

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
RAD: 9-126923- -13	FECHA: 2013-10-17 13:09:56
DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 23
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
CARLOS ORLANDO MAYA RODRIGUEZ

Asunto: Radicación: 9-126923- -13
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 19686
 Folios: 23

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2008/004570				
Fecha de Presentación Internacional	08/04/2008				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 518 a 1186	09/Noviembre/2009
Reivindicaciones:	Folios 1187 a 1222	09/Noviembre/2009
	Folios 1297 a 1330	09/Noviembre/2009
	Folios 1373 a 1408	29/Octubre/2010
	Folios 1422 a 1429	21/Octubre/2010
Dibujos:	Folios 1224 A 1229	09/Agosto/2013
Oposiciones:	Folios 1366-1356	Lafrancol 24/Junio/2010
Respuesta del solicitante:	Folios 1373 a 1408	29/Octubre/2010
Prioridad:	US 60923164	11/Abril/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

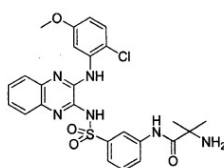
3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiaron la totalidad de las reivindicaciones tal y como fueron modificadas.

Título de la solicitud: “Uso de compuestos inhibidores de quinaxolina de PI3K-ALFA para el tratamiento del cáncer”.

El problema planteado en esta solicitud consiste en la necesidad de proporcionar un compuesto que inhibe la actividad enzimática de lípido quinasa y la resultante modulación de las actividades celulares (tales como proliferación, diferenciación, apoptosis) en combinación anti cáncer.

Para resolver este problema técnico la solicitud en estudio proporciona una composición farmacéutica caracterizada porque comprende el compuesto:



o un isomero simple o un tautómero de éste, donde el compuesto puede ser una sal farmacéuticamente aceptable y en forma adicional puede ser un hidrato y en forma adicional puede ser un solvato de éste, en combinación con uno o más agentes quimioterapéuticos seleccionados del grupo formado por platino, taxano, rapamicina y erlotinib.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 31/337; A61K 31/517; A61K 31/555; A61P 35/00; A61K 31/4985 y A61K 31/498

4. De la solicitud de patente

Título

El título no corresponde con el objeto de la solicitud porque de acuerdo con la modificación presentada la solicitud reivindica composiciones, por lo cual se sugiere al solicitante hacer la corrección necesaria considerando el sustento presentado en el capítulo descriptivo.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1- 5 no son claras porque presentan términos como: “uno o más”, lo que no limita el número de posibles combinaciones entre los agentes anticancerígenos.

La reivindicación 4 no es concisa, porque presentan una excesiva complejidad, lo cual dificulta determinar la extensión del objeto que se busca proteger.

La reivindicación 1 no es clara ya que habla de platino el cual es un elemento químico, se sugiere eliminarlo de la reivindicación o corregirla de acuerdo a lo sustentado en la descripción.

Las reivindicaciones 1-5 no están sustentadas en la descripción porque sugieren composiciones farmacéuticas que no se encuentran como tal en el capítulo descriptivo. Teniendo claro que una composición es sustentada de acuerdo a la proporción de ingredientes y al diseño del sistema de entrega. Este Despacho aclara a la solicitante que la solicitud hace referencia a una combinación de moléculas a diferentes intervalos de tiempo, lo que claramente no es considerado una composición farmacéutica sino un método de tratamiento.

5. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 4, grupos inventivos. Los grupos inventivos están definidos por las características esenciales de los ingredientes combinados y considerando que ninguno de ellos es nuevo e inventivo no existe un concepto común que pueda vincular todas las modalidades reclamadas y que por otra parte existe una inconsistencia entre lo que se reivindica y lo que se encuentra debidamente soportado en la descripción, toda vez que el sustento se asocia a métodos de tratamiento basados en la administración de los ingredientes activos y no en el diseño de un sistema de entrega farmacéutico que involucre físicamente la combinación de los principios activos, por lo anterior, este Despacho encuentra que existen cuatro grupos inventivos no relacionados a saber:

1° Composiciones que contienen el compuesto N-(3-{{3-{{2-cloro-5-(metoxi) fenil} amino} quinoxalin-2-yl) amino} sulfonil}-4-metilfenil)-2-methylalaninamida combinado con taxol.

2° Composiciones que contienen el compuesto N-(3-{{3-{{2-cloro-5-(metoxi) fenil} amino} quinoxalin-2-yl) amino} sulfonil}-4-metilfenil)-2-methylalaninamida combinado con carboplatino.

3° Composiciones que contienen el compuesto N-(3-{{3-{{2-cloro-5-(metoxi) fenil} amino} quinoxalin-2-yl) amino} sulfonil}-4-metilfenil)-2-methylalaninamida combinado con rapamicina.

4° Composiciones que contienen el compuesto N-(3-{{3-{{2-cloro-5-(metoxi) fenil} amino} quinoxalin-2-yl) amino} sulfonil}-4-metilfenil)-2-methylalaninamida combinado con erlotinib.

Se sugiere presentar las solicitudes divisionales que considere necesarias, o limitar la solicitud inicial a una sola invención considerando para ello el sustento de la memoria descriptiva y eliminando de ella las otras invenciones que no cuentan con el sustento debido para las composiciones.

6. Análisis de las Oposiciones

Argumentaciones de Lafranco

Dice el opositor que esta solicitud no cumple con los requisitos de patentabilidad, novedad, nivel inventivo, aplicación industrial. Y agrega que los métodos para el tratamiento de cáncer por medio de la administración de un compuesto de la fórmula (I), en combinación con otros agentes anticancerígenos, son prácticas y eventos totalmente conocidos en la industria farmacéutica, luego no es pertinente conceder un beneficio patentario.

El opositor anexó las fichas técnicas de “The Index Meck” para el caso del principio activo Docetaxel e indica que la molécula se encuentra bajo conocimiento

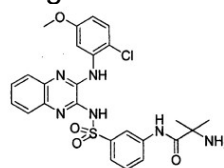
público y ostenta beneficios patentarios concedidos por la Oficina Europea de Patentes EP253738 y Estados Unidos de América US4814470. De igual manera, indica la primera patente concedida para Carboplatino y señala que ostenta beneficios patentarios concedidos por Holanda NL7307863 y Estados Unidos de América US4140707. Asimismo, el opositor señala que Cetuximab fue publicado por primera vez en el documento WO9640210. Finalmente, para la molécula Trastuzumab señala que obtuvo derechos patentarios concedidos por Estados Unidos de América US5677171 y fue publicado en WO99216553. Por lo anterior, el opositor concluye que no puede existir beneficio patentario para reivindicaciones de composiciones y combinaciones farmacéuticas útiles en el tratamiento del cáncer y que comprenden moléculas plenamente identificadas y utilizadas. Y agrega que la solicitud de la referencia pretende proteger mediante derechos patentarios objetos que no son consideraciones invenciones, dado que en su opinión la mezcla (combinaciones) de productos (moléculas) conocidos y su variación, bien sea en uso, forma, dimensión o materia, no se consideran invenciones por cuanto no son susceptibles de aplicación industrial, nivel inventivo y novedad.

Respuestas del Solicitante

El solicitante no da respuesta a la oposición presentada por la sociedad LaFranco S.A.

Respuesta del Examinador Técnico

Con relación a los argumentos presentados por la sociedad opositora LAFRANCO S.A, y frente a los documentos anexados, es decir, las fichas técnicas que aparecen reportadas en “The Merck Index” para las moléculas anticancerígenas, este Despacho concluye que en efecto los principios activos: docetaxel, carboplatino, cetuximab y Trastuzumab se encuentran bajo conocimiento público. A pesar de lo anterior estos documentos no permiten desvirtuar la materia reivindicada dado que no sugieren la combinación de



cualquiera de dichos activos con el compuesto: razón por la cual los argumentos de la sociedad opositora no prosperan como elementos que permitan desvirtuar la patentabilidad de la materia objeto de estudio.

En lo que corresponde a la opinión del opositor en cuanto tiene que ver con la no patentabilidad de las invenciones de combinación este Despacho se permite señalar que con la combinación o mezcla de medios o elementos conocidos, se impone en primer lugar la tarea al examinador para establecer si lo que se trata de patentar es una combinación o una simple agregación de elementos. Y que se debe diferenciar la combinación de elementos de la simple agregación. En la primera se debe determinar si se produjo el efecto de la sinergia de los elementos de forma tal que se genera uno nuevo con propiedades distintas, de tal manera que el nuevo elemento sólo es resultado de dicha combinación sin que puedan determinarse los elementos por separado; en la segunda los elementos se mantienen intactos en cuanto a su efecto fundamental y, por lo tanto, son claramente distinguibles unos de otros, es decir, no hay una sinergia o combinación intrínseca de los elementos. Y para determinar esto se debe realizar el debido comparativo con el estado de la técnica disponible al momento de la fecha de prioridad reivindicada, por lo que no se puede suponer a priori que dichas combinaciones siempre será objeto de negación de la patente.

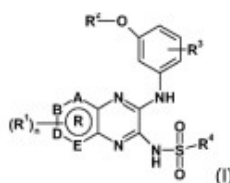
7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: abril 11 de 2007, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D2	Drug Resistance Updates 5 (2002) 234–248	“Activation of the PI3K/Akt pathway and chemotherapeutic resistance2	Octubre 2002
D ¹	WO2007023186	“Pyrazine derivates and use as PI3K inhibitors”	01/marzo/2007

El documento D1 divulga compuestos inhibidores de PI3K/Akt en combinación con agentes anticancerígenos.

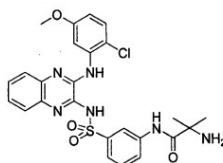
El documento D² divulga compuestos inhibidores de PI3-K de fórmula (I), el proceso de obtención de los mismos y composiciones que los contienen.



8. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en “una composición farmacéutica caracterizada porque comprende el compuesto

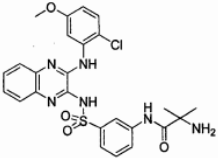


o un isómero simple o un tautómero de éste, donde el compuesto puede ser una sal farmacéuticamente aceptable y en forma adicional puede ser un hidrato y en forma adicional puede ser un solvato de éste, en combinación con uno o más agentes quimioterapéuticos seleccionados del grupo formado por platino, taxano, rapamicina y erlotinib” Radicado No. 09-126923-00012-0000 del 21 agosto del 2013.

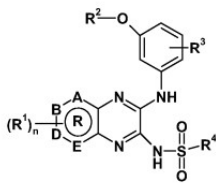
Comparación de las características técnicas esenciales, con las del estado de la técnica:

1D2 http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2007023186A1&KC=A1&FT=D&ND=3&date=20070301&DB=EPODOC&locale=en_EP .http://ac.els-cdn.com/S1368764602001206/1-s2.0-S1368764602001206-main.pdf?_tid=46f7983a-1950-11e3-bcc7-00000aacb361&acdnat=1378732013_4a0721a207cd2af2af2142d158aadb27

D1. http://ac.els-cdn.com/S1368764602001206/1-s2.0-S1368764602001206-main.pdf?_tid=46f7983a-1950-11e3-bcc7-00000aacb361&acdnat=1378732013_4a0721a207cd2af2af2142d158aadb27

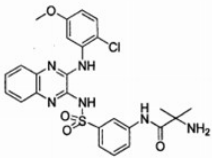
Características esenciales	D2	D1
Com posición farm acéutica caracterizada:	Com binación inhibidor PI3K con agente anticancerígeno	Com posición farm acéutica (Pág. 32 Línea 10)
Un com puesto	inhibidores de fosfoinositido 3-quinasa	Form ula general (I) Pág.17
		Com puesto 103 (Pág. 121 Ej.103) y com puesto 144 (Pág.145)
en com binación agente quim ioterapéuticos carboplatino, taxano, rapam icina y erlotinib	Cisplatino, rapam icina, plaquitaxel (pág. 240-241)	No se menciona

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 puesto que el documento D2 divulga compuestos inhibidores de PI3K/Akt en combinación con agentes anticancerígenos. Por su parte el documento D1 divulga composiciones farmacéuticas caracterizadas porque presentan compuestos de fórmula I (Pág.1):



los cuales presentan actividad inhibitoria sobre la enzima fosfoinositido 3 kinasa (Pág.2 Línea.10) usados para el tratamiento y profilaxis de desórdenes autoinmunes, inflamatorios, cardiovasculares, cáncer, entre otros (Pág.2 Línea.1-9).

La diferencia entre la invención, definida en la reivindicación 1 y las combinaciones divulgadas en el documento D2 consiste en la combinación de un agente anticancerígeno con un inhibidor de específico de PI3K de fórmula:



La diferencia entre la invención, definida en la reivindicación 1 y las composiciones divulgadas en D1 consiste en la combinación con otro agente anticancerígeno.

El efecto que logra al combinar un agente cancerígeno con un inhibidor de PI3K es mejorar la efectividad de estos para el tratamiento del cáncer.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo obtener combinaciones farmacéuticas contra el cáncer basadas en la combinación de un inhibidor alternativo de PI3K con otro agente anticancerígeno.

Sin embargo, la combinación de inhibidores de PI3K con diferentes agentes anticancerígenos para mejorar la eficacia en el tratamiento de diferentes tipos de cáncer, ya se encuentra divulgada en el documento D2 que enseña la

combinación de moléculas inhibitorias de PI3K con diferentes agentes quimioterapéuticos dando como resultado la disminución de la resistencia a la terapia anticancerígena (Pág. 234 Resumen). Lo anterior como resultado de la inhibición de la enzima PI3k, enzima que al ser activada confiere resistencia a la terapia anticancerígena convencional (Pág. 239 Numeral. 7).

El documento D2, además divulga la combinación de Wortmannin un inhibidor de la enzima PI3k con otros agentes anticancerígenos como: plaquitaxel, cisplatino y gemcitabina (Pág.239 Párr.3), dando como resultado un incremento en la apoptosis celular y una disminución el tamaño de los tumores en comparación con el tratamiento monofármaco (Pág. 239 Párr. 3).

En consecuencia, el la persona del oficio normalmente versada en la materia, incluiría compuestos con actividad inhibitoria sobre PI3K del documento D1 en el combinación con otros agentes anticancerígenos, de acuerdo de las enseñanzas del documento D2 para llegar así al objeto de la reivindicación 1 teniendo claro que la inhibición de esta enzima permite que pueda evitarse el mecanismo celular de protección contra la muerte celular, causado por diferente tipos de agentes anticancerígenos(Pág. 235-236 Documento D2). Por lo cual se considera obvia.

En conclusión, la reivindicación 1 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

9. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D2	Drug Resistance Updates 5 (2002) 234–248	1-5	Nivel inventivo
D1	WO2007023186	1-5	Nivel inventivo

10.Respuesta a los argumentos del señor solicitante

Argumentos con respecto al Oficio 8301 del 07 de mayo del 2013. El señor solicitante reitera que hay suficiente divulgación de composiciones farmacéuticas en capítulo descriptivo. Al respecto se aclara que la solicitud claramente no divulga una composición farmacéutica ni el diseño de la misma, y que de acuerdo al capítulo descriptivo es claro que la base de la solicitud es la administración a diferentes intervalos de tiempo de un inhibidor de PI3K con otros agentes anticancerígenos lo que claramente no es considerado una composición farmacéutica sino un método de tratamiento terapéutico, razón por la cual se configura una clara inconsistencia entre la materia reclamada y la divulgación suficiente que permita a la persona versada en la materia su ejecutabilidad. A pesar de que le solicitante mencione en su capítulo descriptivo composiciones, no existe evidencia de que realmente tenga una composición que indique inequívocamente ingredientes, proporciones y formas farmacéuticas.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-10-22

Elaboró: Diego Armando Galvis Sanchez
Revisó: Consuelo Leguizamon Leguizamon

Review

Activation of the PI3K/Akt pathway and chemotherapeutic resistance

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Received 1 October 2002; accepted 10 October 2002

Abstract

The resistance of many types of cancer to conventional chemotherapies is a major factor undermining successful cancer treatment. In this review, the role of a signal transduction pathway comprised of the lipid kinase, phosphatidylinositol 3-kinase (PI3K), and the serine/threonine kinase, Akt (or PKB), in chemotherapeutic resistance will be explored. Activation of this pathway plays a pivotal role in essential cellular functions such as survival, proliferation, migration and differentiation that underlie the biology of human cancer. Akt activation also contributes to tumorigenesis and tumor metastasis, and as shown most recently, resistance to chemotherapy. Modulating Akt activity is now a commonly observed endpoint of chemotherapy administration or administration of chemopreventive agents. Studies performed in vitro and in vivo combining small molecule inhibitors of the PI3K/Akt pathway with standard chemotherapy have been successful in attenuating chemotherapeutic resistance. As a result, small molecules designed to specifically target Akt and other components of the pathway are now being developed for clinical use as single agents and in combination with chemotherapy to overcome therapeutic resistance. Specifically inhibiting Akt activity may be a valid approach to treat cancer and increase the efficacy of chemotherapy. Published by Elsevier Science Ltd.

Keywords: Akt; Kinase; Apoptosis; Chemotherapy; Cancer

1. Introduction

The PI3K/Akt pathway regulates fundamental cellular functions such as cell growth, survival, and movement (reviewed in [Datta et al., 1999](#); [Katso et al., 2001](#)). Inappropriate activation of the PI3K/Akt pathway has been associated with the development of diseases such as diabetes, autoimmunity, and cancer ([Di Cristofano et al., 1999](#); [Hill and Hemmings, 2002](#); [Nicholson and Anderson, 2002](#); [Testa and Bellacosa, 2001](#); [Vivanco and Sawyers, 2002](#)). This link between activation of the PI3K/Akt pathway and cancer makes this pathway an attractive target for therapeutic strategies.

2. Mechanism of activation of the PI3K/Akt pathway

The PI3K family of enzymes is comprised of numerous isoforms that are divided into three classes, I, II, and III. In mammals, the class I PI3Ks are further subdivided into class Ia and Ib based on differences in both structure and function ([Katso et al., 2001](#); [Vanhaesebroeck and](#)

[Waterfield, 1999](#)). Activation of receptor tyrosine kinases (RTK) or G protein-coupled receptors (GPCR), two principal means for activating the PI3K/Akt pathway, stimulates class Ia and Ib PI3K isoforms, respectively ([Cantley, 2002](#); [Katso et al., 2001](#); [Vanhaesebroeck and Waterfield, 1999](#)) ([Fig. 1](#)). The primary function of PI3K family members is to catalyze the phosphorylation of phosphatidylinositol lipids at the 3' position of the inositol ring. This results in the production of D3 phosphorylated phosphoinositides, most importantly phosphatidylinositol-4,5-bisphosphate (PIP₂) and phosphatidylinositol-3,4,5-trisphosphate (PIP₃), which serve as second messengers by binding to and regulating the function of a diverse set of signaling proteins that are downstream of PI3K ([Katso et al., 2001](#); [Toker, 2000](#); [Vanhaesebroeck and Waterfield, 1999](#)). The specific binding of PIP₂ and PIP₃ to some target proteins is mediated through pleckstrin homology (PH) domains present in these target proteins. For kinases that contain PH domains, binding typically causes translocation to the plasma membrane and subsequent activation ([Katso et al., 2001](#); [Vanhaesebroeck and Waterfield, 1999](#)).

Arguably the most important downstream effector of PI3K is the serine/threonine kinase Akt (also called protein kinase B or PKB). Akt was initially characterized as the human homologue of the viral oncogene *v-akt* from the

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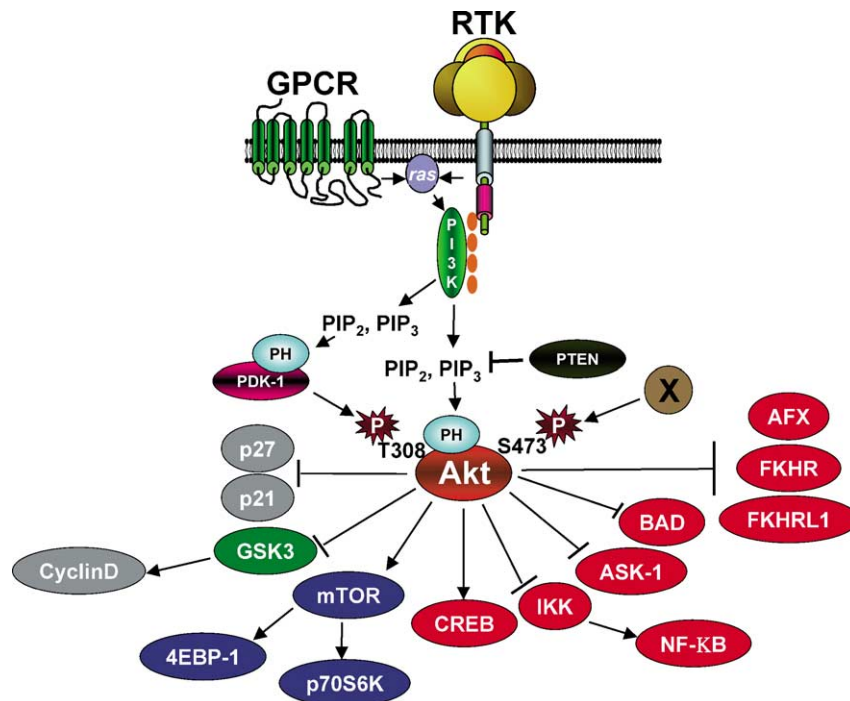


Fig. 1. Overview of the PI3K/Akt pathway. The PI3K/Akt pathway can be activated by receptor tyrosine kinases (RTK), activated Ras, activation of G protein-coupled receptors (GPCR), or inactivation of the tumor suppressor, PTEN. The products of PI3K, PIP₂ and PIP₃, stimulate phosphorylation and activation of Akt. Once active, Akt can control key cellular processes by phosphorylating substrates involved in apoptosis (shown in red), protein synthesis (shown in blue), metabolism (shown in green), and cell cycle (shown in gray). Arrowheads indicate stimulation of downstream substrates; perpendicular lines indicate inhibition of downstream substrates.

transforming retrovirus AKT8 (Bellacosa et al., 1991; Coffey and Woodgett, 1991; Jones et al., 1991). To date, three isoforms of Akt have been identified, Akt1, Akt2 and Akt3, all of which share a high degree of homology at the amino acid level (Datta et al., 1999; Kandel and Hay, 1999). Although the tissue distribution of the three Akt genes is not identical, activation of all three isoforms is similar (Datta et al., 1999; Kandel and Hay, 1999) and is shown in Fig. 1.

Induction of Akt activity is primarily under the control of the phosphoinositide products of PI3K. PIP₂ and PIP₃ bind to the PH domain of Akt, resulting in translocation of Akt to the plasma membrane localization. Subsequently, full activation of Akt requires phosphorylation at two sites, one within the activation loop (T308 for Akt1), and one within the C-terminus (S473 for Akt1) (Datta et al., 1999; Kandel and Hay, 1999; Leslie et al., 2001). Phosphorylation of T308 is carried out by the 3'-phosphoinositide-dependent kinase-1 (PDK-1), whose activity is also regulated by PI3K (Leslie et al., 2001; Toker and Newton, 2000; Vanhaesebroeck and Alessi, 2000). The kinase responsible for S473 phosphorylation has not been identified, and might possibly be Akt itself or a distinct upstream kinase (Datta et al., 1999; Kandel and Hay, 1999; Leslie et al., 2001).

Although Akt is predominantly activated in a PI3K-dependent manner, PI3K-independent mechanisms have also been reported. Agonists of protein kinase A (PKA) or β -adrenergic receptors, as well as changes in intracel-

lular calcium have all been demonstrated to increase Akt activity in a manner that was resistant to administration of PI3K inhibitors (Datta et al., 1999; Kandel and Hay, 1999; Vanhaesebroeck and Alessi, 2000). However, other studies have shown that Akt activation by PKA agonists and increases in calcium levels are sensitive to PI3K inhibition (Vanhaesebroeck and Alessi, 2000). Further work is necessary to clarify these discrepancies.

In addition to activation by RTK or GPCR, Akt can also be activated by many forms of cellular stress. These forms of cellular stress include heat shock, administration of ultraviolet light, ischemia, hypoxia, hypoglycemia, and oxidative stress (Chen et al., 2001; Crossthwaite et al., 2002; Ma et al., 2001; Martindale and Holbrook, 2002; Ouyang et al., 2000; Sakurai et al., 2001; Shaw et al., 1998). Activation of Akt in response to cellular stress may be a generalized, compensatory protective mechanism activated by the cell to escape death, which is relevant because cancer cells likely perceive chemotherapy as a cellular insult, and many forms of chemotherapy exert their cytotoxic effects through the generation of reactive oxygen species.

Once active, Akt controls cellular functions through phosphorylation of downstream substrates (Fig. 1). Akt controls cellular survival through phosphorylation of proteins such as Bad and ASK-1, cell cycle by phosphorylation of cyclin-dependent kinase inhibitors such as p21 and p27 (Blume-Jensen and Hunter, 2001; Diehl et al., 1998; Liang

et al., 2002; Medema et al., 2000; Shin et al., 2002; Testa and Bellacosa, 2001; Viglietto et al., 2002; Zhou et al., 2001), cell metabolism by phosphorylation of glycogen synthase kinase-3 (GSK-3) (Cross et al., 1995a; Lawlor and Alessi, 2001), gene transcription by phosphorylation of transcription factors such as FKHR family members, and protein synthesis by phosphorylation of proteins such as mTOR (Nave et al., 1999; Sekulic et al., 2000).

3. Regulation of the PI3K/Akt pathway

Whereas the production of 3' phosphoinositides by PI3K positively stimulates the activity of the PI3K/Akt pathway, Akt activity is negatively regulated by phosphatases that dephosphorylate phosphoinositides at the 3' or 5' positions and a recently described Akt binding protein, CTMP (carboxy terminal modulating protein). Negative regulation of the PI3K/Akt pathway is mainly accomplished by the dual function lipid and protein phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10), which dephosphorylates the D3 position of PIP₂ and PIP₃, thereby counteracting the activity of PI3K (Vazquez and Sellers, 2000). Although PTEN is both a protein and lipid phosphatase, its tumor suppressor function is due to its lipid phosphatase activity. A recent study by Stocker et al. has shown that the major consequence of decreased PIP₃ levels by PTEN is the control of Akt activity (Stocker et al., 2002).

In addition to PTEN, negative regulation of Akt activity is also exerted by a phosphatase called SHIP (SH2-containing inositol phosphatase), which dephosphorylates PIP₃ at the 5' position (Leslie et al., 2001). The importance of SHIP in regulating the PI3K/Akt pathway in vivo is unclear, however, because unlike PTEN heterozygous knock out mice, SHIP knockout mice do not develop tumors (Helgason et al., 1998; Liu et al., 1999). However, the lifespan of these mice is shortened, which limits the ability to detect tumor development. In addition to inhibition by phosphatases, Akt activity can also be inhibited by the binding of CTMP, a newly discovered protein that binds to the C terminus of Akt at the plasma membrane (Maira et al., 2001).

4. Role of Akt in cellular survival and tumor biology

The balance between cellular survival and apoptosis can determine whether a damaged cell becomes carcinogenic and whether an established tumor will respond to therapy. Because Akt is the prototypic kinase that promotes cellular survival, cellular survival is the cellular process controlled by Akt that has been studied most intensely.

Akt regulates cellular survival through phosphorylation of downstream substrates that indirectly or directly control the apoptotic machinery (Fig. 1). Akt exerts indirect control of apoptosis through regulation of transcription. Phosphorylation of the forkhead family of transcription factors

(AFX, FKHR, and FKHRL1) by Akt inhibits transcription of pro-apoptotic genes such as *Fas-L*, *IGFBP1* and *Bim* (Datta et al., 1999; Nicholson and Anderson, 2002). Conversely, phosphorylation of CREB or IKK (which releases NF- κ B from inhibition) by Akt increases transcription of pro-survival genes (Du and Montminy, 1998; Ozes et al., 1999; Romashkova and Makarov, 1999). Akt can also directly regulate the apoptotic machinery by phosphorylating and inactivating pro-apoptotic proteins such as BAD, which controls release of cytochrome c from mitochondria, and ASK1 (apoptosis signal-regulating kinase-1), a mitogen-activated protein kinase involved in stress- and cytokine-induced cell death (Datta et al., 1997; del Peso et al., 1997; Kim et al., 2001; Zha et al., 1996).

Three separate lines of evidence support the hypothesis that Akt activity plays an important role in tumorigenesis and tumor growth. *First*, studies in transgenic mice that overexpress constitutively active Akt in a tissue-specific manner show that Akt, in concert with other genetic alterations, contributes to tumor formation in tissues such as skin, breast, lymph node, ovary, and brain (Ackler et al., 2002; Holland et al., 2000; Hutchinson et al., 2001; Jones et al., 2000; Malstrom et al., 2001; Moorehead et al., 2001; Orsulic et al., 2002; Parsons et al., 2001; Segrelles et al., 2002). The phenotype of PTEN heterozygous knockout mice was somewhat similar, in that increased Akt activity and a high incidence of tumors in colon, testes, thyroid, prostate, breast, endometrium, liver and hematopoietic cells were observed (reviewed in Kandel and Hay, 1999; Vivanco and Sawyers, 2002).

Second, Akt activation is an early cellular response to carcinogen exposure and may be an important step in environmental carcinogenesis. Our laboratory has recently shown that treatment of normal human lung epithelial cells with a tobacco-specific carcinogen such as NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) increases Akt activity and induces an Akt-dependent, partially transformed phenotype (Brognard et al., 2002). Carcinogens such as TCDD and benz(a)pyrene also activate Akt (K.A. West and P.A. Dennis, data not shown), suggesting that activation of the PI3K/Akt pathway may be a common cellular response to carcinogen exposure.

Third, active Akt has been detected in human cancers in vivo. Akt isoform overexpression has been described in breast, colon, ovarian, pancreatic and prostate cancers (Kandel and Hay, 1999; Roy et al., 2002; Vivanco and Sawyers, 2002). Moreover, immunohistochemical studies using phospho-specific antibodies against S473 that only recognize Akt in an active state have shown that Akt activity is detectable in cancers such as multiple myeloma, head and neck cancer, breast cancer, colon cancer, ovarian cancer, and prostate cancer (Alkan and Izban, 2002; Gupta et al., 2002; Hsu et al., 2001; Kanamori et al., 2001; Kurose et al., 2001; Malik et al., 2002; Nakayama et al., 2001; Roy et al., 2002; Sun et al., 2001; Yuan et al., 2000). In an effort to expand the analysis of Akt in human cancers, we have recently

completed an analysis of the PI3K/Akt pathway in 450 tumor samples from eight different tumor types, and have found that Akt is active in 55% of these samples (P.A. Dennis, data not shown). Together, these results demonstrate the significance of Akt activity in tumor promotion and growth, and the potential relevance of Akt as a molecular target.

5. Modulation of the PI3K/Akt pathway by administration of chemotherapy

The acknowledged critical involvement of Akt in cellular survival has led to an intense investigation into the contribution of Akt to tumor cell survival in response to chemotherapy. Numerous groups have reported the effects of chemotherapy on Akt activity in tumor cell lines, as well as immortalized and non-immortalized cells. These studies show that the administration of chemotherapeutic agents commonly results in modulation of the PI3K/Akt pathway. We have summarized a number of these studies in Table 1.

The aggregate results from this collection of studies indicate that the ability of chemotherapy to inhibit Akt activity correlates well with the cytotoxic effects of these agents. For instance, studies with signal transduction inhibitors such as trastuzumab, ZD1859, or GW572016 that target members of the epidermal growth factor receptor (EGFR) showed that these compounds decreased Akt activity and increased cell death in human breast, prostate, ovarian, epidermoid, and head and neck cancer cell lines (Anderson et al., 2001; Cuello et al., 2001; Gupta et al., 2002; Moasser et al., 2001; Normanno et al., 2002; Xia et al., 2002; Yakes et al., 2002). In the reports by Cuello et al., and Yakes et al., utilization of small molecule inhibitors of the PI3K/Akt pathway (LY294002-discussed below) and genetic manipulation of the PI3K/Akt pathway confirmed the dependence of trastuzumab-mediated death on inhibition of Akt (Cuello et al., 2001; Yakes et al., 2002). Yakes et al. showed that trastuzumab administration resulted in modulation of cyclin D and p27 levels, which are downstream of Akt and controlled by Akt (Fig. 1). Moreover, Xia et al. and Anderson et al. verified the effects of trastuzumab in vivo using human xenograft or athymic mouse models, respectively (Anderson et al., 2001; Xia et al., 2002).

Inhibition of Akt activity and increased cytotoxicity was demonstrated in human lung cancer cell lines in response to topotecan, a topoisomerase I inhibitor. Similar results were seen in human ovarian and head and neck cancer cells, or in transformed rat fibroblasts in response to farnesyl transferase inhibitors (FTIs) or depsipeptide, respectively (Fecteau et al., 2002; Gupta et al., 2002; Jiang et al., 2000; Nakashio et al., 2000). In the study by Jiang et al. using FTIs, the ability to inhibit Akt activity and increase cell death was verified by biochemical and genetic approaches (Jiang et al., 2000). Decreases in Akt activity were dependent on FTI-mediated inhibition of PI3K activity (Jiang et al., 2000). In the report using topotecan, genetic methods were employed to inhibit

the PI3K/Akt pathway, and a loss of PI3K, PDK-1, and Bad function correlated with the decrease in Akt activity and subsequent increase in cell death (Nakashio et al., 2000).

Administration of STI571 or the broad-spectrum kinase inhibitor, staurosporine, also attenuated Akt activity and increased apoptosis in a variety of tumor and normal cell lines (Dierov et al., 2002; Fang et al., 2000; Klejman et al., 2002; Sato et al., 2002a; Tenzer et al., 2001; Wan et al., 2002). In the report by Fang et al., STI-571 decreased levels of the pro-survival proteins Bcl-2 and c-IAP (two proteins whose transcription is controlled by Akt activity) in conjunction with inhibition of Akt activity (Fang et al., 2000). Klejman et al. demonstrated that the combination of the PI3K inhibitors, wortmanin or LY294002, with STI571 enhanced the selective cell killing effects of this compound (Klejman et al., 2002). In the work by Tenzer et al. reduced phosphorylation of a downstream Akt substrate (GSK-3) correlated with the effects of PKC412 (a staurosporine derivative) on Akt activity and cell death. These observations were confirmed using genetic approaches to manipulate Akt activity (Tenzer et al., 2001). Interestingly, the Tenzer et al. study also demonstrated that staurosporine administration inhibited Akt activity, but in a manner different from that of LY294002. The mechanism of staurosporine induced inhibition of the PI3K/Akt pathway was elucidated by the work of Sato et al., who used a small molecule inhibitor, genetic approaches, and in vivo assays to demonstrate that staurosporine directly inhibits the activity of PDK-1 (Sato et al., 2002a).

Although most reports have shown that chemotherapy decreases Akt activity, there is also evidence that chemotherapeutic agents can increase Akt activity. In most cases, increases in Akt activity in response to chemotherapy are transient and are followed by subsequent decreases in activity (Table 1). For example, work from our laboratory showed that in several human breast cancer cell lines, addition of trastuzumab, tamoxifen, or doxorubicin increased Akt activity at early time points, prior to the detection of apoptosis (Clark et al., 2002). This early increase in Akt activity was exploitable, in that the addition of a PI3K inhibitor to any of these agents greatly potentiated apoptosis. The initial increases in Akt activity seen with trastuzumab or tamoxifen were followed by decreases at later times, which is consistent with other reports (Clark et al., 2002; Cuello et al., 2001; Normanno et al., 2002; Yakes et al., 2002). In contrast, a subsequent decrease in Akt activity was not observed with doxorubicin. The basis for the different kinetics of Akt activation by these agents is unclear.

Other reports have also shown increases in Akt activity in response to chemotherapy. Treatment of human ovarian cancer cell lines with cisplatin increased Akt activity without measurable cytotoxicity (Asselin et al., 2001; Hayakawa et al., 2002). Hayakawa and colleagues used small molecule inhibitors and genetic approaches to verify that the induction of Akt activity by cisplatin was responsible for the observed chemotherapeutic resistance (Asselin et al., 2001; Hayakawa

Table 1

Chemotherapeutic agents that modulate activity of the PI3K/Akt pathway

Chemotherapy	Cellular system	Effect on Akt	Reference
Anthracyclins			
Doxorubicin	MB468, ZR571, MB231 cells	Stimulation	Clark et al. (2002)
Daunorubicin	U937 cells	Stimulation	Plo et al. (1999)
Signaling inhibitors			
Trastuzumab	MB468, ZR751, MB231, MCF-7, BT-474 SKBR-3, MB453, MD361 cells	Stimulation/inhibition Inhibition	Clark et al. (2002) Cuello et al. (2001), Normanno et al. (2002), Yakes et al. (2002)
ZD1839	BT-474, SKBR-3, MD361, T47D cells SQ20B cells A431, SKOV3 cells DU145 cells	Inhibition	Normanno et al. (2002) Gupta et al. (2002) Moasser et al. (2001) Anderson et al. (2001)
GW572016	BT-474, HN5 cells	Inhibition	Xia et al. (2002)
Tamoxifen	MCF-7, ZR571, MB231 cells	Stimulation/inhibition	Clark et al. (2002)
STI571	CML-BC (blast crisis), K562, BaF3-TP cells	Inhibition	Dierov et al. (2002), Fang et al. (2000), Klejman et al. (2002)
Topoisomerase inhibitors			
Topotecan	A549 cells	Inhibition	Nakashio et al. (2000)
Etoposide	NIH3T3 cells	Stimulation	Tang et al. (2001)
DNA damaging agents			
Cisplatin	Caov-3, A2780 cells	Stimulation/inhibition	Asselin et al. (2001)
Nucleoside analogues			
Gemcitabine	PK1 and PK8 cells BT474 and MB453 cells	No effect	Ng et al. (2001), Ng et al. (2000) Nelson and Fry (2001)
Microtubule agents			
Paclitaxel	SW626; OVCA433 cells	Stimulation Inhibition	Mabuchi et al. (2002) Del Bufalo et al. (2002)
cdk inhibitors			
Staurosporine	Ishikawa cells NIH 3T3 cells	Inhibition Stimulation	Wan et al. (2002) Tang et al. (2001)
PKC412	HT1080, N-17 and COS-7 cells	Inhibition	Tenzer et al. (2001)
UCN-01 (7-hydroxystaurosporine)	Myr Akt-Rat1a and Rat1a cells	Inhibition	Sato et al. (2002a)
Farnesyltransferase inhibitors			
FTI-277	NIH3T3, Akt-transformed NIH3T3 cells	Inhibition	Jiang et al. (2000)
L744,832	OVCAR-3, A2780, COS-7, SQ20B cells	Inhibition	Gupta et al. (2002)
Histone deacetylase inhibitors			
FR901228 (depsipeptide)	ras-10T 1/2 cells	Inhibition	Fecteau et al. (2002)
Sodium butyrate	KM20 (human colon cancer cells)	Stimulation	Wang et al. (2002)

Representative agents from virtually every class of chemotherapy modify activity of the PI3K/Akt pathway. In most cases, inhibition of the pathway coincides with cytotoxicity. In selected studies where genetic manipulation of the system was performed, inhibition of the pathway was responsible for mediating cytotoxicity (see text for details). Stimulation/inhibition indicates a biphasic response of stimulation followed by inhibition.

et al., 2002). They further demonstrated that genetic alteration of the Akt downstream substrate Bad could sensitize the cells to chemotherapy-induced cell death (Asselin et al., 2001; Hayakawa et al., 2002; Cheng et al., 2002). In a separate set of studies, the addition of paclitaxel to human ovarian carcinoma cells increased Akt activity and was correlated with low levels of cell death (Mabuchi et al., 2002). Mabuchi et al. used small molecule inhibitors as well as genetic alterations of both Akt and the downstream Akt substrate, Bad, to corroborate their results of paclitaxel-induced Akt activation and increased chemotherapeutic resistance (Mabuchi et al., 2002). These results with paclitaxel are in contrast to another report that showed decreases in Akt activity in response to paclitaxel. Possible explanations for this discrepancy

include the timepoint at which Akt activity was assayed and the p53 status of the cell lines (Del Bufalo et al., 2002).

Finally, in some cases chemotherapy fails to affect Akt activity. In a series of studies in human pancreatic adenocarcinoma cells and breast cancer cells that have constitutively high Akt activity, addition of gemcitabine failed to affect Akt activity or elicit cell death (Nelson and Fry, 2001; Ng et al., 2000, 2001). Whether any effects on Akt or cellular survival would be observed at different time points is unknown. Taken together, the results from these reports show that modulation of the PI3K/Akt pathway is a common observation with the administration of chemotherapy, and suggest that in some cases, modulation of Akt activity may be directly responsible for the response to chemotherapy.

6. Modulation of the PI3K/Akt pathway by administration of chemopreventive agents

In addition to being modulated by agents designed to treat established cancers, Akt may also be targeted by several bioactive compounds derived from natural products that prevent cancer in different model systems. Treatment of prostate cancer cells with the compound indole-3-carbinol (IC3), a bioactive compound found in Brassica vegetables, induced apoptosis in an Akt-dependent manner (Chinni and Sarkar, 2002). In these studies, Chinni and Sarkar demonstrated that the effects of IC3 were due to decreased levels and activity of EGFR, as well as PI3K (Chinni and Sarkar, 2002). This is interesting considering that upregulation of EGFR is a common characteristic of human cancers (Salomon et al., 1995). Similar to the effects of administering IC3, administration of green or black tea polyphenols inhibited the growth of breast cancer or prostate cancer cells, respectively (Klein and Fischer, 2002; Pianetti et al., 2002). In both sets of studies, decreased cell growth was dependent on the ability of these compounds to downregulate Akt activity (Klein and Fischer, 2002; Pianetti et al., 2002).

In addition to IC3 or tea polyphenols, various dietary flavonoid compounds (polyphenolic derivatives abundant in many edible plants) have been shown to affect Akt signaling. For instance, nobiletin and luteolin inhibit Akt-dependent stimulation of NF- κ B gene transcription in human fibrosarcoma cells and murine macrophages, respectively (Sato et al., 2002b; Xagorari et al., 2001). In the report using luteolin, administration of luteolin was correlated with decreased Akt activity and phosphorylation of the downstream Akt substrate IKK κ , which functions in the regulation of NF- κ B activity (Xagorari et al., 2001). These results are interesting in light of the fact that Akt-dependent control of NF- κ B gene transcription has been implicated in Akt-mediated survival signaling and likely plays a role in the survival of tumor cells (discussed above). In the study by Sato et al., administration of nobiletin decreased tumor cell invasion as well as Akt activity (Sato et al., 2002b). Finally, Yoshizumi et al. showed that the flavonoid, quercetin, decreased angiotensin-induced Akt activity and cell growth in rat aortic smooth muscle cells (Yoshizumi et al., 2001). This is relevant because the PI3K inhibitor, LY294002 (discussed below), is a derivative of quercetin (Vlahos et al., 1994). Given that administration of standard chemotherapy agents is usually associated with at least mild toxicity, the possible use of non-toxic, natural compounds to target the PI3K/Akt pathway and prevent cancer is attractive.

7. Inhibiting the PI3K/Akt pathway to overcome chemotherapeutic resistance

Because activation of the PI3K/Akt pathway confers chemotherapeutic resistance and conventional chemotherapies and chemopreventive agents commonly affect the PI3K/

Akt pathway, new compounds are being developed to directly inhibit this pathway by targeting key regulatory proteins such as PI3K, Akt or mTOR. Several of these new compounds have emerged over the past few years and hold potential for new effective therapies. Used alone or in combination with existing therapies, these inhibitors of the PI3K/Akt pathway will be developed to exploit activation of the PI3K/Akt pathway within cancer cells and/or enhance the efficacy of existing chemotherapeutic agents.

7.1. Classical PI3K inhibitors

7.1.1. Wortmannin

Wortmannin is a fungal metabolite and a potent inhibitor of type I PI3K (Powis et al., 1994). At nanomolar concentrations, wortmannin irreversibly inhibits PI3K by binding to the p110 catalytic subunit. When tested in a large panel of protein kinases, wortmannin was found to have high selectivity for inhibiting PI3K (Davies et al., 2000). Wortmannin has antitumor activity in vitro and in vivo studies, with an IC₅₀ range for inhibition of PI3K from 2 to 4 nM (Powis et al., 1994; Schultz et al., 1995). Although wortmannin possesses high specificity for inhibiting PI3K at nanomolar doses, other enzymes, especially those with PI3K domains, are also inhibited by wortmannin at higher doses. Wortmannin has been shown to inhibit myosin light chain kinase, phospholipase C, phospholipase D, and phospholipase A₂, and DNA-dependent protein kinase (DNA-PK) at similar or higher concentrations needed to inhibit PI3K itself (Nakanishi et al., 1992; Bonser et al., 1991; Boulton et al., 2000; Cross et al., 1995b). Therefore, studies performed with wortmannin must be examined for the dose of wortmannin employed. Moreover, results obtained with small molecule inhibitors such as wortmannin should be confirmed by using genetic approaches to inhibit the pathway.

Wortmannin has activity in vitro and in vivo. In multiple in vitro studies, pretreatment with wortmannin, or simultaneous treatment with wortmannin and a growth factor, potentially inhibited the proliferative response and/or promoted apoptosis by inhibiting PI3K activity, as measured by in vitro kinase assays (reviewed in Katso et al., 2001). Based upon the potent inhibitory effect of wortmannin in vitro assays, additional studies in animal models have been conducted to test the efficacy of wortmannin in inhibiting tumor growth in vivo. Xenograft studies in SCID mice with human or mouse mammary carcinoma cells, or human pancreatic carcinoma cells, have demonstrated the ability of wortmannin to reduce tumor size when administered daily (Lemke et al., 1999; Schultz et al., 1995). When given to SCID mice bearing tumors from pancreatic cancer cells, wortmannin was found to inhibit Akt phosphorylation by 50% relative to vehicle control (Ng et al., 2001). Similarly, tumor burden from human breast cancer cells was reduced by 97 or 85% compared to controls after 14 days of 1 mg/kg wortmannin administration by oral gavage beginning 1 day or 7 days, respectively, after tumor implantation (Lemke et al., 1999). In these studies,

no significant difference in PI3K activity was observed between wortmannin-treated tumor homogenates compared to control tumor homogenates, thus raising the possibility of nonspecific effects of wortmannin. However, in cell culture, wortmannin treatment significantly inhibited PI3K activity in these cells. With discontinued use of wortmannin, growth rates of wortmannin-treated tumors were similar to controls, suggesting a cytostatic mechanism of wortmannin. Therefore, in some systems, wortmannin can inhibit the PI3K/Akt pathway and reduce cell proliferation and/or tumor growth.

Although these studies suggest that blocking the PI3K/Akt pathway with wortmannin might be a valuable approach to treat cancer, one disadvantage to the use of wortmannin is its stability in an aqueous environment. Wortmannin is soluble in organic solvents, which may limit its use in clinical trials. Currently, water-soluble wortmannin conjugates are being developed to circumvent this issue, but some of these conjugates inhibit PI3K activity to a lesser extent compared to non-conjugated wortmannin (Varticovski et al., 2001). Additional studies are required to determine the effectiveness of these new compounds in xenograft animal models.

Although inhibition of the PI3K/Akt pathway by wortmannin alone may inhibit cell proliferation, promote apoptosis, and/or inhibit tumor growth, its effects may be further enhanced by combining wortmannin with radiation or traditional chemotherapeutic drugs (Rosenzweig et al., 1997; Sarkaria et al., 1998; Kim et al., 2000). Wortmannin can potentiate the effect of ionizing radiation by partially inhibiting the function of DNA-PK, which promotes repair of DNA double strand breaks (Boulton et al., 2000; Boulton et al., 1996; Sarkaria et al., 1998). Most studies using wortmannin as a DNA-PK inhibitor have used micromolar concentrations, which are much higher concentrations than are needed to inhibit PI3K itself; however, some recent studies have been able to use nanomolar concentrations of wortmannin with radiation and still observe the enhancement of the combined therapy (Edwards et al., 2002; Sarkaria et al., 1998).

Similar to its effects in combination with radiation, the effectiveness of chemotherapeutic drugs is also enhanced by the addition of wortmannin. Both in vivo and in vitro studies using a human ovarian cancer model with paclitaxel administration have shown an increase in apoptosis and decrease in tumor growth when wortmannin was added to the paclitaxel treatment, compared to either wortmannin or paclitaxel treatment alone (Hu et al., 2002). Similarly, induction of apoptosis by cisplatin therapy was also enhanced when wortmannin was added to a resistant ovarian carcinoma cell line, compared to either treatment alone (Asselin et al., 2001). In studies with human pancreatic adenocarcinoma cell lines, gemcitabine-induced apoptosis was markedly enhanced by the addition of wortmannin (Ng et al., 2000). The combination of gemcitabine and wortmannin in a human colon cancer cell line also produced a marked increase in apoptosis compared to either treatment alone, as well as the combinations of wortmannin plus sodium butyrate or 5-fluorouracil

(Wang et al., 2002). In these studies listed, the potentiation of apoptosis caused by wortmannin was associated with decreased levels of phosphorylated Akt in wortmannin-treated cells. Most recently, Klejman et al. showed that treatment of bone marrow from healthy volunteers or patients with chronic myelogenous leukemia with STI571 (Gleevec) and wortmannin inhibited cell growth and induced apoptosis in the cells derived from the leukemia patients, whereas normal bone cells were unaffected by the combined treatment (Klejman et al., 2002). When given singly, STI571 or wortmannin inhibited the phosphorylation of Akt and p70^{S6K} and induced apoptosis in CML cells. In combination, STI571 and wortmannin greatly potentiated apoptosis, which was associated with an increase in caspase-3 activation compared to either compound alone. In all these studies, wortmannin, when used in nanomolar concentrations, was able to enhance the effectiveness of a chemotherapeutic drug. Thus, the combination of a PI3K inhibitor such as wortmannin with conventional chemotherapies may provide better treatment for various types of cancer that have become resistant to standard therapies.

7.1.2. LY294002

The flavonoid derivative, LY294002, is also a potent inhibitor of PI3K. LY294002 is a competitive and reversible inhibitor of the ATP binding site of PI3K, with an IC₅₀ about 500-fold higher than wortmannin at 1.4 μ M (Sanchez-Margalet et al., 1994). LY294002 has no inhibitory effect against several ATP-requiring enzymes such as PI4K, EGF receptor tyrosine kinase, c-src, MAP kinase, S6 kinase, diacylglycerol kinase, protein kinase A, protein kinase C, and ATPase (Vlahos et al., 1994). However, LY294002 has been shown to be an antagonist of the aryl hydrocarbon receptor (AHR), which is a ligand-activated transcription factor (Guo et al., 2000). The binding of LY294002 to AHR prevents transcriptional activation, thus preventing gene expression. Although similar concentrations of LY294002 can inhibit PI3K function and work as an AHR antagonist, the inhibitory effect of LY294002 on AHR appears to be unrelated to its ability to inhibit PI3K (Guo et al., 2000).

LY294002 has been widely used to inhibit PI3K in vitro and to assign PI3K-dependence to various cellular functions. In a melanoma cell line, LY294002 treatment caused a G1 phase cell cycle arrest, which was associated with increased p27 levels, inhibition of CDK4 and CDK2, and inhibition of retinoblastoma protein hyperphosphorylation (Casagrande et al., 1998). In studies with non-small cell lung cancer cells performed in our laboratory, LY294002 treatment inhibited Akt phosphorylation and activity and induced apoptosis in cells with constitutively active Akt (Brognard et al., 2001). When combined with individual chemotherapies used in the treatment of non-small cell lung cancer or irradiation, LY294002 greatly enhanced chemotherapy- or radiation-induced apoptosis and potentiated radiation-induced decreases in clonogenic cell growth in cells with high Akt levels. LY294002 was ineffective

in cells with low levels of Akt activity (Brognard et al., 2001). We obtained similar results when we assessed a series of breast cancer cell lines for response to LY294002. LY294002 greatly potentiated apoptosis caused by doxorubicin, trastuzumab, paclitaxel, etoposide, or tamoxifen (Clark et al., 2002). In a separate study investigating small cell lung cancer cells, Krystal et al. showed that LY294002 treatment potentially inhibited Akt phosphorylation induced by stem cell factor and insulin-like growth factor treatment, with an average IC_{50} of $5 \mu M$ (Krystal et al., 2002). Inhibition of Akt phosphorylation was associated with an inhibition of cell growth. In the studies mentioned above, the results obtained with LY294002 were confirmed by genetically inhibiting the pathway with dominant negative mutant forms of Akt. The fact that transfection of Akt mutants yielded similar results as LY294002 administration suggests that the effects of LY294002 are due to disruption of the PI3K/Akt pathway, and highlight the potential of LY294002 as a therapeutic agent.

Relatively few *in vivo* studies have been conducted to demonstrate the efficacy of LY294002 on the inhibition of tumor growth. In human colon cancer xenografts in SCID mice, daily intravenous administration of LY294002 from day 20 to 40 suppressed tumor growth and induced apoptosis (Semba et al., 2002). In addition to suppressing tumor growth, LY294002 treatment also reduced the appearance of mesenteric dissemination nodules compared to control animals. Interestingly, intraperitoneal administration of LY294002 did not attenuate tumor growth as much as intravenous administration. LY294002-treated animals also exhibited less phosphorylated Akt compared to control animals, as determined by immunohistochemical analysis (Itoh et al., 2002; Semba et al., 2002). In a different xenograft study using athymic, immunodeficient mice, daily intraperitoneal administration of LY294002 for 3 weeks inhibited the growth of ovarian carcinoma tumors (Hu et al., 2000a). The mean tumor burden in the LY294002-treated animals was reduced by approximately 65% compared to control animals, with less ascites noted in the LY294002-treated animals. Furthermore, morphological studies demonstrated that LY294002 treatment induced apoptosis *in vivo*. Although LY294002 treatment markedly reduced the growth of the tumors, 80% of the LY294002-treated mice developed dry and scaly skin. Within 3 days after the cessation of LY294002 treatment, however, the skin of the mice returned to normal, consistent with LY294002 being a reversible inhibitor of PI3K.

LY294002 has also been combined with traditional chemotherapeutic agents *in vivo*. A subsequent study by Hu et al. using their athymic mice ovarian cancer model system demonstrated that combining paclitaxel with LY294002 resulted in decreased tumor growth and ascites. More importantly, these studies used LY294002 at a lower dose with paclitaxel, and these mice showed no signs of dermatological side effects (Hu et al., 2002). Therefore, not only does LY294002 enhance the effectiveness of pacli-

taxel, but a lower dose of LY294002 may be effective and also less toxic. Similar to wortmannin, the combination of LY294002 with various cytotoxic drugs or radiation enhances the effectiveness of these treatments, and highlights the therapeutic potential of targeting this pathway.

7.2. Novel PI3K inhibitors

Both wortmannin and LY294002 inhibit PI3K activity by targeting the ATP binding domain of the p110 subunit. Because of difficulties with stability, solubility, and toxicity of these compounds, efforts are underway to develop new inhibitors of the PI3K/Akt pathway. Recent studies with analogues of naturally occurring phosphoinositol lipids show that many of them effectively inhibit PI3K activity, though the precise mechanisms they employ to inhibit PI3K are under current investigation.

7.2.1. Alkylphospholipids

Alkylphospholipids (ALK) are a class of ether lipids with potent antineoplastic and antiproliferative activities (Crul et al., 2002; Hilgard et al., 1997). ALK interfere with the metabolism of phospholipid constituents of the cell membrane, reduce PLC-mediated inositol (1,4,5)-triphosphate and calcium release, and inhibit PKC (Crul et al., 2002). The lead compound for this class of agents is the alkylphosphocholine analogue, D-21266, also known as perifosine (Hilgard et al., 1997). Perifosine was developed as a less toxic form of ALK, because the parent analogue, miltefosine, was found to induce severe gastrointestinal toxicity when administered orally in human clinical trials (Hilgard et al., 1997; Patel et al., 2002). *In vitro*, perifosine has shown stronger anti-neoplastic effects against melanoma, lung, prostate, colon, and breast cancer cells, compared to miltefosine (Crul et al., 2002). Unfortunately, recent results from a Phase I clinical trial with perifosine showed similar toxicities as those that were observed previously with miltefosine, namely adverse gastrointestinal effects when perifosine was administered by mouth daily (Crul et al., 2002). Different dosing schedules with perifosine are now being considered for future clinical trials. In addition, a Phase I trial exploring the combined toxicities and effects of perifosine and radiotherapy will soon open for accrual.

7.3. Novel Akt inhibitors

7.3.1. Phosphatidylinositol (PI) ether lipid analogues

Targeting the binding of ATP to a kinase has been the traditional approach to developing kinase inhibitors, but this approach has been plagued by the development of compounds with low specificity. In an effort to develop effective Akt inhibitors and improve specificity, investigators have sought to inhibit the binding of PIP_2 and PIP_3 to the pleckstrin-homology (PH) domains of Akt. Molecularly modeling the interaction of PIP_2 with the PH domain

of Akt has led to rationale development of novel phosphatidylinositol ether lipid analogues that were designed to inhibit this interaction (Hu et al., 2001; Hu et al., 2000b; Qiao et al., 1998; Rong et al., 2001). Treatment of multiple cancer cell lines with these PI analogues has demonstrated that several analogues can impair PI3K and Akt kinase activities and inhibit cancer cell growth (Hu et al., 2001; Hu et al., 2000b; Qiao et al., 1998). One of the first generation analogues, DPIEL, was found to inhibit PI3K with an IC_{50} of $14.8 \pm 5.6 \mu M$, and to block the growth of a human colon cancer cell line with an IC_{50} of $2.1 \mu M$ (Hu et al., 2001). When injected intraperitoneally, DPIEL also inhibited tumor cell growth in xenograft model systems (Hu et al., 2000b; Qiao et al., 1998), which prompted toxicology and solubility studies evaluating DPIEL as a potential drug. Unfortunately, DPIEL was acid labile when given orally. When given intravenously, DPIEL induced massive hemolysis. These data will limit consideration of this compound for clinical use (Egorin et al., 2002). Newer generation analogues bearing different substitutions have demonstrated the ability to selectively block Akt activation without affecting upstream kinases such as PDK-1, or other signaling pathways such as the MAPK pathway (Kozikowski et al., 2002). Moreover, these compounds are most toxic to cell lines with high endogenous levels of Akt activity. Additional studies will be required to evaluate the effectiveness of these compounds in other systems, to establish toxicity profiles, and to characterize the mechanism of Akt inhibition.

7.4. mTOR inhibitors

As an alternative to targeting PI3K or Akt itself, other investigators have chosen to inhibit the PI3K/Akt pathway by targeting kinases downstream of Akt such as the mammalian target of rapamycin (mTOR (or FRAP)). mTOR is an evolutionarily conserved member of the phosphatidylinositol kinase related kinase family (Fingar et al., 2002), and inhibition of PI3K by either wortmannin or LY294002 suppresses mTOR activity (Brunn et al., 1996). mTOR is a direct target of Akt and functions as a molecular sensor regulating protein synthesis based upon the availability of nutrients such as amino acids (Nave et al., 1999). Both mTOR and PI3K activity, via independent and parallel pathways, modulate the activity of at least two kinases that control protein synthesis; namely, $p70^{S6K}$, a ribosomal kinase, and 4E-BP1, which binds and inhibits the eukaryotic initiation factor-4E (Braun-Dullaeus et al., 2001; Brunn et al., 1996; Fingar et al., 2002) (Fig. 1). $p70^{S6K}$ and 4E-BP1 have been demonstrated to be direct targets of mTOR. By controlling protein synthesis, $p70^{S6K}$ and 4E-BP1 also control cell growth and hypertrophy (Fingar et al., 2002), which are obviously important processes for tumor growth. Thus, even more distal steps in the PI3K/Akt pathway may have the potential to be exploited to treat cancer.

7.4.1. Rapamycin

The prototypic inhibitor of mTOR, rapamycin, is a macrocyclic lactone antibiotic that induces a G_1 cell cycle arrest in *Drosophila*, yeast, and mammalian cells (Schmelzle and Hall, 2000). Rapamycin forms a complex with FKBP12 (FK506-binding protein), which specifically binds to mTOR and inhibits its function (Neshat et al., 2001). The inhibition of mTOR inhibits $p70^{S6K}$ and allows continued inhibition by 4E-BP1, thereby leading to a growth arrest at the G_1 phase of the cell cycle. Rapamycin also prevents cyclin-dependent kinase activation, inhibits the retinoblastoma protein (pRb) phosphorylation, and accelerates cyclin D1 turnover, all of which can also lead to a mid-to-late G_1 cell cycle arrest (Albers et al., 1993; Hidalgo and Rowinsky, 2000; Hosoi et al., 1999). Initial in vitro studies with rapamycin demonstrated the sensitivity of several tumor cell lines to the growth inhibition and apoptotic effects of rapamycin (Hosoi et al., 1999; Sekulic et al., 2000; Seufferlein and Rozengurt, 1996). Rapamycin potently inhibited the growth of cell lines derived from rhabdomyosarcomas, glioblastomas, neuroblastomas, small cell lung cancers, osteosarcomas, pancreatic cancers, breast cancers, prostate cancers, and B-cell lymphomas (Huang and Houghton, 2001). In animal models, treatment with rapamycin also produced a profound antiproliferative effect in several tumor types (Hidalgo and Rowinsky, 2000; Seufferlein and Rozengurt, 1996).

Rapamycin has been assessed in in vivo tumor model systems. Within 4 h of administration of rapamycin by intraperitoneal injection in CBA mice bearing human colon adenocarcinoma or rhabdomyosarcoma xenografts, a rapid association of 4E-BP1 with eIF4E was observed (Dudkin et al., 2001). Rapamycin treatment (5 or 20 mg/kg) led to the dephosphorylation of 4E-BP1 at Thr⁷⁰, which was highly correlated with inhibition of tumor growth (Dudkin et al., 2001). Toxicity studies in mice have shown that single doses of rapamycin of 100 mg/kg do not result in any obvious drug toxicity; however, daily treatment of 20 mg/kg 5 days per week for more than 2 weeks was associated with weight loss and dermatitis (Geoerger et al., 2001). In human clinical trials, minimal systemic toxicity was observed in Phase III studies designed to evaluate rapamycin as an immunosuppressant. The favorable outcome of these trials led to FDA approval of rapamycin for use as an immunosuppressant.

7.4.2. CCI-779

A disadvantage to the use of rapamycin as an effective chemotherapeutic agent is its poor solubility and chemical stability (Hidalgo and Rowinsky, 2000; Huang and Houghton, 2001). Recently, several ester analogues of rapamycin have been developed with improved aqueous solubility and stability. CCI-779 has been designed for intravenous injection and RAD001 has been designed for oral administration (reviewed in Huang and Houghton, 2001). Preclinical studies with CCI-779 in heterozygous PTEN knockout mice showed that this analogue reduces tumor size and $p70^{S6K}$ activity without affecting Akt activity

Table 2
Compounds that directly inhibit the PI3K/Akt pathway

Compound	Mode of action	Stage of development
Wortmannin	Irreversible binding to the ATP domain of p110 subunit of PI3K	Preclinical
LY294002	Reversible binding to the ATP domain of p110 subunit of PI3K	Preclinical
Perifosine	Disruption of phospholipid metabolism	Phase I clinical trials
PI analogues	Inhibition of binding of PIP2/PIP3 to PH domain of Akt	Preclinical
Rapamycin	Forms complex with FKBP12 and binds and inhibits mTOR	FDA approved as immunosuppressant
CCI-779	Forms complex with FKBP12 and binds and inhibits mTOR	Phase I clinical trials

Multiple steps in the PI3K/Akt pathway may be targeted to inhibit activation of the pathway and thereby potentially increase chemotherapeutic efficacy. Individual compounds, their putative mechanisms of action, and stage of development is listed (see text for details).

(Podsypanina et al., 2001). In several PTEN deficient human myeloma cell lines with high levels of constitutively active Akt, treatment with CCI-779 at concentrations below 1 nM inhibited cell growth by inducing a G₁ arrest (Shi et al., 2002). In contrast, myeloma cells expressing wild type PTEN were greater than 1000-fold more resistant to CCI-779 treatment (Shi et al., 2002).

Selective sensitivity to CCI-779 based on inactive PTEN (or active Akt) has also been observed in vivo. In studies performed by Neshat et al., treatment with CCI-779 selectively reversed the growth of prostate cancer cell xenografts bearing mutant, but not wildtype, PTEN (Neshat et al., 2001). In nu/nu mice bearing breast cancer xenografts, CCI-779 inhibited the growth of MDA-MB-468 tumors that lack PTEN, but not MDA-MB-435 tumors that have wildtype PTEN (Yu et al., 2001). Immunoblot analysis of Akt demonstrated high levels of phosphorylated Akt in MDA-MB-468 cells, whereas only minimal phosphorylation of Akt was observed in MDA-MB-435 cells. Thus, this study found a strong correlation between mutant PTEN status, Akt activation and sensitivity to CCI-779.

Not all tumors are responsive to rapamycin or CCI-779. Mutations of mTOR or FKBP12 can prevent binding of rapamycin, creating rapamycin resistance, as well as mutations in downstream mTOR proteins such as S6K 1 or 4E-BP1 (Hosoi et al., 1999; Huang and Houghton, 2001). One means to circumvent resistance to mTOR inhibitors might be to combine these agents with traditional forms of chemotherapy. In fact, Geoerger et al. showed that in addition to inhibiting tumor growth when administered alone, CCI-779 could be combined with chemotherapy to enhance inhibition of tumor growth (Geoerger et al., 2001). This enhancement of growth inhibition might have implications for improving clinical outcome for patients whose tumors are resistant to mTOR inhibitors. Clinical relevance is more germane for this class of inhibitors of the PI3K/Akt pathway, because one mTOR inhibitor is FDA approved (rapamycin), and analogues such as CCI-779 are in Phase II clinical trials (Table 2).

Taken together, these studies investigating new approaches to directly target the PI3K/Akt pathway have shown that it is feasible to inhibit this pathway, and that inhibition of the pathway at different points can nonetheless result in

cell death or inhibition of tumor growth. Induction of cell death or inhibition of tumor growth can be observed when these compounds are used singly or used in combination with other, more traditional forms of therapy. Whether any of these compounds might be truly useful in the management of patients with cancer awaits further study.

8. Conclusions/future directions

Activation of the PI3K/Akt pathway confers resistance to chemotherapy in vitro and in vivo, and modulation of the pathway is a consequence of administration of many types of chemotherapeutic and chemopreventive agents. In some systems, inhibition of Akt appears to be necessary and sufficient for chemotherapy-induced cytotoxicity. The mechanism of chemotherapy-induced inhibition of the PI3K/Akt pathway is a subject of intense investigation. Whether chemotherapy targets this pathway in vivo is unclear, but activation of Akt can now be measured in human tumors, and this question will likely be answered in the context of ongoing clinical trials. A critical question regarding the importance of this pathway is that irrespective of the prevalence of active Akt in human tumors, does activation of Akt have predictive or prognostic value for patients with cancer?

Until that answer is known, the detection of active Akt in human tumors is furiously driving the development of compounds directed against components in the pathway. Can a therapeutic index be achieved when such a ubiquitous pathway is targeted? Perhaps a basis for a potential therapeutic index can be attributed to increased reliance on pathways that promote cellular survival by cancer cells exposed to forms of cellular stress that are known to activate Akt. With new components being identified in the PI3K/Akt pathway almost weekly, which component is the best target? Early generation compounds targeted at proximal proteins in the pathway such as Akt itself seem effective, but have not yet been evaluated in mouse models and their utility has not been demonstrated. More mature compounds such as CCI-779 are already in Phase II clinical trials. The results from these trials should provide insight into the utility of inhibiting the PI3K/Akt to treat cancer and overcome chemotherapeutic resistance.

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