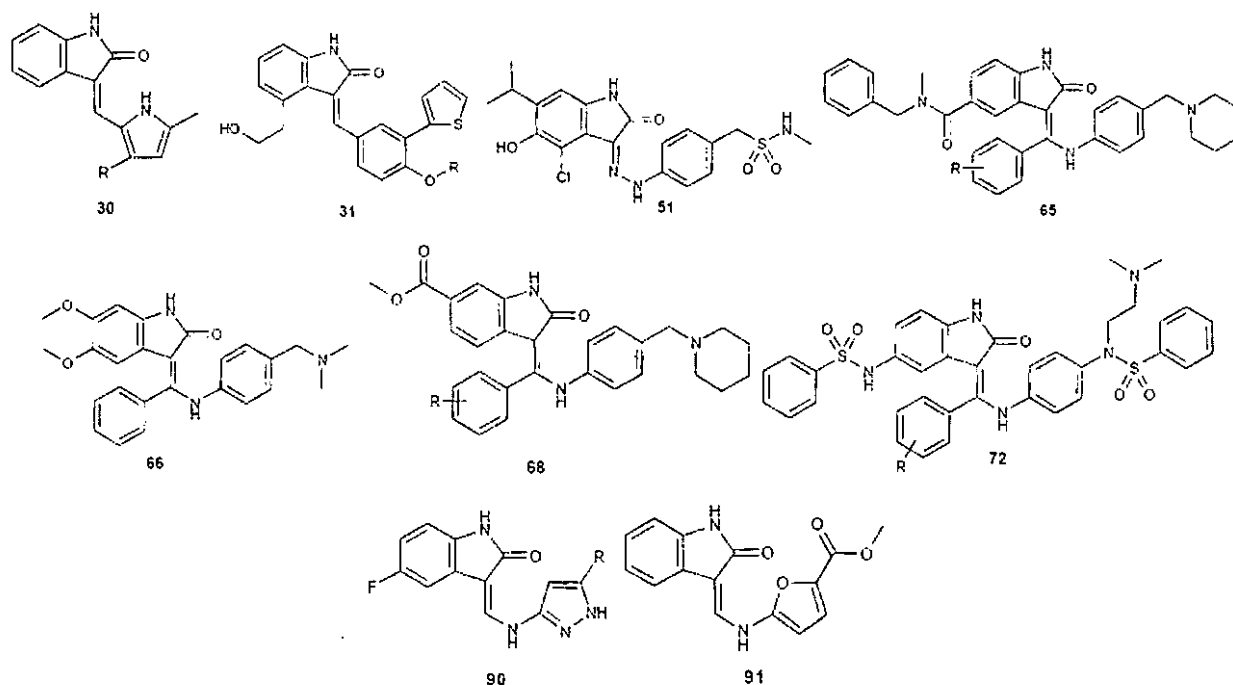


Teniendo en cuenta las anteriores diferencias estructurales, una persona medianamente versada en la materia no habría encontrado sugerencia o enseñanza alguna en D1 para desarrollar el compuesto aquí reivindicado, con alguna esperanza razonable sobre su actividad para inhibir FAK, puesto que el compuesto que reporta mayor actividad en D1 sugiere patrones de sustitución en las posiciones 2, 4 y 5 drásticamente diferentes. De hecho, siguiendo las enseñanzas de D1 los compuestos reportados que presentan mayor actividad, incluyen los mismos grupos heteroarilo en posiciones 2 y 4 (Ejemplos 56, 57 y 66) o un grupo aminoindazol en posición 2, y un aminofenilo sustituido por un cloro (Ejemplo 74) o un un aminofenilo sustituido por un etoxi (Ejemplo 373). Ninguno de los compuestos con mayor actividad en D1 enseñan o siquiera sugieren que los dos grupos heteroarilo que sustituyen las posiciones 2 y 4 del núcleo central (X en la Fórmula I de D1) pueden corresponder a una fracción 5-amino-2-indolin-2-ona, y menos aún que dichas posiciones podrían estar sustituidas por un grupo piridina sustituido por un grupo N-metil-N-metanosulfonamida a través de un grupo aminoetileno, como se reclama en el compuesto de la presente invención.

iii) De otra parte, D4 divulga ciertos compuestos que presentan un grupo indolin-2-ona, que presentan las siguientes estructuras:

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Sin embargo, a pesar de que todos estos compuestos, efectivamente presenten un grupo indolin-2-ona, **nada** en D4 enseña o siquiera sugiere cómo sintetizar el compuesto N-metil-N-(3-([2-(2-oxo-2,3-dihidro-1H-indol-5-ilamino)-5-trifluorometil-pirimidin-4-ilamino]-metil)-piridin-2-il)-metanosulfonamida, como se reivindica en la presente invención. De hecho, es importante señalar que **ninguno** de los anteriores compuestos presenta siquiera un anillo pirimidina, y menos aún, un grupo piridina sustituido por una fracción N-metil-N-metanosulfonamida a través de un grupo aminoetileno. De hecho, los compuestos 65, 66, 68, 72, 90 y 91 unen los diferentes anillos heterociclos a través de un puente C=C-NH, así como los compuestos 30 y 31 lo hacen a través de un puente C=C, y el compuesto 51 a través de un puente C=N-NH.

Teniendo en cuenta las características estructurales de los compuestos de D4, la persona medianamente versada en la materia se habría inclinado a incluir un grupo alquilideno en la posición 3 del núcleo indolin-2-ona, lo cual no sucede en el compuesto de la presente invención.

iv) Determinado aquello que es diferente con respecto al arte previo, es muy importante que quién examine la materia de la solicitud de patente la analice como un todo. No es dable a quien examina una patente simplemente desglosar sus elementos separadamente y luego empezar a buscar referencias en el estado del arte que los incluyan, como si de armar un rompecabezas con piezas aisladas, o una colcha de retazos, se tratara.

En el presente caso, el Examinador concluye que la simple mención de ciertos compuestos en un artículo que recopila información sobre compuestos terapéuticamente efectivos como anticancerígenos, y que presentan aisladamente un anillo indolin-2-ona, es motivación suficiente

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para que la persona medianamente versada en la materia los incluyera en los compuestos divulgados en D1. Sin embargo, el Examinador omite el grupo que sustituye la posición 3 del anillo indolin-2-ona de dichos compuestos, el cual puede ser un grupo C=C-NH, C=C o C=N-NH a través del cual se unen otros grupos heteroarilos.

Es claro que el Examinador no evalúa el arte previo como un todo. Por el contrario, el Examinador se limita a señalar un grupo aislado en los compuestos de D4 sin determinar, por ejemplo, qué motivación tendría la persona medianamente versada en la materia para tomar en cuenta compuestos que presenta diferencias estructurales drásticas respecto a la presente invención; de hecho, **ninguno** de los compuestos señalados por el Examinador presentan siquiera un grupo pirimidina en su estructura. Es importante entonces preguntarse si también serían motivación suficiente para el técnico medio todos los compuestos anticancerígenos que presenten un grupo pirimidina, así sus patrones de sustitución en cada posición sean diferentes.

Contrario a lo evaluado por el Examinador, considero respetuosamente que las enseñanzas de D1 y/o D4, solos o en combinación, alejarían a la persona medianamente versada en la materia del compuesto de la presente invención, ya que dichos documentos lo habrían inclinado a desarrollar compuestos estructuralmente diferentes. Por lo cual, la presente invención reviste nivel inventivo.

Por último, me permito presentar adjunto a este memorial el Anexo 1 “Antitumor activity and pharmacology of a selective focal adhesión kinase inhibitor, PF-562,271”, en el cual se presentan los resultados obtenidos por el compuesto de la presente invención (denominado en el artículo PF-562,271) en diferentes ensayos que demuestran su actividad antitumoral mediante la inhibición de la FAK. El compuesto de la presente invención es un inhibidor potente de la actividad catalítica de las quinasas FAK (Focal Adhesion Kinase) y Pky2 (FAK2) que presenta un IC₅₀ de 1,5 y 1,4 nM, respectivamente, y presenta una selectividad frente a otras quinasas evaluadas de entre 20 y 100 veces más (ver Tabla 1, página 1939, en donde se resume el perfil *in vitro* del compuesto PF-562,271). Adicionalmente, el compuesto aquí reivindicado demostró una alta eficacia antitumoral en diferentes modelos de xenoinjertos de cáncer de próstata, seno, páncreas y colon, los cuales se evidencian en la Tabla 2, página 1942, y en las páginas 1938 y 1939.

Los anteriores resultados farmacológicos que muestra el compuesto de la presente invención, permiten concluir que no solo presenta una estructura química imposible de prever a partir del arte previo, sino que presenta un efecto técnico farmacológico sorprendente e inesperado. Según

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todo lo anterior, y como lo mencioné en respuesta al primer examen de fondo, la presente invención cumple con lo establecido por el Manual Andino de Patentes (MAP), particularmente, que las invenciones en el campo de la química deben ser consideradas como inventivas cuando los compuestos reclamados tienen una estructura inesperada (no predecible por la persona medianamente versada en la materia) y proporcionan efectos mejorados o inesperados³, por lo cual, de nuevo, reviste nivel inventivo.

6. Por todo lo anterior, considero que las objeciones presentadas por el Examinador en su examen de fondo No. 2171 han sido resueltas, y por consiguiente solicito respetuosamente que el Despacho tenga por cumplidos los requisitos de patentabilidad y proceda con la concesión de la patente.

Atentamente,



CARLOS R. OLARTE
C.C. No. 79.782.747 de Bogotá
T.P. 74.295

Anexos:

- Capítulo Reivindicatorio Modificado.
- Anexo 1: Roberts, W. *et al.* "Antitumor activity and pharmacology of a selective Focal Adhesión Kinase inhibitor, PF-562,271". Cancer Research. Vol 68. Pages 1935-1944. 2008.

³ Manual Andino de Patentes. (2004). Sección 10.8.1 (disponible en <http://www.comunidadandina.org/public/patentes.pdf>).

CAPITULO REIVINDICATORIO MODIFICADO
(Tercer Examen de Fondo)

1. Un compuesto que es N-metil-N-(3-{[2-(2-oxo-2,3-dihidro-1H-indol-5-ilamino)-5-trifluorometil-pirimidin-4-ilamino]-metil}-piridin-2-il)-metanosulfonamida o una sal farmacéuticamente aceptable del mismo.
2. Una composición farmacéutica que comprende un vehículo farmacéuticamente aceptable y N-metil-N-(3-{[2-(2-oxo-2,3-dihidro-1H-indol-5-ilamino)-5-trifluorometil-pirimidin-4-ilamino]-metil}-piridin-2-il)-metanosulfonamida o una sal farmacéuticamente aceptable del mismo

Cancer Research

Antitumor Activity and Pharmacology of a Selective Focal Adhesion Kinase Inhibitor, PF-562,271

Walter Gregory Roberts, Ethan Ung, Pamela Whalen, et al.

Cancer Res 2008;68:1935-1944. Published online March 13, 2008.

Updated Version	Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-07-5155
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2008/06/19/68.6.1935.DC1.html

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Antitumor Activity and Pharmacology of a Selective Focal Adhesion Kinase Inhibitor, PF-562,271

Walter Gregory Roberts, Ethan Ung, Pamela Whalen, Beth Cooper, Catherine Hulford, Christofer Autry, Daniel Richter, Earling Emerson, Jing Lin, John Kath, Kevin Coleman, Lili Yao, Luis Martinez-Alsina, Marianne Lorenzen, Martin Berliner, Michael Luzzio, Nandini Patel, Erika Schmitt, Susan LaGreca, Jitesh Jani, Matt Wessel, Eric Marr, Matt Griffor, and Felix Vajdos

Pfizer Oncology, Groton, Connecticut

Abstract

Cancer cells are characterized by the ability to grow in an anchorage-independent manner. The activity of the non-receptor tyrosine kinase, focal adhesion kinase (FAK), is thought to contribute to this phenotype. FAK localizes in focal adhesion plaques and has a role as a scaffolding and signaling protein for other adhesion molecules. Recent studies show a strong correlation between increased FAK expression and phosphorylation status and the invasive phenotype of aggressive human tumors. PF-562,271 is a potent, ATP-competitive, reversible inhibitor of FAK and Pyk2 catalytic activity with a IC_{50} of 1.5 and 14 nmol/L, respectively. Additionally, PF-562,271 displayed robust inhibition in an inducible cell-based assay measuring phospho-FAK with an IC_{50} of 5 nmol/L. PF-562,271 was evaluated against multiple kinases and displays $>100\times$ selectivity against a long list of nontarget kinases. PF-562,271 inhibits FAK phosphorylation *in vivo* in a dose-dependent fashion (calculated EC_{50} of 93 ng/mL, total) after p.o. administration to tumor-bearing mice. *In vivo* inhibition of FAK phosphorylation ($>50\%$) was sustained for >4 hours with a single p.o. dose of 33 mg/kg. Antitumor efficacy and regressions were observed in multiple human s.c. xenograft models. No weight loss, morbidity, or mortality were observed in any *in vivo* experiment. Tumor growth inhibition was dose and drug exposure dependent. Taken together, these data show that kinase inhibition with an ATP-competitive small molecule inhibitor of FAK decreases the phospho-status *in vivo*, resulting in robust antitumor activity. [Cancer Res 2008;68(6):1935–44]

Introduction

A hallmark of cancer is the ability for cells to proliferate regardless of adhesion to a substrate (1). Mounting evidence suggests that focal adhesion kinase (FAK) plays an essential role in the ability of cells to grow in an adhesion-independent manner (2). FAK is a nonreceptor tyrosine kinase that transduces signaling from a diverse group of stimuli (e.g., integrins, cytokines, chemokines, and growth factors) to control a variety of cellular pathways and processes, including cell proliferation, migration, morphology, and cell survival (3, 4). Consistent with these cellular functions and

implicating the importance of FAK in tumor progression, exogenous expression of the tumor suppressor gene PTEN results in dephosphorylation of FAK (5). FAK is found at elevated levels in most human cancers, particularly as cancers transform into highly invasive metastases (6). This makes disruption of FAK activity and function an exciting and novel approach to targeted anticancer therapy against virtually any solid tumor (7).

FAK is localized at sites of contact with the extracellular matrix (ECM; focal adhesions) and serves to transduce signals from integrin receptors and enhance signaling by growth factor receptors (8, 9). The transmembrane integrin receptors are important for linking the ECM proteins with the cellular actin cytoskeleton and the nucleus to regulate cell morphology, tissue architecture, and attachment-induced gene expression (10). FAK activity enhances signaling of many receptor tyrosine kinases that are themselves viable anticancer targets, such as insulin-like growth factor receptor (IGF-IR) (11), the crbB family (12), and angiogenesis receptors (13). Given that integrin signaling is mediated through FAK catalytic activity (14), a small molecule inhibitor of FAK could be considered a nonantibody approach to inhibit integrin signaling. Interestingly, loss of FAK activity using conditional knockout mice disrupted angiogenesis in late-stage embryos but did not affect vasculogenesis (15), suggesting that FAK inhibition may also result in an antiangiogenic effect (16). These data are consistent with the role of FAK in signal transduction of known angiogenic integrins as well as the fact that overexpression of FAK in endothelial cells promotes angiogenesis (17, 18).

Pyk2 is the only other member of the FAK family with 48% amino acid identity (19). However, the role of Pyk2 in tumorigenesis is not well-established. Although FAK is expressed in most tissues and cell types, Pyk2 seems to have more limited tissue distribution (hematopoietic cells, vascular smooth muscle, endothelium, spleen, kidney, and central nervous system; ref. 19). Unlike the lethal FAK knockout (20), Pyk2 knockout mice develop normally except that they exhibit defective macrophage migration and have increased bone density (21). Interestingly and importantly for prostate and breast cancer patients with or without bone metastasis, dual FAK/Pyk2 inhibitors resulted in prevention of bone resorption and increased bone formation while inhibiting the growth of bone metastases (22, 23).¹

PF-562,271 represents an unprecedented approach to treating cancer through FAK inhibition. Moreover, FAK inhibition is

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Walter Gregory Roberts, Oncovia Therapeutics, 100 Plaza Court #479, Groton, CT 06340. E-mail: robertswg@oncoviarx.com.

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doi:10.1158/0008-5472.CAN-07-5155

¹ C.M. Bagi, C.J. Andresen, W.G. Roberts. Dual inhibitor of FAK and Pyk2 protein kinase has positive effects on lytic bone tumors in nude rats—Implications for bone metastases. Manuscript in submission, 2008.

expected to affect both tumor and endothelial cells. With its novel target and dual antitumor and antiangiogenesis mechanisms of action, PF-562,271 offers the potential to enhance the current armamentarium of cancer therapeutics as a single agent or in combination with cytotoxics or other targeted agents.

Materials and Methods

Chemical synthesis. PF-562,271 was identified through a combination of rational design, high throughput kinase activity screens, and structure-activity relationships as previously described (24).

Expression. The FAK catalytic domain (FAKcd) protein used for crystallization experiments was expressed with an NH₂-terminal 6XHis-tag and comprises residues 410 to 689 after thrombin cleavage (FAK sequence with residue 410 changed from a Pro to a Gly). FAKcd was expressed in Sf9 insect cells using the pFastBac expression vector (Invitrogen Corp.).

Purification. Cell paste was resuspended in 400 mL buffer A [50 mmol/L HEPES (pH 7.0), 500 mmol/L NaCl, 0.1 mmol/L Tris (2-carboxy-ethyl)-phosphine-HCl (TCEP), and Complete, EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics)]. The cells were lysed and cell debris was removed by centrifugation at 4°C for 35 min at 28,000 × *g*. The supernatant was incubated with Ni-NTA Superflow (QIAGEN GmbH) with gentle rocking at 4°C for 1 h and then subjected to centrifugation at 700 × *g* in a swinging bucket rotor. The supernatant was discarded and the Ni-NTA resin was resuspended in 20.0 mL of buffer A and transferred to an XK-16 column connected to a fast protein liquid chromatography (GE Healthcare Bio-Sciences Corp.). The Ni-NTA resin was washed with five-column volumes of buffer A and eluted off the column with a step gradient of buffer A containing 300 mmol/L imidazole. The eluted fractions were exchanged into buffer B [25 mmol/L HEPES (pH 7.0), 400 mmol/L NaCl, and 0.1 mmol/L TCEP]. After buffer exchange, high-activity thrombin (Calbiochem of Merck KGaA) was added to the pooled fractions at a 1:300 (w/w) ratio and incubated overnight at 13°C to remove the NH₂-terminal His-tag. The reaction mixture was loaded back onto the Ni-NTA column equilibrated with buffer A, and the flow-through was collected. The flow-through was concentrated down to 1.7 mL and loaded directly onto a Superdex 200 HiLoad 16/60 prep grade column (GE Healthcare Bio-Sciences Corp.) equilibrated with buffer C [10 mmol/L HEPES (pH 7.5), 200 mmol/L ammonium sulfate, and 0.1 mmol/L TCEP]. The protein was stored frozen in aliquots at -80°C in buffer C.

Crystallization. The FAKcd/PF-562,271 complex was crystallized using hanging drop vapor diffusion in 24-well VDX plates (Hampton Research). The protein was mixed 1:1 with reservoir solution containing 15% polyethylene glycol 8K, 0.1 mol/L HEPES (pH 7.5), 0.2 mol/L (NH₄)₂SO₄. The concentration of PF-562,271 was 10 μmol/L. Crystals grew to 0.08 × 0.08 × 0.02 mm over 3 to 5 d.

Data collection and structure determination. X-ray diffraction data for FAKcd/PF-562,271 was collected on a FRED rotating anode X-ray generator outfitted with a HTC image plate detector system (Rigaku/MSK, Inc.). After transfer to a cryoprotectant consisting of 80% reservoir solution + 20% ethylene glycol + 10 μmol/L compound, crystals were flash cooled in liquid nitrogen. All images were processed using HKL2000 (25). Data statistics are reported in Supplementary Data.

The FAKcd/PF-562,271 structure was determined by the molecular replacement method, using the program MOLREP (26), using as a search model a FAKcd monomer structure derived from an ongoing structure-based drug design program. Refinement of the structures was performed using the program REFMAC (27), Arp/Warp (28, 29), and Xfit (30). Various data, coordinate, and map manipulations were performed using programs from the CCP4 suite (29).

Recombinant kinase assay and enzyme kinetics. All *in vitro* assays used for identification of a FAK inhibitor have been previously described (31). Briefly, purified-activated FAK kinase domain (amino acid 410-689) was reacted with 50 μmol/L ATP and 10 μg per well of a random peptide polymer of Glu and Tyr, p(Glu/Tyr), in kinase buffer [50 mmol/L HEPES (pH 7.5), 125 mmol/L NaCl, and 48 mmol/L MgCl₂] for 15 min.

Phosphorylation of p(Glu/Tyr) was challenged with serially diluted compound at 1/2-Log concentrations starting at a top concentration of 1 μmol/L. Each concentration was tested in triplicate. Phosphorylation of p(Glu/Tyr) was detected with a general antiphospho-tyrosine (PY20) antibody followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody. HRP substrate was added, and absorbance readings at 450 nm were obtained after addition of stop solution (2 mol/L H₂SO₄). IC₅₀ values were determined using the Hill-Slope Model. Broad kinase selectivity profiling was performed in house and by using the KinaseProfiler Selectivity Screening Service available through Upstate Biotechnology.²

Cell-based FAK phosphorylation assay. The GeneSwitch Inducible System from Invitrogen was used for the cell assay (32). Stable A431 epithelial carcinoma clones were generated to express either wild-type V5-tagged FAK protein or mutant FAKY397F V5-tagged protein under the inducible regulation of mifepristone. Stable clones were grown in DMEM 10% fetal bovine serum, 750 μg/mL Zeocin, and 50 μg/mL Hygromycin. One day before running the FAK cell ELISA, A431 FAK wild-type cells were seeded in growth medium in 96-well U-bottomed plates. After 4 to 6 h at 37°C, 5% CO₂, FAK expression was induced with 0.1 μmol/L mifepristone. Uninduced controls were included. Anti-V5- or anti-FAK-coated plates were blocked in 3% bovine serum albumin (BSA)/0.5% Tween for 1 h at room temperature. Cells were treated with 1/2-Log serial dilutions starting at a top concentration of 1 μmol/L for 30 min at 37°C, 5% CO₂. Lysates from cells treated with indicated concentrations of compound were prepared in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, 1 mmol/L NaF, and protease inhibitors] and transferred to the anti-V5- or anti-FAK-coated plates to capture total induced or total FAK protein. Antiphosphospecific FAKY397 was used to detect autophosphorylated FAKY397 followed by secondary reporter antibody. HRP substrate was added, and plates were read at 450 nm/L. IC₅₀ values were determined using the Hill-Slope Model. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed to determine compound cytotoxicity.

Animals for *in vivo* studies. Athymic female mice (CD-1 Nu/Nu, ~20 grams) were used for all *in vivo* studies. Mice were obtained from Charles River Laboratories and housed in specific pathogen-free conditions, according to the guidelines of the Association for the Assessment and Accreditation for Laboratory Animal Care, International. All *in vivo* studies were carried out under approved institutional experimental animal care and use protocols.

Ex vivo ELISA. Female athymic mice were injected with 1 × 10⁶ U87MG human glioblastoma cells on day 1. On day 9, when tumors were ~300 mm³, the mice received compound or vehicle (5% Gelucire 44/14 in sterile water; Gattefossé) p.o. For pharmacokinetic/pharmacodynamic (PK/PD) analysis, blood and tumor samples were collected from each animal (*n* > 4 mice per group per time point) into heparinized vacutainers and liquid N₂, respectively, at the indicated times postdose. Blood and tumors were harvested for evaluation of drug levels and tumor-associated phospho-FAK and total FAK. Plasma concentrations of PF-562,271 were determined using reverse phase high performance liquid chromatography with mass spectrometric (MS/MS) detection. Tumors were homogenized in 1 mL lysis buffer per 200-mg tumor [lysis buffer: 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1% Glycerol, 1% Triton X-100, 1.6 mmol/L Na₃VO₄, 10 mmol/L NaF, 25 mg/L Soy Bean Trypsin Inhibitor, EDTA-free complete Protease Inhibitor Tablets], spun 5 min at 14,000 rpm, and the supernatant were aliquoted to 96-well polypropylene plates on dry ice. Total protein concentration was determined using BSA protein assay (Pierce). Ninety-six-well goat-anti-rabbit ReactiBind plates (Pierce) were blocked with 100 μL per well cold blocking buffer (TBS, 0.1% Tween 20, and 3% BSA) for 60 min on a plate shaker at room temperature. The blocking buffer was replaced with 0.5 μg anti-pFAK397 in 100 μL cold blocking buffer per well and incubated for 60 min at room temperature with

² http://www.upstate.com/discovery/services/kp_overview.q

agitation. Plates were washed with TBS-T (TBS, 0.1% Tween 20) before addition of tumor lysate (500 µg total protein in lysis buffer without protease inhibitors) and incubated 2 h at room temperature with agitation. Captured pFAK was detected with anti-FAK Ab and then incubated with 15 ng HRP-anti-IgG per well (in blocking buffer) for 30 min at room temperature. The plates were washed as above and phosphotyrosine quantitated using 3, 3', 5, 5'-tetramethylbenzidine as described above. All phospho-FAK inhibition data are analyzed by comparing PF-562,271-treated tumors to vehicle-treated tumors.

Prediction of efficacious concentration. Blood and tumor samples were collected at each time point postadministration of PF-562,271 for determination of blood drug concentration and FAK phosphotyrosine reduction. The relationship between compound concentration and FAK phosphotyrosine reduction has been explored in pharmacologic models (tumor-bearing athymic mice) with pooled experimental data from multiple individual studies. FAK phosphotyrosine reduction correlates well with blood concentrations of PF-562,271 in athymic mice and follows a simple E_{max} pharmacodynamic model:

$$E = \frac{E_{max} \times C}{EC_{50} + C}$$

where E is the measured response (FAK phosphorylation reduction), E_{max} is the maximum response, EC_{50} is the concentration of PF-562,271 required for half-maximal reduction of FAK phosphorylation, and C is the blood concentration of PF-562,271.

Tumor growth inhibition studies. All tumor cell lines were obtained from American Type Culture Collection (ATCC) and propagated by standard tissue culture procedures in the medium suggested by ATCC. Exponentially growing cells were trypsinized and resuspended in sterile PBS and inoculated s.c. (1×10^6 cells per mouse in 200 µL) into the right flank of mice. Animals bearing tumors of $\sim 150 \text{ mm}^3$ in size were divided into groups receiving either vehicle (5% Gelucire) or PF-562,271 (diluted in vehicle), and dosed by p.o. gavage. Animal body weight and tumor measurements were obtained every 2 d. Tumor volume (mm^3) was measured with Vernier calipers and calculated using the formula: length (mm) $\times$ width (mm) $\times$ width (mm) $\times$ 0.5. Percent growth inhibition was calculated as previously described (33). For all tumor growth inhibition experiments, 8 to 10 mice per dose group were used. A Student's t test was used to determine the P value.

Microscopy and immunohistochemistry of tumor apoptosis and microvascular density. Mice bearing H125 tumors were treated for 3 d with PF-562,271 (12–50 mg/kg, twice daily, p.o.). For microvascular density analysis, mice bearing U87MG tumors were used and treated for 3 d with a C4 sulfone derivative of PF-562,271 (50 mg/kg, daily, p.o.). After treatment, tumors were excised and quick frozen in OCT medium. Seven-micrometer sections were cut and processed for immunohistochemical detection of apoptosis using a commercially available terminal deoxynucleotidyl-transferase-mediated dUTP nick-end (TUNEL) kit (ApopTag; Millipore) or rat antimurine endothelial MECA32 Ab (anti-endothelin; PharMingen). Tissue sections were counter stained with hematoxylin or methyl green and examined using a Zeiss Axiophot microscope at $\times 20$ with a reticule grid. All discreet, positively stained apoptotic cells or vascular profiles, with or without lumina, were counted in 10 ($\times 200$) fields from multiple sections of each tumor. Fields were randomly chosen throughout the entire section. For each time point and dose, four mice were evaluated. A Student's t test was used to determine the P value.

Results

Synthesis and physical chemical properties. PF-562,271 is a methane sulfonamide diaminopyrimidine that is Rule of 5 compliant, demonstrating favorable physical chemical properties (Fig. 1). X-ray crystallographic analysis confirms that PF-562,271 binds in the ATP-binding cleft of FAK, forming two of the three "canonical" H-bonds between the inhibitor and main-chain atoms in the kinase hinge region (Fig. 1B; for crystallographic details, see

Supplementary Materials). The amino-oxindole moiety extends out from the active site cleft along the hinge region backbone, where the oxygen atom forms a hydrogen bond to the side chain of Arg 426. The amino-methyl pyridinyl sulfonamide group projects out from the active site toward the activation loop region, forming interactions with residues in the "Asp-Phe-Gly (DFG) motif" of FAK and inducing the formation of a short stretch of α -helix including the residues around the "DFG motif" (Fig. 1C). The primary driver for this seems to be the favorable H-bonding interaction that is established between one of the sulfonamide oxygen atoms and the backbone NH of D564. Consistent with the fact that the FAKcd used for X-ray crystallography was uniformly nonphosphorylated (data not shown), the activation loop region of the kinase (residues 571–584) is completely disordered.

Inhibition of FAK tyrosine kinase activity, selectivity, and autophosphorylation in intact cells. PF-562,271 is a potent ATP-competitive, reversible inhibitor of FAK and Pyk2 kinase, with a IC_{50} of 1.5 nmol/L (0.7 ng/mL) and 13 nmol/L (7 ng/mL), respectively (Table 1). This compound shows Michaelis-Menton kinetics, consistent with it being an ATP-competitive and reversible inhibitor of the enzyme (Fig. 1D). PF-562,271 was evaluated in a number of kinase screens and panels and displays $>100\times$ selectivity against all tested enzymes, except for some cyclin-dependent kinase (cdk) cyclin complexes (Table 1). Although PF-562,271 was shown to be a 30- to 120-nmol/L (15.2 to 60.1 ng/mL) inhibitor of cdk2/E, cdk5/p35, cdk1/B, and cdk3/E in recombinant enzyme assays, in cell-based assays evaluating the role of cdks, a 48-hour exposure of 3.3 µmol/L PF-562,271 was required to alter cell cycle progression (Fig. 2). Furthermore, confirmatory studies failed to detect any PF-562,271 inhibitory activity against cdk5/p35 enzyme. The cell-based assay exploits the mechanism of the GeneSwitch system to exogenously control the expression and subsequent phosphorylation of FAK and the kinase-dependent autophosphorylation site at residue Y397. PF-562,271 is potent in an inducible cell-based assay measuring phospho-FAK with a IC_{50} of 5 nmol/L (2.5 ng/mL; Table 1). Comparative cell assays suggest that PF-562,271 is ~ 4 -fold less potent for Pyk2 than FAK (data not shown). These cell-based data show an even greater selectivity for FAK (and Pyk2) relative to other kinase targets (e.g., cdks). Therefore, PF-562,271 is an excellent molecule to inhibit FAK activity in animal models of disease.

Inhibition of FAK phosphorylation in tumors. After demonstration of FAK inhibition and selectivity *in vitro*, the ability of PF-562,271 to modulate FAK activity *in vivo* was evaluated using tumor-bearing mice. As shown in Fig. 3A and B, p.o. administration of PF-562,271 to tumor-bearing mice inhibited FAK phosphorylation in tumors in a dose- and time-dependent manner. Percent inhibition of phosphorylated FAK is relative to vehicle-treated control animals. Maximal pFAK inhibition of 78% was achieved 1 hour after a 33 mg/kg p.o. dose. However, *in vivo* inhibition of FAK phosphorylation was sustained ($>50\%$) for >4 hour with this single p.o. dose. By contrast, although a 10 mg/kg dose approached a maximal inhibition of 70%, $>50\%$ pFAK inhibition was only sustained for <2 hour. FAK phosphorylation inhibition relative to total blood concentration of PF-562,271 resulted in a calculated EC_{50} of 93 ng/mL (total; Fig. 3B).

Evaluation of *in vivo* efficacy, C_{max} , or C_{ave} . In an effort to better understand the pharmacologic inhibition of FAK and the relationship to tumor growth inhibition, experiments were designed to evaluate different dosing regimens, i.e., daily dosing, twice daily dosing, and continuous coverage using osmotic minipumps. These experiments allow the comparison between C_{max} ,

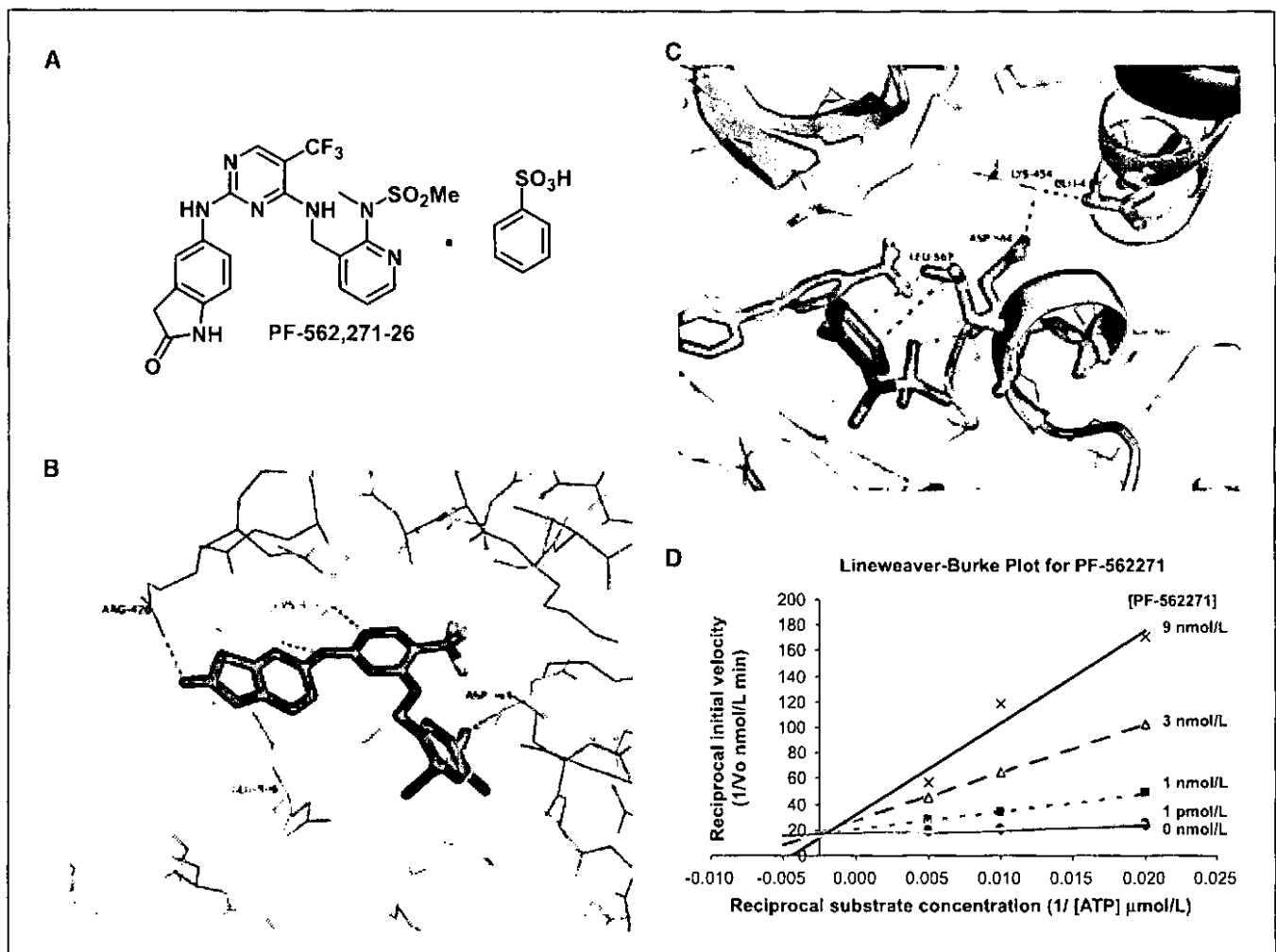


Figure 1. A, chemical name: N-Methyl-N-(3-[[2-(2-oxo-2,3-dihydro-1H-indol-5-ylamino)-5-trifluoromethyl-pyrimidin-4-ylamino]-methyl]-pyridin-2-yl)-methanesulfonamide; molecular formula: $C_{21}H_{23}F_3N_7O_3S \cdot C_6H_6O_3S$; molecular weight: 507.50 (free base); 665.68 (besylate salt). B, the crystal structure of PF-562,271 (green) bound to the active site of FAK (cyan). Dashed lines, canonical backbone hydrogen bonds to Cys-502, as is the hydrogen bond between the oxindole O atom and Arg-426. Glu-506 and Asp564 are labeled for reference. Residues 428 to 435, which fold over the active site upon substrate or ligand binding, have been omitted for clarity. C, closeup view of the methyl-sulfonamide interaction with the DFG region of FAK. Orange dashed lines, hydrophobic interactions between Leu-567 and the pyridinyl moiety of PF-562,271. Note the hydrogen bond between the sulfonamide O atom and the backbone NH of Asp-564. D, a Double-Reciprocal Plot (Lineweaver-Burke) of velocity versus ATP concentration (50 to 200 nmol/L) at varying PF-562,271 concentrations (1 pmol/L to 9 nmol/L) demonstrating competitive inhibition. PF-562,271 competitively inhibits FAK at equivalent V_{max} rates over the concentration range tested: VM, ATP = 0.059 nmol/L min; VM, 1 pmol/L = 0.060 pmol/L min; VM, 1 nmol/L = 0.049 nmol/L min; VM, 3 nmol/L = 0.037 nmol/L min; and VM, 9 nmol/L = 0.032 nmol/L min.

C_{ave} and C_{min} and the relationship to attaining maximal efficacy, providing the critical PK/PD linkage of FAK inhibition to antitumor activity in these preclinical models. A direct comparison between daily and twice daily dose while keeping the total daily dose equivalent provides either a high C_{max} with 4 hours of >50% target inhibition or a lower C_{max} but extended target inhibition (Fig. 3C; Table 2). Whereas the 50 mg/kg daily dose resulted in a C_{max} of 157 ng/mL (free), the corresponding tumor growth inhibition was only 45% with 37% inhibition of pFAK after 15 days of dosing in the PC-3M human prostate xenograft model. Contrast these results to the 61% tumor growth inhibition and 27% pFAK inhibition observed in the same model for the same number of dosing days while splitting the dose to provide two daily doses of 25 mg/kg (still 50 mg/kg/day) and only reaching a C_{max} of 25 ng/mL. To further evaluate extended coverage/inhibition of the target, osmotic mini-pumps loaded with PF-562,271 were implanted into tumor-bearing mice (Fig. 3D; Table 2). In this case, the mini-pumps

only maintained 0.7 ng/mL (free) blood levels but did so continuously for 24 hours. This results in a lower overall inhibition of FAK phosphorylation in tumors but for extended periods of time compared with p.o. dosing (Table 2). Importantly, the tumor growth inhibition using mini-pumps was identical to the 50 mg/kg twice daily dosing regimen that resulted in a C_{max} of 273 ng/mL (free; Fig. 3D).

Breadth and amount of antitumor activity with p.o. dosing. The pharmacokinetics, inhibition of phosphorylated FAK, and antitumor efficacy of PF-562,271 was evaluated in the following human s.c. xenograft models: PC-3M (prostate), BT474 (breast), BxPc3 (pancreatic), LoVo (colon), U87MG (glioblastoma), and H125 and H460 (lung; Table 2). Dose-dependent tumor growth inhibition was observed in all models. Maximum tumor inhibition for PC-3M, BT474, BxPc3, and LoVo ranged from 78% to 94% inhibition for the group with regressions in up to 50% of a given dose group (Table 2).

Regressions were observed in PC-3M, BT474, BxPc3, and LoVo models at doses of 25 to 50 mg/kg twice daily, corresponding to C_{max} (free) ranges of 78 to 885 ng/mL, C_{ave} (free) of 14 to 40 ng/mL, and inhibition of phospho-FAK of 31% to 76% for >4 hours. No weight loss, morbidity, or death was observed in any tumor growth inhibition (TGI) experiment (up to 50 mg/kg twice daily $\times$ 29 days or 100 mg/kg daily $\times$ 25 days). All data are based on 6 to 10 animals per dose, and experiments were completed at least twice. After dosing, animals were euthanized, blood and tumor were analyzed for drug concentration (PK), and tumors were evaluated for phospho-FAK (PD).

In vivo mechanism of action. The primary mechanism by which FAK inhibits tumor growth is believed to be through the induction of anoikis (i.e., anchorage-dependent apoptosis). To test whether inhibition of FAK activity in tumors induces apoptosis, mice bearing H125 lung xenografts were treated with PF-562,271 twice daily for 3 or 10 days to evaluate apoptosis or tumor growth inhibition, respectively. Apoptosis cannot be evaluated at the end of TGI experiments because tumors are too necrotic to obtain reliable staining data. At a dose of 25 mg/kg, apoptosis was 2-fold greater in treated tumors compared with vehicle-treated control tumors on day 3 (Fig. 4). There was no increase in apoptosis at doses of 12.5 mg/kg at day 3, although tumor growth was inhibited 44% by day 10. FAK inhibition may also result in an antiangiogenic effect due to its linkage to α_v integrin signal transduction. As shown in Fig. 4D, U87MG tumors treated with a PF-562,271 derivative (50 mg/kg, twice daily, p.o.) resulted in ~50% decrease in microvascular density after only 3 days of treatment.

Table 1. *In vitro* profile of PF-562,271

	IC ₅₀			IC ₅₀	
	nmol/L	ng/mL		nmol/L	ng/mL
FAK	1.5	0.8	GSK3 α	101	51.2
Pyk2	13	6.6	GSK3 β	133	67.4
FAK CELL	5	2.5	IGF-IR	>500	>254
Abl	>1,000	>507	IKK α	>1,000	>507
Aurora-A	145	73.5	IKK β	>1,000	>507
CDK1/cyclinB	58	29.4	JNK1 α 1	>1,000	>507
CDK2/cyclinA	227	115.1	Lck	408	206.9
CDK2/cyclinE	30	15.2	MAPK1	340	172.4
CDK3/cyclinE	47	23.8	MEK1	>1,000	>507
CDK4	>1,000	>507	p70S6K	>1,000	>507
CDK5/p35	120	60.8	PDGFR β	>3,000	>1,521
CDK6/cyclinD3	>1,000	>507	PKA	620	314.3
CDK7/cyclinH/MAT1	97	49.2	PKB α	>1,000	>507
CHK1	>1,000	>507	PKB β	>1,000	>507
CHK2	>1,000	>507	PKB γ	>1,000	>507
EGFR	>1,000	>507	PKC α	>1,000	>507
EphB2	>1,000	>507	PKC β II	>1,000	>507
FGFR3	>1,000	>507	PKC δ	>1,000	>507
Flt3	97	49.2	CSrc	797	404.1
Fms	>1,000	>507	TrkB	>2,500	>1,268
Fyn	377	191.1	Yes	294	149.1

Abbreviations: JNK, c-Jun-NH₂-kinase; PDGFR, platelet-derived growth factor receptor.

Discussion

In this report, we characterize the pharmacology of a selective small molecule inhibitor of FAK, PF-562,271. These preclinical pharmacology studies helped determine the appropriate utility of this compound resulting in the advance to human clinical trials for the treatment of cancer. PF-562,271 has been administered to >60 patients in the phase I setting. Although no objective responses were reported, several patients have experienced prolonged disease stabilization of 6 months or longer, including some beyond 1 year. These include patients with a variety of tumor types including sarcoma, renal, lung, lacrimal, and prostate cancers. Moreover, patients have shown a metabolic response by 18 F-fluoro-2-deoxyglucose-positron emission tomography (PET) scan criteria. For example, one patient with stage IV ovarian cancer showed a metabolic response by PET scan criteria, with a reduction in the standardized uptake value signal of a splenic metastasis by 46%, 2 weeks after taking PF-562,271 (34).

Crystallographic analysis of PF-562,271 with the catalytic domain of FAK combined with the Scatchard analysis confirms that the compound binds in the ATP-binding pocket consistent with it being an ATP-competitive and reversible inhibitor of FAK (35). The most remarkable feature of the crystal structure of FAKed/PF-562,271 is the conformation of the residues comprising the conserved "DFG motif," which adopt a helical conformation. This helical conformation is driven primarily by interactions between the methane sulfonamide moiety and the backbone NH of Asp 564, as well as hydrophobic interactions between the side chain of Leu 567 and the pyridinyl moiety of PF-562,271. In addition to perturbing the conformation of the activation loop, this helix forces the side chain of Asp 564 upwards and away from the ATP-binding cleft, where it interacts with the conserved Lys 454-Glu 471 salt bridge. This Lys-Glu salt bridge is a key feature of activated protein kinases, correctly orienting the "C-helix" of the kinase and establishing the proper orientation of the NH₂- and COOH-terminal loops for catalysis. Because Asp 564 is believed to play an important role in catalysis by coordinating a Mg²⁺ ion and helping to stabilize the β - and γ - phosphates of Mg-ATP (36), the repositioning of the Asp 564 side chain would presumably abolish ATP binding by removing this critical interaction. In addition to increased potency, this sulfonamide interaction pocket may explain to some extent the exquisite selectivity of PF-562,271 for FAK because the activation loop region is the most plastic and idiosyncratic structure in the otherwise well-conserved protein kinase topology. Finally, although helical segments in the activation loop region have been seen in other protein kinases, including CDK2, Src, Hck, MAP/ERK kinase (MAPK), and epidermal growth factor receptor (EGFR; refs. 37–39), the structure reported here is the first to include directly the catalytically important "DFG motif." It will be interesting to determine if the DFG helix observed here functions in an analogous manner to these other kinases in contributing to an autoinhibited state of the enzyme.

PF-562,271 showed selectivity against a wide panel of kinases, as well as nanomolar potency in a cell-based assay of FAK activity. There have been previous reports of inhibitors with cross-reactivity against FAK, but what distinguishes PF-562,271 from these other inhibitors is the potency for FAK and the selectivity against other kinases, especially IGF-IR, IR, and src (40). The moderate activity of this compound against some cdk in recombinant kinase assays was insufficient to translate into cellular effects expected of a cdk inhibitor (41). Given that the no-effect concentration for inhibition

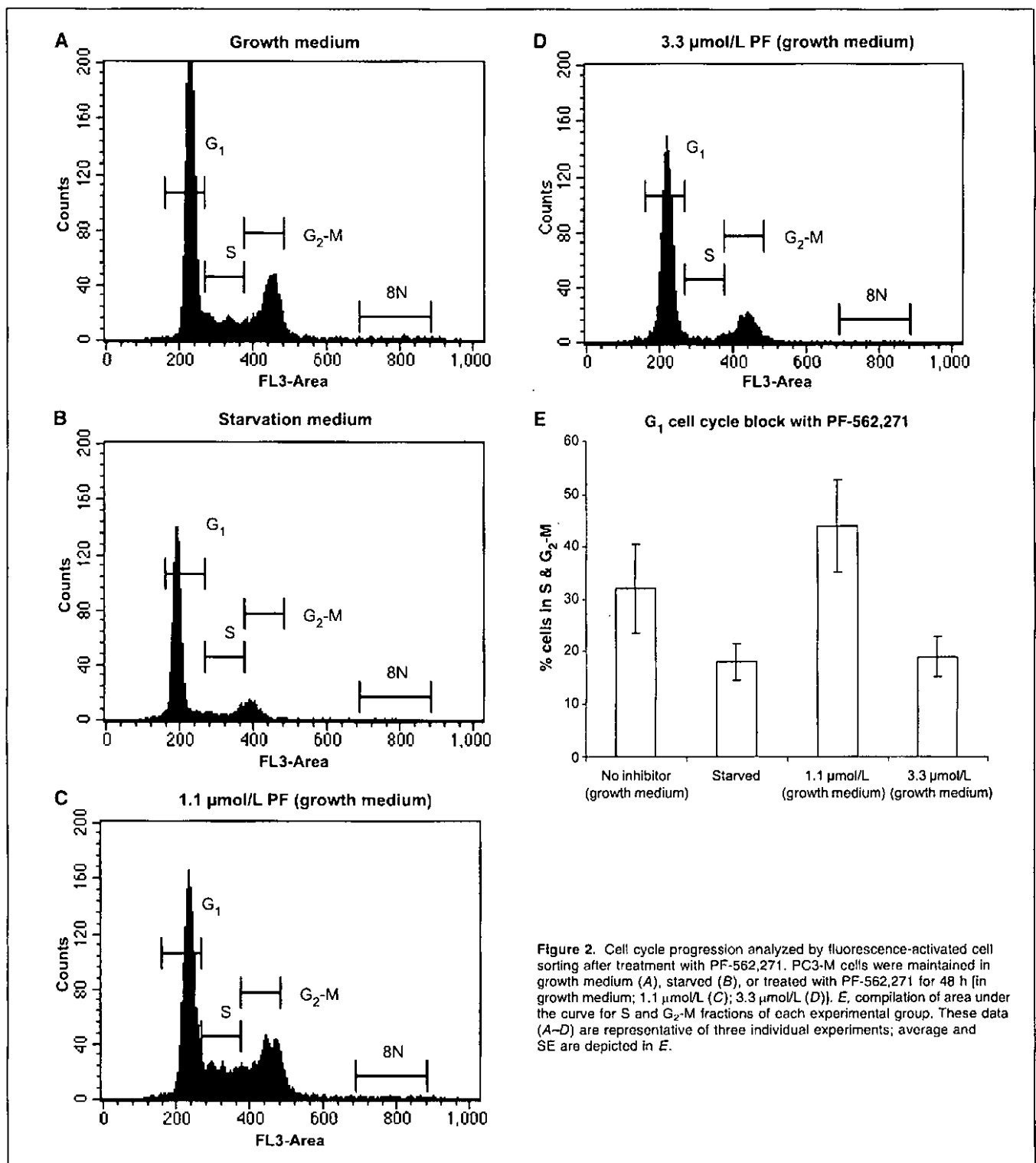


Figure 2. Cell cycle progression analyzed by fluorescence-activated cell sorting after treatment with PF-562,271. PC3-M cells were maintained in growth medium (A), starved (B), or treated with PF-562,271 for 48 h [in growth medium; 1.1 $\mu\text{mol/L}$ (C); 3.3 $\mu\text{mol/L}$ (D)]. E, compilation of area under the curve for S and G_2 -M fractions of each experimental group. These data (A–D) are representative of three individual experiments; average and SE are depicted in E.

of cell-based cdk activity ($>1,100$ ng/mL) is far in excess of the FAK *in vivo* and *in vitro* IC_{50} (*in vivo* C_{max} , 69 nmol/L; *in vitro* C_{max} , 1 nmol/L), PF-562,271 is not considered to be a pharmacologically relevant inhibitor of cdk *in vitro* or *in vivo*. When activated, FAK becomes phosphorylated at Y397, which is a binding site for src; thus, both catalytically active molecules are colocalized within focal

adhesions (42). Src, FAK, and integrin signaling have long been implicated in cancer biology. Given the catalytic capacity of both FAK and src (43), it is imperative that PF-562,271 is selective for FAK to accurately elucidate the role of FAK and correctly interpret antitumor effects. Because PF-562,271 (and a closely related compound, PF-573,228) showed substantial kinase selectivity for FAK compared with

other kinases, it is now possible to dissect the specific role of FAK ATP catalysis directly (31). More importantly, PF-562,271 has the appropriate pharmacologic properties for clinical testing in humans. This molecule is not only an excellent tool for understanding the role of FAK *in vitro* and *in vivo* but also the appropriate molecule to test the hypothesis in the clinical disease setting.

A rigorous *in vivo* PK/PD evaluation was completed for PF-562,271. The compound is well-absorbed with maximal blood levels occurring between 30 minutes and 2 hours after p.o. administration. Maximal pharmacodynamic modulation occurs simultaneously with maximal pharmacokinetic exposure in the blood regardless of dose or number of repeated doses. Measured PK is accurately modeled using *in vitro* and *in vivo* calculation of absorption, distribution, metabolism, and excretion, demonstrating a well-behaved and predictable *in vivo* pharmacology.

The ability to modulate FAK phosphorylation after p.o. administration of PF-562,271 was evaluated in multiple tumor types, but for routine assessment, the U87MG model was used. This was due to the robust and consistent growth of the tumor, allowing for an efficient drug discovery platform, as well as the elevated expression and activation of FAK in these tumor xenografts. However, inhibition of phospho-FAK was observed in every tumor model to varying degrees. TGI in the nonclinical

models was shown with both intermittent and sustained exposures. It is interesting and clinically relevant to note that transient maximal inhibition of FAK activity never provided the greatest antitumor efficacy. This is clearly evident when twice daily dosing is compared with daily dosing and most strikingly shown when mini-pumps are used. Twice daily dosing and mini-pump experiments (steady state concentration) resulted in greater TGI in multiple models compared with daily dosing (equivalent total daily dose), suggesting C_{ave} and the time above the C_{ave} are more relevant to efficacy than C_{max} . These data clearly show that the area under the curve for target modulation and not maximal inhibition yields maximal antitumor efficacy. This characteristic has been observed with other receptor tyrosine kinase inhibitors (33). The mini-pump model also better mimics the clinical dosing regimen, where repeated dosing will result in a sustained steady-state exposure of the compound over the entire dosing period (34).

PF-562,271 was evaluated in multiple tumor xenograft models to show breadth of activity, although these models do not predict clinical efficacy nor do they adequately represent the clinical tumor types. The xenograft models were chosen due to increased FAK expression and/or FAK activation (44–46). It is important to note that although FAK expression and activation are elevated in these

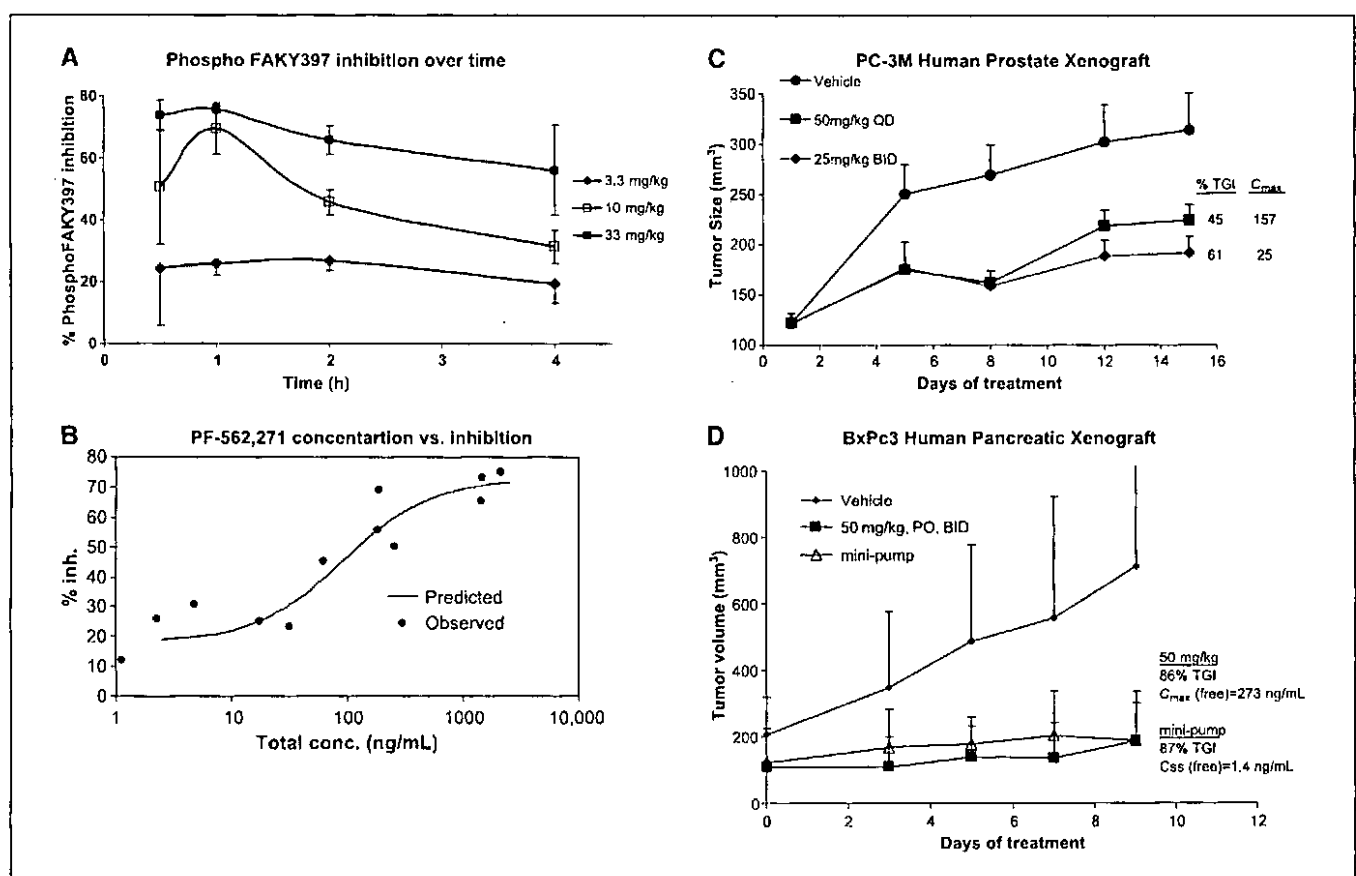


Figure 3. Pharmacokinetic (A) and pharmacodynamic (B) inhibition of phospho-FAK after p.o. administration of PF-562,271 to U87MG-bearing mice. The compound shows dose-, time-, and concentration-dependent inhibition of tumor-associated phosphorylated FAK. Percent inhibition of phosphorylated FAK is relative to vehicle-treated control animals. Tumor growth inhibition with PF-562,271 of PC3-M (C) and BxPc3 (D) xenografts. Keeping the total daily dose constant, twice daily administration (BID) results in greater tumor growth inhibition compared with once daily dosing while having a considerably lower C_{max} . D, mini-pumps, which continuously deliver compound for the entire dosing period, generated comparable tumor inhibition as 50 mg/kg twice daily. C_{max} is ng/mL free fraction. C_{ss} , steady state concentration. A, average and SE; $n = 4$ to 6 animals per group. B, individual plasma concentrations from multiple animals ($n = 3$ –4) receiving increasing dose, all taken at the approximate T_{max} (~1 h). C and D, average and SD; $n = 8$ to 10 animals per group.

models, tumor growth in these models is not primarily driven by FAK expression nor due to activating mutations in FAK. Regardless of the multitude of activated pathways (e.g., EGFR, PDGFR, VEGFR, and src) and disrupted/inactive tumor suppressors (e.g., PTEN), PF-562,271 showed robust antitumor activity as a single agent in every model at well-tolerated doses achieving low to moderate blood levels. Moreover, regressions were observed in most models with up to 50% of a dose group resulting in regressions. This level of antitumor activity was established for repeated dosing of up to 29 days with no decrease in body weight or animal activity, demonstrating that PF-562,271 is very well-tolerated and potent in these models.

The function of FAK as a transducer of integrin engagement is well-established (3). Cells detached from matrix *in vitro* undergo anoikis (attachment-mediated apoptosis) with a concomitant decrease in FAK activity (1). To evaluate the molecular changes associated with the inhibition of FAK *in vivo* and evaluate a potential mechanism behind the dramatic antitumor efficacy, we measured the amount of apoptosis after treatment with PF-562,271. Consistent with the association of FAK signaling and apoptosis (47), tumors had a significant increase in apoptotic bodies after p.o. administration of PF-562,271 when compared with vehicle-treated animals. Increased apoptosis was observed

with increased individual and cumulative dose, aligned with pharmacokinetic and pharmacodynamic modulation of FAK activity. Interestingly, on favorable sections, apoptotic vascular cells could be identified (data not shown). Moreover, microvascular density was decreased by 50% in U87 tumors after FAK inhibition, consistent with the linkage of FAK to $\alpha_v\beta_3$ integrin signaling and previous reports of FAK expression in angiogenic blood vessels (48). Therefore, although FAK inhibition is primarily an antitumor approach, it should also be considered antiangiogenic. Induction of apoptosis occurred as early as 3 days of dosing. At this time point, there is no obvious distinction in tumor growth between vehicle- and PF-562,271-treated animals, suggesting that apoptosis may be one of the earliest functional changes after FAK catalytic inhibition *in vivo*. These results suggest that inhibitors of FAK activity could limit tumor growth and metastasis through induction of tumor and endothelial cell apoptosis and inhibition of migration.

Broadly speaking, pharmacologic anticancer therapy consists of cytotoxics and targeted agents (small molecules and biologics). Unfortunately, the majority of patients treated with these agents eventually progress. In addition, toxicities associated with these agents often preclude adequate treatment. In this report, the *in vivo* pharmacology of a highly selective and potent inhibitor of

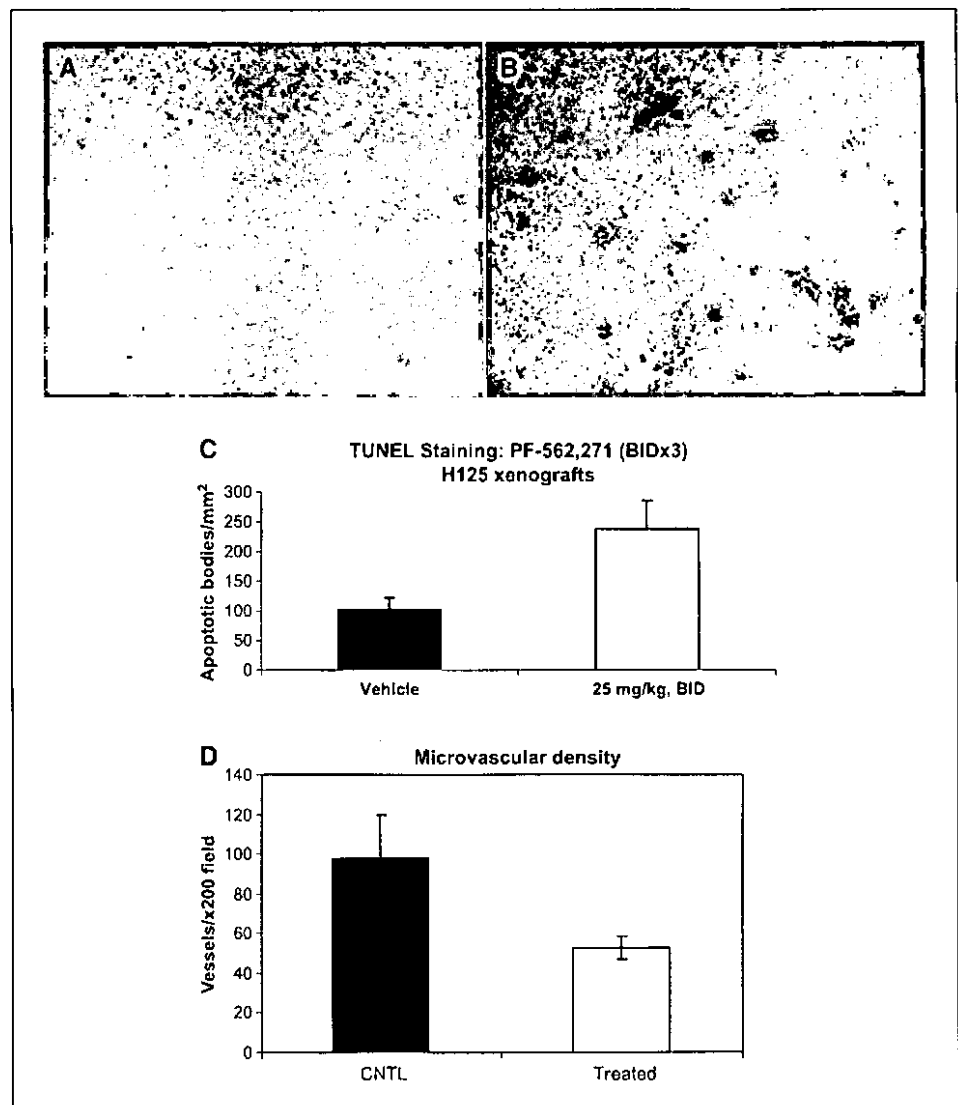
Table 2. *In vivo* profile of PF-562,271

Tumor growth inhibition studies

PK/PD		<i>EC</i> ₅₀			<i>EC</i> ₅₀ free			
U87		93 258			18.6 51.6			
Tumor	Dose (mg/kg)	TGI (%)	Regressions	Blood sample time	Total blood conc. (ng/mL)	Free conc. (ng/mL)	<i>C</i> _{ave} free* (ng/mL)	PD [†] (% inh)
BxPC3	50 BID	86 [‡]	3/10	1 h, d 10	1,364	273	ND	31 [‡]
	100 QD	71 [‡]		1 h, d 10	3,524	705	ND	59 [‡]
	Pump	57 [‡]		1 h, d 10	3.4	0.7	0.7 [§]	37 [‡]
LoVo	25 QD	73 [‡]	2/6	1 h, d 25	388	78	ND	ND
	50 QD	75 [‡]		1 h, d 25	952	190	ND	ND
	25 BID	76 [‡]		1 h, d 25	271	54	ND	ND
U87MG	50 BID	85 [‡]	1/5	1 h, d 25	323	65	ND	ND
	50 BID	44		1 h, d 9	2,500	500	ND	78 [‡]
	100 QD	47 [‡]		2 h, d 10	4,425	885	ND	ND
H460	50 QD	51 [‡]	0/8	1 h, d 9	3.6	0.7	0.7 [§]	45 [‡]
	50 QD	42		1 h, d 10	2,060	412	ND	35 [‡]
	25 BID	44		ND	ND	ND	ND	ND
H125	50 BID	67 [‡]	3/7	ND	ND	ND	ND	ND
	50 BID	78*		0.5 h, d 15	479	96	16	52 [‡]
	25 BID	61 [‡]		0.5 h, d 15	127	25	4.5	27
PC3M	50 QD	45 [‡]	3/8	0.5 h, d 15	783	157	19	37 [‡]
	12.5 BID	37		0.5 h, d 29	147	29	3	50 [‡]
	25 BID	59 [‡]		1 h, d 29	578	116	14	55 [‡]
BT474	50 BID	94 [‡]	4/8	1 h, d 29	900	180	40	76 [‡]

Abbreviations: Conc, concentration; inh, inhibition; QD, daily; BID, twice daily; ND, not determined.
*Calculated from AUC0-t(last)/h.
†% inhibition of phosphorylated FAK in the tumor.
‡Statistically different from vehicle control samples calculated using a Student's *t* test with a *P* value of <0.05.
§Steady state concentration.

Figure 4. Apoptosis induction as measured by TUNEL staining in H125 tumor xenografts after 3 d of twice daily dosing with 25 mg/kg of PF-562,271 (A, vehicle; B, treated). Corresponding tumor growth inhibition after 10 d of dosing was 44% and 67% for 25 and 50 mg/kg, respectively. C, graph below shows quantification of sections. D, decrease in tumor microvascular density was observed to be ~50% after 3 d of 50 mg/kg twice daily treatment with 50 mg/kg of a C4 sulfone derivative of PF-562,271. CNTL, control. Bars, average and SD.



FAK catalytic activity, PF-562,271, is described. The novel mode of inhibition of FAK, characterized by the inhibitor-induced "DFG helix," resulted in profound antitumor activity across a wide variety of tumor types while being well-tolerated. PF-562,271 showed well-behaved pharmacology *in vivo* with a robust PK/PD relationship. PF-562,271 shows the selectivity and pharmacology that has allowed it to be a first in class inhibitor presently in clinical testing for the treatment of cancer.

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