UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE MEDICINA

BIOLOGIA EXPERIMENTAL

CALCITRIOL O SUS ANÁLOGOS AUMENTAN LA ACTIVIDAD ANTINEOPLÁSICA DE GEFITINIB, LAPATINIB Y NERATINIB EN CÉLULAS DE CÁNCER DE MAMA

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

SEGOVIA MENDOZA MARIANA

TUTORA PRINCIPAL DE TESIS: DRA. ROCÍO ÁNGELES GARCÍA BECERRA
FACULTAD DE MEDICINA, UNAM

COMITÉ TUTOR: DR. ALFONSO DUEÑAS GONZÁLEZ
FACULTAD DE MEDICINA, UNAM

DR. ÁNGEL ALFONSO ZARAIN HERZBERG
FACULTAD DE MEDICINA, UNAM

MÉXICO, CD. MX., NOVIEMBRE 2016.

UNAM
POSGRADO
Ciencias Biológicas

COORDINACIÓN

Lic. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que el Subcomité de Biología Experimental y Biomedicina del Posgrado en Ciencias Biológicas, en su sesión ordinaria del día 22 de agosto de 2016, aprobó el jurado para la presentación del examen para obtener el grado de **DOCTORA EN CIENCIAS** de la alumna **SEGOVIA MENDOZA MARIANA** con número de cuenta **510007939**, con la tesis titulada **"CALCITRIOL O SUS ANÁLOGOS AUMENTAN LA ACTIVIDAD ANTINEOPLÁSICA DE GEFITINIB, LAPATINIB Y NERATINIB EN CÉLULAS DE CÁNCER DE MAMA"**, realizada bajo la dirección de la **DRA. ROCÍO ÁNGELES GARCÍA BECERRA:**

Presidente:	DRA. ERIKA PATRICIA RENDÓN HUERTA
Vocal:	DRA. GISELA CEBALLOS CANCINO
Secretario:	DR. ÁNGEL ALFONSO ZARAIN HERZBERG
Suplente:	DR. ALEJANDRO ZENTELLA DEHESA
Suplente:	DR. ALFONSO DUEÑAS GONZÁLEZ

Sin otro particular, me es grato enviarle un cordial saludo.

A t e n t a m e n t e
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Cd. Universitaria, Cd, Mx., a 06 de octubre de 2016

DRA. MARÍA DEL CORO ARIZMENDI ARRIAGA
COORDINADORA DEL PROGRAMA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

AGRADECIMIENTOS

A la Universidad Nacional Autónoma de México (UNAM) y al Posgrado en Ciencias Biológicas por brindarme la oportunidad de crecer tanto en lo profesional como en lo personal y en un futuro poder retribuir un poco a las demás personas.

Al Consejo Nacional de Ciencia y Tecnología (CONACyT) por la beca otorgada para la realización de este proyecto.

Al Comité Tutoral asignado; **Dr. Alfonso Dueñas González y el Dr. Ángel Alfonso Zarain Herzberg,** por sus valiosas aportaciones para mejorar este proyecto.

A mi Directora de Tesis, la Dra. Rocío Ángeles García Becerra por el apoyo brindado, por creer en mí, por caminar conmigo durante estos cuatro años y por ser un ejemplo a seguir.

AGRADECIMIENTOS

Al Dr. Heriberto Prado García por ser un gran apoyo en las etapas de mi vida profesional y por brindarme su tiempo, amistad y confianza.

Al Dr. Mauricio Reginato por apoyarme durante mis estudios de doctorado, por su amabilidad y calidez que recibí en su laboratorio.

A todos los compañeros del laboratorio de Biología de la Reproducción, **Janice, Nancy Noyola, Nancy Santos, Mitzi, Andrea, Gaby, Davo,** por hacer una relación armónica dentro del mismo.

Al Dr. David Barrera Hernández por su amistad, apoyo y cariño que me brindó durante mi doctorado.

A mis amigos **David Salomón Dozal Domínguez y Gaby Guerrero Palomo** por las experiencias y consejos compartidos en esta etapa.

A mis amigos, **Adriana, Sofía, Isela, Aidee y al Sr. Luis** por compartir experiencia en nuestra segunda casa que es el laboratorio.

Al Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ) y a todas las personas que lo conforman por brindarme un gran apoyo para la realización de este trabajo, pero también porque aquí crecí en muchos aspectos.

DEDICADO A……..

Mis padres, ***José Luis Segovia Balmes*** *y* ***Ma. Elena Mendoza Díaz****, por trascender y crecer conmigo, por creer en mí, apoyarme a conseguir mis sueños, ser mi refugio, mi soporte, por impulsarme y apoyarme siempre para seguir adelante en otra etapa de mi vida. En especial a mi padre por su ejemplo de fortaleza, paciencia y perseverancia y a mi madre por su enorme calidez, la cual ha cimentado en mí.*

A mi hermana ***Regina Segovia Mendoza****, la cual ha sido siempre un gran apoyo e inspiración. Así como un ejemplo a seguir porque siempre ha sabido salir avante en todos los proyectos que se ha planteado y por su enorme cariño. También por creer en mí y por hacerme partícipe e incluirme en las nuevas facetas de su vida que incluyen a su esposo* ***Gerardo Tovar*** *y a su hijo* ***Leonardo Tovar Segovia****.*

ÍNDICE

I. ÍNDICE DE FIGURAS

II. ÍNDICE DE TABLAS

III. ABREVIATURAS

ATP	trifosfato de adenosina
C	calcitriol
CI	concentración inhibitoria
CK	citoqueratina
DNA	ácido desoxirribonucléico
E	EB1089
EGFR	receptor del factor de crecimiento epidérmico
ERK	cinasas extracelulares
G	gefitinib
GAPDH	gliceraldehído 3 fosfato deshidrogenasa
GDP	difosfato de guanosina
GTP	trifosfato de guanosina
HER2	receptor del factor de crecimiento epidérmico tipo 2
HER3	receptor del factor de crecimiento epidérmico tipo 3
HER4	receptor del factor de crecimiento epidérmico tipo 4
I	porcentaje de inhibición celular
IC	índice de combinación
IGFR	receptor del factor de crecimiento insulínico
ITC	inhibidores de residuos de tirosina cinasa
JAK	cinasas janus trasductoras de la señal
Kd	kilodaltones
L	lapatinib
MAPK	cinasas activadas por mitógenos
N	neratinib

PCR	reacción en cadena de la polimerasa
PI3K/Akt	fosfatidil inositol 3 cinasa/serina treonina cinasa
PLC	fosfolipasa C
RE	receptor de estrógenos
RNA	ácido ribonucléico
RP	receptor de progesterona
STAT	cinasas activadoras de la transcripción
VEGFR	receptor del factor de crecimiento del endotelio vascular
VDR	receptor de vitamina D

RESUMEN

El cáncer de mama es la neoplasia maligna más frecuente en mujeres y representa un problema de salud pública a nivel mundial y nacional. La sobreexpresión de los miembros de la familia del receptor del factor de crecimiento epidérmico (EGFR), EGFR/HER1, HER2, HER3 Y HER4, y la activación de sus vías de señalización se han asociado con el incremento de la proliferación celular, supervivencia, adhesión y migración. Por lo tanto, el desarrollo de diversas moléculas inhibidoras de la actividad tirosina cinasa de estos receptores como el gefitinib, el lapatinib y el neratinib representa una buena alternativa terapéutica.

Por otro lado, el calcitriol, el metabolito más activo de la vitamina D, y algunos de sus análogos han sido considerados como importantes agentes antineoplásicos, debido a que inducen apoptosis y arresto del ciclo celular en células de cáncer de mama. Con la finalidad de incrementar las tasas de respuesta global, mejorar el tiempo libre de la progresión y prolongar la supervivencia global, actualmente se están investigando tratamientos combinados en el cáncer de mama.

En el presente trabajo se evaluó el efecto y se determinó el mecanismo de acción de la combinación de gefitinib, lapatinib o neratinib con el calcitriol o sus análogos en células de cáncer de mama que expresen al EGFR y/o al HER2.

La presencia de los diferentes receptores y la fosforilación de diversas proteínas fueron evaluadas por Western blot. Los efectos antiproliferativos de los diferentes compuestos solos o en combinación fueron evaluados a través de la cuantificación del contenido de ADN. La expresión génica se evaluó mediante PCR en tiempo real. Los niveles de las proteínas BIM y caspasa 3 así como fases del ciclo celular se analizaron por citometría de flujo. La formación de colonias se evaluó con el método de agar suave.

Todos los antineoplásicos utilizados inhibieron la proliferación celular de manera dependiente de la concentración. Las combinaciones de los inhibidores de residuos de tirosina cinasa con el calcitriol o sus análogos fueron más eficaces para inhibir el crecimiento celular. La expresión de los genes EGFR y Bcl-2 fue disminuida por el

tratamiento combinado del gefitinib con el calcitriol o el EB1089, mientras que la expresión de BIM fue inducida por la combinación de estos fármacos. Además, la fosforilación de MAPK y AKT fue inhibida en mayor medida en las células de cáncer de mama tratadas con la combinación de los inhibidores de residuos de tirosina con el calcitriol o su análogo. Los tratamientos combinados indujeron cambios en el perfil del ciclo celular, incrementaron la apoptosis celular, el porcentaje de fragmentos celulares y la expresión de la caspasa 3.

La combinación de los inhibidores de residuos de tirosina cinasa con el calcitriol o sus análogos sintéticos resultó en mayor efecto antiproliferativo y apoptótico. El mecanismo implicado en estos eventos es a través de la inhibición de la fosforilación de ERK y Akt, lo que conllevó a la modulación de los mediadores de apoptosis, BIM y Bcl-2, resultando en la activación de la caspasa 3 y la inducción de muerte celular.

Nuestros resultados sugieren que la adición del calcitriol o del EB1089 a terapias dirigidas como gefitinib, lapatinib y neratinib podría ser útil para pacientes con cáncer de mama que sobreexpresen el EGFR.

ABSTRACT

Breast cancer is the most common malignancy in women and represents a public health problem at global and national level. Overexpression of the family members of epidermal growth factor receptor (EGFR), EGFR / HER1, HER2, HER3 and HER4, and activation of their signaling pathways have been associated with increased cell proliferation, survival, adhesion and migration. Therefore, the development of several inhibitory molecules from tyrosine kinase activity of these receptors as gefitinib, lapatinib and neratinib represents a good therapeutic alternative.

Moreover, calcitriol, the most active metabolite of vitamin D, and some of its analogs have been considered important antineoplastic agents, because they induce apoptosis and cell cycle arrest in breast cancer lines. In order to increase overall response rates, improve time to progression and prolong overall survival, they are currently being investigated in combination therapy in breast cancer.

n this work, we evaluated the effect and mechanism of action of the combination of gefitinib, lapatinib or neratinib with calcitriol or its analogues in breast cancer cell lines that express EGFR and / or HER2.

The presence of these receptors and phosphorylation of various proteins were evaluated by Western blot. The antiproliferative effects of various compounds alone or in combination were evaluated by quantitation of DNA content. Gene expression was assessed by real time PCR. BIM protein levels, caspase 3 and cell cycle phases were analyzed by flow cytometry. Colony formation was evaluated using soft agar method.

All antineoplastic used inhibited cell proliferation in a concentration-dependent manner. Combinations of tyrosine kinase inhibitors with calcitriol or its analogues were more effective for inhibiting cell growth. EGFR and Bcl-2 gene expression was reduced by the combined treatment with gefitinib plus calcitriol or EB1089, whereas BIM expression was induced by the combination of these drugs. Furthermore, AKT and MAPK phosphorylation was inhibited in a greater manner in breast cancer cells treated with the combination of tyrosine kinase inhibitors with calcitriol or its analog. The combined treatments induced

changes in cell cycle profile, increased apoptosis, the percentage of cell fragments and the expression of caspase 3.

The combination of tyrosine kinase inhibitors with calcitriol or its synthetic analogs resulted in greater antiproliferative and apoptotic effect. The mechanism involved in these events is through inhibition of phosphorylation of ERK and Akt proteins, which led to modulating mediators of apoptosis, BIM and Bcl-2, resulting in the activation of caspase 3 and induction of cell death.

Our results suggest that the addition of calcitriol or EB1089 to targeted therapies gefitinib, lapatinib and neratinib could be useful for patients with breast cancer that overexpress the EGFR and / or HER2.

1. INTRODUCCIÓN

A nivel mundial el cáncer de mama es la primera causa de muerte e incidencia por neoplasias malignas en la mujer [1, 2]. En México según el Instituto Nacional de Estadística y Geografía (INEGI) ocupa el primer lugar en defunciones en mujeres en edad reproductiva y constituye un problema importante de salud pública [3]. Entre los factores de riesgo que contribuyen a desarrollar cáncer de mama se encuentran factores genéticos, biológicos y hormonales, así como la deficiencia de la vitamina D [4].

De acuerdo al perfil de expresión génica el cáncer de mama se divide principalmente en tres grandes grupos; receptor de estrógenos y progesterona (RE, RP, respectivamente) positivo, el receptor del factor de crecimiento epidérmico tipo 2 (HER2) positivo y el triple negativo.

Un factor importante en el desarrollo y la resistencia a los tratamientos del cáncer de mama es la sobreexpresión de los miembros de la familia del receptor del factor de crecimiento epidérmico (EGFR), EGFR/HER1, HER2, HER3 y HER4. La unión de factores de crecimiento a los EGFRs induce la formación de homo- y heterodímeros, lo que da lugar a la fosforilación en los residuos tirosina cinasa de cada receptor. Consecuentemente, diversas vías de señalización como PI3K/Akt y MAPK son activadas. Estos eventos promueven proliferación celular, diferenciación e inhibición de la apoptosis. Con la finalidad de bloquear las vías de señalización activadas por los EGFRs se han desarrollado nuevas estrategias terapéuticas como el empleo de inhibidores de residuos de tirosina cinasa como el gefitinib, el lapatinib y el neratinib [5-7].

El metabolito activo de la vitamina D es el calcitriol, el cual tiene importantes propiedades antineoplásicas en células de cáncer de mama a través de unirse a su receptor (VDR). La mayoría de los carcinomas mamarios expresan al VDR, lo cual correlaciona con un mayor tiempo libre de la enfermedad en comparación con los pacientes con tumores negativos al mismo [8-11]. Lo anterior sugiere que la presencia y función del VDR puede ser considerada como un blanco molecular importante en cáncer de mama. De hecho, varios análogos sintéticos del calcitriol han sido desarrollados con el objetivo de mejorar su actividad antiproliferativa y reducir sus efectos calcémicos [12].

En estudios clínicos en pacientes con cáncer de mama han mostrado que la combinación de calcitriol con agentes citotóxicos como el cisplatino o agentes antihormonales, mejora la respuesta al tratamiento [13, 14].

En el presente trabajo se evaluó el efecto antiproliferativo y antiapoptótico del calcitriol y dos análogos en combinación con (gefitinib, lapatinib y neratinib) en cáncer de mama EGFR y HER2 positivo.

2. GENERALIDADES DEL CÁNCER

2.1 Definición

El cáncer se define como una enfermedad multifactorial o como un conjunto de enfermedades caracterizadas por la transformación y malignización de una célula normal [15], esta condición es propiciada y mantenida por una serie de alteraciones que sufre la célula conocidas como “las marcas del cáncer” entre las que se encuentran: la autosuficiencia de señales de crecimiento, insensibilidad a las señales antiproliferativas, potencial replicativo ilimitado, sustento de angiogénesis, invasión tisular y evasión de apoptosis [16].

2.2. Incidencia

En los últimos años el cáncer se ha establecido como una de las principales causas de muerte en el mundo representando el 13% de las defunciones mundiales. Los datos estadísticos de la Organización Mundial de la Salud en el año 2011 muestran que el cáncer es la principal causa de muerte a nivel mundial. Actualmente, a esta patología se le atribuyen 7.6 millones de defunciones y se pronostica que para el año 2030 supere los 13.1 millones [17].

En México en 2013, del total de defunciones de la población de 20 años y más, 13.6% se debieron a algún tumor y de estas, 93.6% a los tumores malignos. Por sexo, del total de defunciones por cáncer, 48.8% ocurren en varones y 51.2%, en mujeres. [18]

Cabe mencionar, que en el caso del sexo femenino el cáncer de mama es la neoplasia más común. A continuación, se mencionan algunas de sus características.

3. CÁNCER DE MAMA

3.1 Generalidades

En México el cáncer de mama es la neoplasia maligna más común en mujeres y es la segunda causa de muerte después del cáncer de pulmón, de acuerdo a estadísticas del INEGI [18].

Año tras año más de un millón de mujeres son diagnosticadas con esta enfermedad y esto es causante de más de 410,000 muertes [19]. Lo anterior representa un problema de salud pública de importancia y es por ello que existen múltiples investigaciones dirigidas contra esta patología.

Actualmente, la terapia del cáncer de mama pretende dirigirse a blancos moleculares proteicos específicos de acuerdo al perfil de expresión molecular del mismo.

3.2 Clasificación

El carcinoma mamario es una enfermedad heterogénea que se ha clasificado en 3 grupos de acuerdo a sus patrones de expresión génica, **Fig. 1**. A continuación se mencionan algunas características de cada grupo [20, 21]:

- Luminal: Grupo caracterizado por la elevada expresión del RE, del RP, así como de las citoqueratinas (CKs) 8 y 18. Puede subdividirse en:
  - Luminal A: El cual es diferenciado y presenta bajos niveles de expresión del marcador de proliferación celular Ki67.
  - Luminal B: Es pobremente diferenciado y los niveles de expresión de Ki67 son elevados.
- HER2: Grupo caracterizado por la sobrexpresión del HER2 y mantiene poca o nula expresión del RE.
- Basal o triple negativo: Este grupo no expresa ni el RE, RP o HER2, sin embargo, presenta elevada expresión de las CKs 5, 6 y 17.

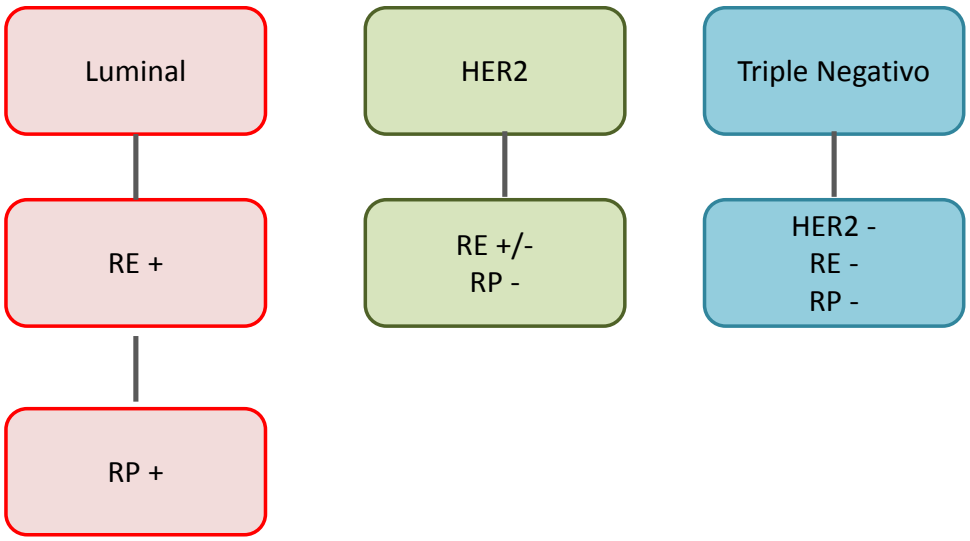


Fig 1. **Clasificación molecular del cáncer de mama**. Fenotipo luminal u hormonal caracterizado por la presencia de los receptores de estrógeno y progesterona. Cáncer de mama fenotipo HER2 positivo, el cual puede tener la presencia o ausencia del receptor de estrógenos (RE) y carece de la presencia del receptor de progesterona (PR). Cáncer de mama triple negativo, el cual no tiene la presencia de ninguno de los receptores anteriormente mencionados.

La clasificación de diferentes tipos de cáncer de mama, se debe a múltiples desregulaciones génicas que favorecen la expresión elevada de proteínas que participan en el desarrollo de cáncer, un ejemplo de ello, son los receptores membranales que transducen señales estimulando el crecimiento celular. Por ejemplo, el HER2 el cual esta amplificado de 10 a 100 veces más en el carcinoma mamario en comparación con el tejido de mama no tumoral [22].

Este trabajo se enfocará en el cáncer de mama que expresa a los miembros de la familia EGFR, por lo que a continuación se describen algunas características de esta familia.

4. FAMILIA DE RECEPTORES DEL FACTOR DE CRECIMIENTO EPIDÉRMICO

4.1 Descripción y estructura

La familia EGFR comprende cuatro receptores con actividad de tirosina cinasa (EGFR o HER1, HER2, HER3 y HER4), los cuales están expresados en una amplia variedad de

tejidos como el epitelial, el mesenquimal o el neuronal y tienen un papel fundamental durante el desarrollo embrionario [23, 24]. Estas proteínas están constituidas por una región extracelular que consta de 4 dominios, una región transmembranal y una región intracelular, la cual contiene el dominio con actividad catalítica y el carboxilo terminal donde se encuentra la subunidad regulatoria, **Fig. 2** [25]. El HER3 es el único miembro de la familia que carece de dicha actividad debido a polimorfismos de aminoácidos fundamentales para esta función, sin embargo, recientemente se ha reportado que este receptor si posee actividad de tirosina cinasa, aunque su función es alrededor de 100 veces menos potente que la de cualquier otro miembro [26]. Cabe resaltar, que la actividad catalítica de los receptores es primordial para que lleven a cabo sus funciones biológicas y de hecho se le ha considerado como diana molecular para la inhibición de la activación de estos receptores.

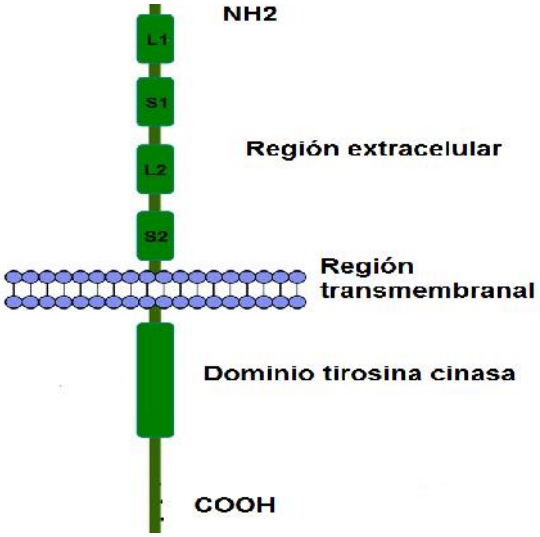


Fig. 2. **Estructura de la familia EGFR**. Representación esquematizada de la región extracelular que comprende cuatro dominios (L1, S1, L2, S2), la transmembranal y la intracelular que contiene el dominio con actividad de tirosina cinasa y el carboxilo terminal.

4.2 Mecanismo de acción

La familia EGFR actúa a través de la formación de homo y heterodímeros posterior a la unión de su ligando, lo cual conlleva a la fosforilación de los mismos receptores y de otras moléculas implicadas en la cascada de señalización, culminando en procesos como

proliferación, supervivencia celular, adhesión, migración, diferenciación y angiogénesis [27]. El mecanismo de acción de estos receptores se menciona a continuación:

1) Unión del ligando
2) Dimerización y fosforilación del receptor
3) Formación de un complejo multiproteico a través de proteínas con dominios de reconocimiento a tirosinas fosforiladas (dominios SH2) y proteínas adaptadoras (Grb2).
4) Activación de cascadas de señalización
5) Amplificación de la señalización
6) Modulación de procesos biológicos

4.3 Vías de señalización activadas por la familia EGFR

Los miembros de la familia EGFR pueden activar las siguientes vías de señalización: cinasas activadas por mitógenos (MAPK) denominadas también como cinasas reguladas por señales extracelulares (ERK), la cinasa 3 fosfoinositol / proteína cinasa B (PI3K/Akt), cinasas janus transductoras de la señal y activadoras de la transcripción (JAK-STAT) y fosfolipasa C gamma (PLCγ), entre otras **Fig. 3**. De manera general, las vías principalmente estudiadas son la MAPK y la PI3K/Akt, las cuales se describen a continuación [28].

4.3.1 Vía de las MAPK

En la vía de las MAPK existe un complejo de proteínas acopladoras (Shc/Grb2/SOS) que sirve como intermediario entre las moléculas activadas por los receptores de la familia EGFR y las proteínas cinasas ERK. Tras la unión de factores de crecimiento a sus respectivos receptores, el complejo Shc/Grb2/SOS permite la activación de la proteína Ras, una GTPasa, que media el intercambio de difosfato de guanosina (GDP) por guanidin trifosfato (GTP), lo cual induce el cambio conformacional de Ras y permite reclutar a Raf a

la membrana celular, una cinasa con actividad de serina / treonina. Esta proteína fosforila y activa a las cinasas ERK 1 y 2 (las cuales tiene un peso molecular de 42 y 44 Kd, respectivamente), mismas que presentan también actividad enzimática y fosforilan residuos de serina / treonina y tirosina de otras proteínas y al mismo tiempo se internalizan en el núcleo celular presentando actividad de factores de transcripción, los cuales regulan la expresión génica y tienen efectos en la modulación de la apoptosis y la progresión celular **Fig. 3** [29, 30].

4.3.2 Vía PI3K/Akt

La vía de la PI3K/Akt participa principalmente en la supervivencia y metabolismo celular, además, actúa de manera estrecha con la vía de las MAPK [31]. Es importante destacar que la cinasa PI3K está constituida por dos subunidades: una catalítica (110 Kd) y una reguladora (85 Kd), la cual contiene dominios SH2 (reconocimiento de tirosinas fosforiladas). La vía PI3K/Akt puede ser activada por dos mecanismos: El reconocimiento de alguna tirosina fosforilada de los receptores membranales por la subunidad regulatoria de PI3K o por el complejo multiproteico Shc/Grb2/SOS que activa a Ras la cual induce la translocación y activación de la subunidad catalítica de PI3K. Una vez que PI3K se encuentra activa fosforila al fosfatidil inositol 3,4 difosfato (PIP2), convirtiéndolo en un segundo mensajero, fosfatidil inositol 3,4,5, trifosfato (PIP3) el cual conduce a la activación de la cinasa Akt, que activa múltiples blancos moleculares **Fig. 3** [32].

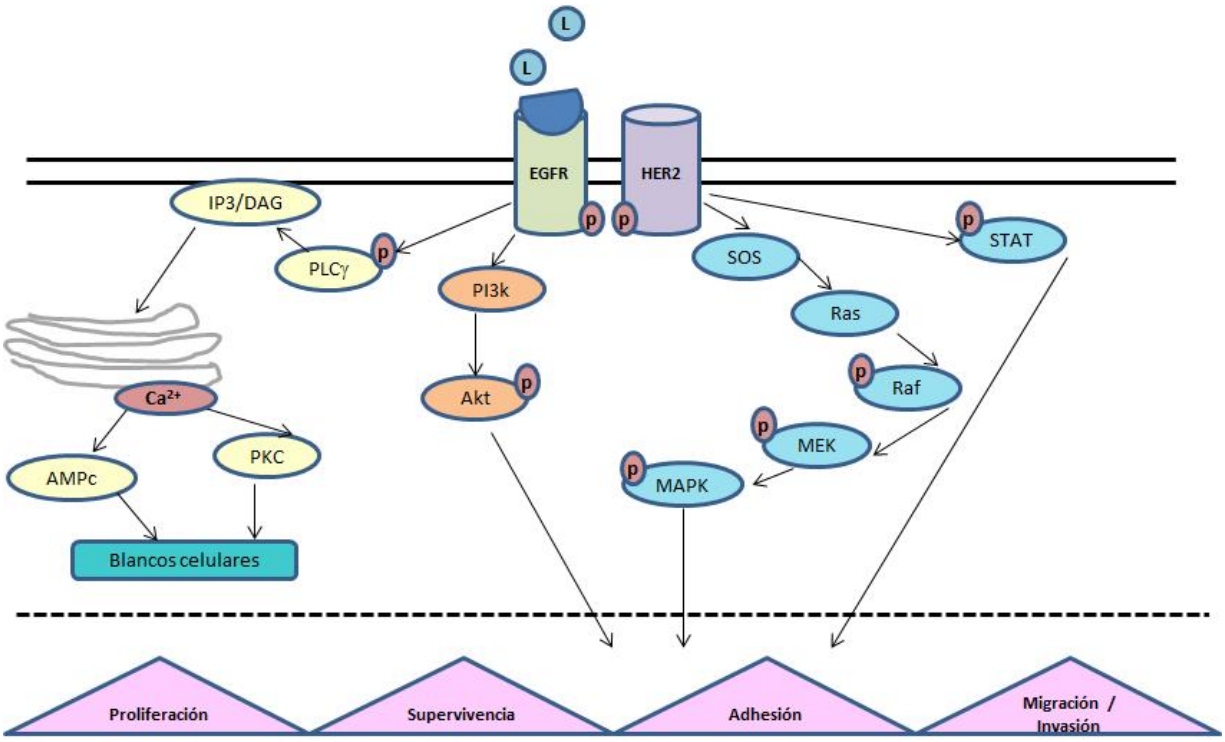


Fig. 3. **Vías de señalización activadas por la familia EGFR.** Posterior a la unión del ligando (L), la familia de receptores EGFR forma homo y heterodímeros, una vez unidos estos receptores se fosforilan (p) y activan diversas cascadas de señalización como las MAPK, PI3K/Akt, JAK-STAT y PLCγ que tienen impacto en diversos efectos celulares.

5. EGFR EN CÁNCER DE MAMA

La expresión del EGFR se encuentra del 15 – 45 % en tumores mamarios y se asocia con el incremento de la proliferación celular, la inestabilidad genómica y la co-expresión del HER2 [33]. La sobreexpresión del EGFR y su alteración de número de copias están frecuentemente correlacionadas con cáncer de mama agresivo y resistente a la terapia, como el cáncer de mama negativo al RE y tumores positivos al HER2 [34, 35] [36]. Su presencia se atribuye también con elevado riesgo de recurrencia y mal pronóstico en pacientes [37]. En modelos preclínicos de cáncer de mama, la presencia de este receptor permite la transformación y malignización de células de ratón, lo que produce un aumento en la proliferación y resistencia a la apoptosis [38].

5.1 EGFR como blanco terapéutico

El cáncer de mama triple negativo es altamente proliferativo y metastásico, generalmente posee elevada expresión del EGFR [39]. En este sentido diversos reportes de investigación están dirigidos al estudio del bloqueo de su función con la finalidad de mejorar la sobrevida en pacientes [40]. Dentro de los fármacos que se han utilizado actualmente para bloquear la acción de este receptor se encuentran; el anticuerpo monoclonal cetuximab así como los inhibidores de la actividad de residuos de tirosina cinasa (ITC) de esta familia de receptores, como el gefitinib, el erlotinib y el neratinib, todos ellos en esquemas de combinación con quimioterapia [41].

6. HER2 EN CÁNCER DE MAMA

El HER2 es considerado como un proto-oncogén que se encuentra comúnmente sobreexpresado en varios tipos de tumores incluyendo el carcinoma mamario, donde su expresión se presenta en el 20-30% de todos los fenotipos de cáncer de mama y se le ha asociado como un factor predictivo de mal pronóstico y de comportamiento tumoral agresivo [42, 43]. En la **Tabla 1** se muestra el porcentaje de sobreexpresión del HER2 en diferentes tumores, por lo cual se considera como blanco terapéutico.

Tabla 1. Sobreexpresión del HER2 en diferentes neoplasias

Neoplasia	% de sobreexpresión del HER2
Cáncer de mama	30
Cáncer de ovario	25
Cáncer de endometrio	10
Cáncer de pulmón	9

Por otra parte, a diferencia de los otros receptores de esta familia, no se ha identificado un ligando específico para este receptor, sin embargo, es conocido que su co-receptor preferente para formar heterodímeros es el EGFR [44, 45]. Además, el HER2 modula la activación de los otros miembros de la familia EGFR a través de regular la unión de sus ligandos [46]. Lo anterior explica como en ausencia de un ligando propio esta proteína puede activarse debido a la acción cooperativa entre receptores induciendo proliferación, migración y diferenciación, entre otros procesos.

6.1 HER2 como blanco terapéutico

La sobreexpresión del HER2 en cáncer de mama permite la activación constitutiva de vías inducidas por los factores de crecimiento fungiendo como un modulador oncogénico. Debido a la dependencia que representa la presencia del HER2 y de otros miembros de la familia EGFR para la proliferación y supervivencia de células de cáncer de mama, el desarrollo de diversos tratamientos para inhibir la acción de estas proteínas se ha convertido en un objetivo primordial de la terapia en esta patología. Actualmente, dos tipos importantes de terapias contra el HER2 están siendo utilizadas en la clínica: los anticuerpos humanizados contra el HER2 y los ITC de esta familia de receptores.

En este trabajo nos enfocaremos en el mecanismo de acción de los ITC, describiendo algunas de sus principales características a continuación.

7. INHIBIDORES DE RESIDUOS DE TIROSINA CINASA

Los ITC son una de las terapias prometedoras para los pacientes con cáncer que presentan sobreexpresión de proteínas de la familia EGFR. De manera general, estos fármacos presentan homología con el trifosfato de adenosina (ATP), necesario para la activación de la actividad catalítica de los miembros de la familia EGFR, lo cual les permite competir por el sitio catalítico de los receptores. Una vez unidos a este sitio inhiben la fosforilación y la subsecuente transducción de señales, lo cual activa el proceso de apoptosis e inhibición de

la proliferación celular [47]. Los ITC mayormente estudiados en cáncer de mama son: el lapatinib, el neratinib y el gefitinib [48].

7.1 Lapatinib

El lapatinib en combinación con el antineoplásico capecitabina es el único tratamiento aprobado por la Administración de Alimentos y Medicamentos de Estados Unidos para pacientes con cáncer de mama HER2 positivo en estado avanzado resistentes a trastuzumab [49]. Este fármaco se ha descrito como un inhibidor dual y reversible del sitio catalítico del EGFR y del HER2 [50]. El lapatinib ha mostrado inhibir la proliferación de células de cáncer de mama por diversos mecanismos, entre los que se incluyen, incrementar la fase subG1 del ciclo celular, reducir la fosforilación de EGFR, HER2, Akt y de las proteínas ERK [51]. También, el lapatinib induce el incremento del inhibidor de la ciclina p21 [52] y modula la expresión de proteínas involucradas en la apoptosis, induciendo la proteína pro-apoptótica BIM y reduciendo la expresión de la proteína anti-apoptótica survivina [53]. Además de los mecanismos previamente mencionados, el lapatinib se ha utilizado para el tratamiento de cáncer de mama debido a que inhibe la señalización cruzada entre el HER2 y el receptor del factor de crecimiento insulínico tipo I (IGFR-1), también ha mostrado inhibir la forma trunca del HER2 (p95HER2), estos dos últimos son mecanismos descritos de resistencia a la terapia de primera línea, el trastuzumab [54, 55].

7.1.1 Toxicidad y Resistencia

El lapatinib no ha mostrado tener efectos secundarios severos, la diarrea, salpullido, náuseas, dolor abdominal y fatiga son los principales síntomas causados por este fármaco [11].

Al rededor del 20 - 35 % de los pacientes con cáncer de mama metastásico resistente a trastuzumab responden al lapatinib [56, 57], y al igual que el trastuzumab, el periodo de administración antes de generar resistencia es de alrededor de un año [58]. Por otra parte, la

combinación del lapatinib con el trastuzumab se ha evaluado en pacientes con cáncer de mama HER2 positivo y mostró efecto sinérgico en la reducción de la masa tumoral [59]. Sin embargo, la búsqueda de nuevos compuestos continúa siendo un reto para esta patología.

Entre los principales mecanismos de resistencia para este fármaco se encuentran [48]:

- Hiperactivación de diversas vías de señalización como PI3K o la vía STAT3 [60-62].
- La inducción de la expresión del RE tras periodos prologados de administración de lapatinib [63].
- La activación constitutiva de las vías de señalización por diversas proteínas involucradas con otros receptores como el receptor del factor de crecimiento de hepatocitos (MET) y la cinasa de sarcoma celular (Src) [64].

7.2 Neratinib

El neratinib es un ITC irreversible cuyos blancos moleculares descritos son el EGFR, el HER2 y el HER4 a los cuales se une covalentemente bloqueando su señalización [65]. Actualmente, el neratinib se encuentra en fases clínicas para el tratamiento de tumores sólidos y de pacientes con cáncer de mama HER2 metastásico, se le considera como una alternativa prometedora para pacientes resistentes al lapatinib [66-69].

Existen pocos reportes sobre el mecanismo de acción del neratinib. Rabindran et al., mostraron que el neratinib inhibió la proliferación de células de cáncer de mama con fenotipo HER2 positivo. Así mismo, el ITC inhibió la fosforilación del EGFR, del HER2, Akt y MEK. Dichos efectos fueron mediados por el incremento de la fase G1-S del ciclo celular a través de la reducción de la proteína del retinoblastoma (pRb), la ciclina D1 y el incremento del inhibidor de ciclinas p27 [65].

7.2.1 Toxicidad y Resistencia

Al igual que el lapatinib, el neratinib no ha mostrado tener efectos secundarios severos, los más comunes reportados en pacientes con tumores sólidos son diarrea, náusea, vómito y fatiga [66, 67, 70].

La búsqueda de mecanismos de resistencia para este fármaco continúa bajo investigación. Seyhan et al., identificaron un grupo de genes involucrados en la resistencia al neratinib, en donde se encuentran factores de trascripción, transportadores de iones, proteínas de ubiquitinación y genes del ciclo celular [38, 48].

7.3 Gefitinib

El gefitinib es otro ITC reversible selectivo para el EGFR [71]. Actualmente, este compuesto se usa para el tratamiento de cáncer de pulmón no microcítico que presenta mutaciones en el EGFR [72]. Además, existen estudios que describen su efecto antineoplásico en cáncer de mama [73, 74].

De manera general, el gefitinib compite por el sitio de unión del ATP del EGFR, una vez unido, bloquea su autofosforilación y la transducción de señal de este receptor [75]. Sin embargo, diversos estudios muestran el efecto antiproliferativo del gefitinib en células de cáncer de mama con fenotipo HER2 positivo en los cuales, este compuesto inhibió la fosforilación de este receptor, disminuyó la fase S del ciclo celular, incrementó los niveles de p27 e inhibió diversas vías de señalización como PI3K/Akt, MAPK y STAT [76, 77]. También, se ha reportado que la sobreexpresión del HER2 en células de carcinoma mamario rige la sensibilidad al gefitinib [78]. No obstante, un estudio de Anido et al., sugiere que la presencia del EGFR en células de carcinoma mamario que sobreexpresan el HER2, es requerida para que gefinitib inhiba el crecimiento celular [43].

7.3.1 Toxicidad y Resistencia

Al igual que los otros dos ITC descritos con anterioridad, los principales efectos secundarios del gefitinib son: diarrea, salpullido, náuseas y vómitos.

Los mecanismos de resistencia al gefitinib reportados en cáncer de pulmón se deben principalmente a mutaciones en el EGFR [79]. Sin embargo, en cáncer de mama la resistencia a este agente no es clara y aparentemente en este tipo de cáncer no se han reportado mutaciones en el EGFR [80]. Existen trabajos que mencionan que las mutaciones en el gen K-Ras participan en los mecanismos de resistencia al gefitinib. En carcinoma mamario se han descrito mutaciones en este gen y algunos trabajos sugieren que éstas pueden comprometer la sensibilidad a este compuesto [81-83]. No obstante, la búsqueda de mecanismos y eficacia del gefitinib en cáncer de mama continúa abierta.

Cabe señalar que los ITC descritos anteriormente, **Fig.4**, han sido ampliamente evaluados en combinación con diversos agentes como antineoplásicos, anticuerpos monoclonales, antihormonales, entre otros [84-86]. Todas estas combinaciones buscan mejorar la multiterapia dirigida contra dianas moleculares específicas.

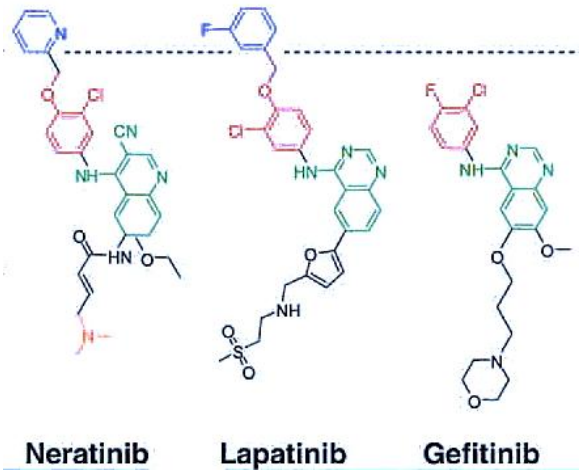


Fig. 4. **Fórmula química desarrollada del neratinib, el lapatinib y el gefitinib**. La figura muestra en verde (quinazolina) y en rojo (anilina) las estructuras base que comparten los inhibidores de residuos de tirosina. En el caso del neratinib y el lapatinib comparten además un anillo aromático.

8. VITAMINA D

8.1 Generalidades

La vitamina D_3 (colecalciferol), es una hormona que deriva del colesterol cuya función fisiológica principal es la regulación del calcio, el transporte del fósforo y la mineralización del hueso. La síntesis del metabolito activo de la vitamina D comienza a partir del 7-dehidrocolesterol en la piel, por efecto de la luz UV sufre una rotura en el anillo B de su estructura (pro-vitamina D) y posteriormente por una isomerización espontánea se forma la vitamina D, la cual sufre dos hidroxilaciones, una en hígado y la otra en riñón, las cuales dan origen a la formación de 25-hidroxivitamina D3 o calcidiol y 1-alfa,25 dihidroxicolecalciferol o calcitriol, respectivamente **Fig 5** [87]. La hidroxilación para la formación del calcitriol puede también llevarse a cabo en otros órganos como en mama, colon y próstata, lo que indica que el calcitriol tiene otras funciones fisiológicas independientemente de la homeostasis del calcio y del fósforo [88].

De manera interesante, este compuesto presenta efectos antiproliferativos, antineoplásicos y de diferenciación en diversos tipos de células tumorales y en cultivos primarios de cáncer de mama[10, 89].

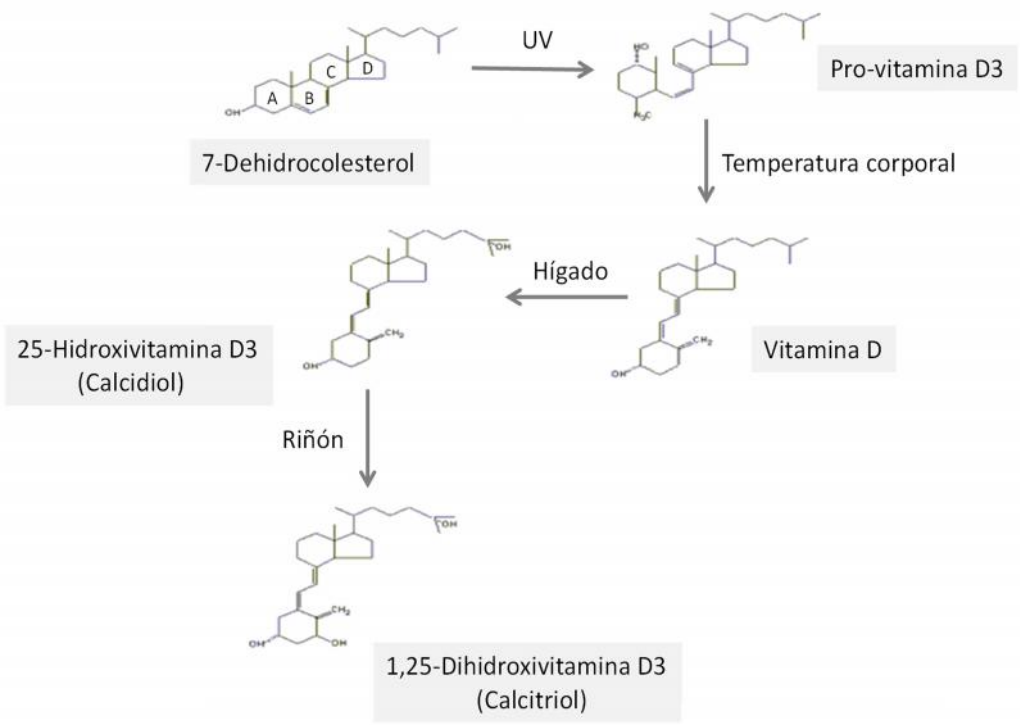


Fig. 5. **Biosíntesis del calcitriol**. El 7-dehidrocolesterol en la piel sufre una rotura en el anillo B de su estructura por efecto de la luz UV e inmediatamente se da una isomerización espontánea para formar la vitamina D, la cual es trasladada al hígado para formar al calcidiol y subsecuentemente al riñon o sitios extrarenales para formar al calcitriol.

8.2 Relación entre el receptor de vitamina D, el calcitriol y el cáncer de mama

Evidencia científica soporta el concepto que altas concentraciones del calcitriol en suero, están asociados con la prevención del desarrollo de cáncer de mama, la supervivencia libre de enfermedad y la reducción de mortalidad [5, 6, 90-92]. Además, diversos estudios han demostrado que los diferentes subtipos de cáncer de mama tienen patrones génicos diferentes y que la expresión del receptor de vitamina D (VDR) así como su función biológica puede ser subtipo específico. En este sentido un estudio demostró una relación estrecha entre el desarrollo de cáncer de mama RE negativo y triple negativo y bajos niveles séricos del precursor del calcitriol. Cabe señalar que en estos fenotipos se ha reportado una deficiencia de calcitriol superior a otros fenotipos [93-95]. Por otra parte, un estudio comparó la frecuencia de cambios genómicos en el VDR en pacientes con cáncer de mama utilizando diversas bases de datos públicas, la conclusión de este trabajo fue que las

alteraciones en el VDR son raras en el cáncer de mama humano, lo cual sugiere que la función de esta proteína tiene un papel preponderante en esta patología [96].

8.3 Efectos antineoplásicos del calcitriol en cáncer

La mayoría de los efectos antiproliferativos del calcitriol son a través de unirse al VDR, una vez unido, el VDR forma heterodímeros principalmente con el receptor retinoico X, los cuales se unen a los elementos de respuesta a la vitamina D (VDREs) en las regiones promotoras de los genes blanco. Estos eventos modulan la inducción o la inhibición de la transcripción génica, de esta manera, más de 60 genes son modulados por el calcitriol [97].

Dentro de las funciones antineoplásicas del calcitriol se encuentran la regulación del ciclo celular a través del arresto en la fase G1, la activación de inhibidores de ciclinas p27 y p21 y la reducción del factor de transcripción c-myc, entre otros [98-100]. También, se ha descrito que el calcitriol inhibe angiogénesis, reduce invasividad celular y la actividad de las metaloproteinasas [101] e incrementa proteínas de adhesión como la E-caderina [102]. El calcitriol también induce la apoptosis intrínseca en diversos modelos tumorales a través de la inhibición de la proteína anti-apoptótica Bcl-2 y la liberación del citocromo c de la mitocondria [103]. Existen otros mecanismos descritos por los cuales el calcitriol ejerce sus funciones antineoplásicas como la inhibición de la fosforilación de la vía de las MAPK [104], la reducción génica y proteica del EGFR [105, 106].

8.4 Efectos del calcitriol en cáncer de mama

Diversos estudios epidemiológicos han descrito una relación inversa entre la deficiencia del calcidiol, precursor del calcitriol, y el riesgo de desarrollar cáncer de mama [5, 6, 107-109]. Además, ratones knock-out para el VDR presentan mayor predisposición a desarrollar cáncer de mama y potenciación de crecimiento tumoral cuando son tratados con estradiol [110]. También, el tratamiento de análogos del calcitriol inhibe el desarrollo de tumores mamarios inducidos con carcinógenos [12]

De manera notable, la expresión del VDR se ha encontrado en la mayoría de fenotipos de tumores mamarios, lo cual correlaciona con mayor tiempo libre de la enfermedad en comparación con los pacientes con tumores negativos al mismo [8, 11, 111]. Además, diversos estudios muestran que el calcitriol inhibe la proliferación de una amplia variedad de líneas y cultivos primarios incluido el carcinoma mamario [112],[10]. Algunos de los efectos antineoplásicos del calcitriol en cáncer de mama son la reducción de la expresión del marcador de proliferación tumoral (Ki67), la desfosforilación de la proteína Rb, la cual permite el arresto del ciclo celular en la fase G1[88], [113]. Recientemente, se reportó que el calcitriol suprime la expresión génica y proteica del canal de potasio Ether-à-go-go, implicado en la oncognesis y proliferación de cáncer de mama [10]. El calcitriol también ha mostrado inhibir la actividad de la cinasa Src, lo cual se ha relacionado con el decremento de la actividad de las cinasas ERK 1/2 dando como resultado la inhibición de la proliferación celular [88]. Estos trabajos sugieren el uso del calcitriol para el tratamiento y prevención del cáncer de mama [114].

Es importante señalar que la mayoría de los estudios han evaluado el papel del calcitriol en carcinoma mamario con fenotipo hormonal [89]. Sin embargo, su efecto en tumores mamarios con fenotipo EGFR o HER2 positivo es pobremente estudiado. Por lo que, el efecto esta hormona en cáncer de mama con estos fenotipos es el principal objetivo de esta investigación.

8.5 Combinaciones clínicas del calcitriol con inhibidores de residuos de tirosina

En la literatura existen solo dos reportes fase I sobre la combinación del calcitriol con el gefitinib en pacientes con tumores sólidos [115, 116]. El objetivo principal de estos estudios fue investigar las dosis máximas toleradas de la combinación del calcitriol y el gefitinib en tumores sólidos sin profundizar en el efecto farmacológico o el mecanismo de acción de dicha combinación. Cabe resaltar que, en el caso particular del cáncer de mama en ambos trabajos incluyeron solo un paciente con esta patología y no mencionaron el fenotipo tumoral. Lo anterior es de consideración debido a que el blanco molecular del gefitinib es el EGFR, además este compuesto presenta su efecto antiproliferativo en células

de cáncer de mama con sobreexpresión del HER2. Por lo que, el fenotipo del tumor es de relevancia para la sensibilidad de esta molécula. Finalmente, ambos trabajos concluyeron que la administración de dosis elevadas de calcitriol (74 μg por semana) produce hipercalcemia como principal efecto secundario.

Considerando el efecto calcémico del calcitriol, estudios en la modificación de los esquemas de administración de la hormona han demostrado disminuir la hipercalcemia [117]. Además, compuestos análogos del calcitriol con menor efecto hipercalcémico se han desarrollado, los cuales mantienen los efectos antiproliferativos del calcitriol en diversos tumores [118, 119].

9. ANÁLOGOS DEL CALCITRIOL CON POTENCIAL TERAPÉUTICO EN CÁNCER DE MAMA

Los análogos del calcitriol con modificaciones estructurales en el carbono 17, donde se ubica la cadena lateral de la molécula, presentan efectos antiproliferativos similares a este compuesto [120, 121], **Fig. 6**. El calcipotriol (MC903) y el EB1089 (secocalcitol) pertenecen a este grupo por lo cual se decidió evaluarlos en este trabajo. A continuación, se mencionan sus principales características.

9.1 Calcipotriol

El calcipotriol presenta un doble enlace en el carbono 22 (C-22 y C-23), un grupo hidroxilo en el C-24 y un anillo ciclopropano que une a los C-25, C-26 y C-27 **Fig. 6**. El calcipotriol es fácilmente catabolizado por lo que presenta una vida media corta (6 minutos), comparado con calcitriol (8-12 hrs) lo cual favorece la reducción del efecto hipercalcémico. El calcipotriol es usado actualmente para lesiones tópicas como psoriasis. En cáncer de mama, el calcipotriol modula los procesos inflamatorios al disminuir los niveles de la interleucina 1 (IL-1) y la IL-6 e incrementar los niveles de los factores de crecimiento transformante beta 1 y 2 (TGFβ1 y TGFβ2), además participa en la diferenciación de queratinocitos [122].

9.1.1 Efectos del calcipotriol en cáncer de mama

En estudios *in vitro* y preclínicos en modelos tumorales de cáncer de mama, el calcipotriol ha mostrado inhibir la proliferación celular y el crecimiento tumoral, sin la generación de hipercalcemia [123].

En lo que respecta a estudios clínicos, el calcipotriol se ha evaluado principalmente en el carcinoma mamario inflamatorio en administración tópica [124]. Sin embargo, el efecto de este compuesto ha sido pobremente estudiado en cáncer de mama con diferentes fenotipos moleculares.

9.2 EB1089

El EB1089 es un análogo del calcitriol que contiene dos dimetilos terminales en el C-25 y dos dobles ligaduras entre C-22 / C-23 y el C-24 / C-25, **Fig. 6**, que lo protegen de la rápida degradación enzimática y le confieren una vida media similar a la del calcitriol [125]. Diversos reportes mencionan que la actividad antiproliferativa del EB1089 es de 100 veces mayor a la del calcitriol en diversas líneas celulares de cáncer.

9.2.1 Efectos del EB1089 en cáncer de mama

En células de cáncer de mama con fenotipo RE positivo, el EB1089 inhibe la proliferación con mayor potencia que el ligando natural a través de inhibir al factor de transcripción c-myc y la expresión de la proteína anti-apoptótica Bcl-2, también incrementa la expresión de p21 e induce arresto del ciclo celular [126, 127]. En estudios *in vivo* este análogo inhibe la progresión tumoral e incluso a dosis elevadas muestra regresión tumoral con efectos hipercalcémicos menores que los del calcitriol [128]. Diversos estudios han evaluado el efecto del EB1089 en células de carcinoma mamario en combinación con anti-estrogénicos [129], antineoplásicos [130] e incluso con radiación [131]. Todos estos trabajos han concluido que la administración del EB1089 con cualquiera de estos agentes incrementa la muerte celular en mayor medida que la administración de cada compuesto por separado.

A nivel clínico los estudios sobre el efecto del EB1089 se enfocan principalmente en evaluar las dosis máximas toleradas [118]. Al igual que el calcipotriol, los efectos y el mecanismo de acción del EB1089 en cáncer de mama fenotipo EGFR o HER2 positivo son desconocidos.

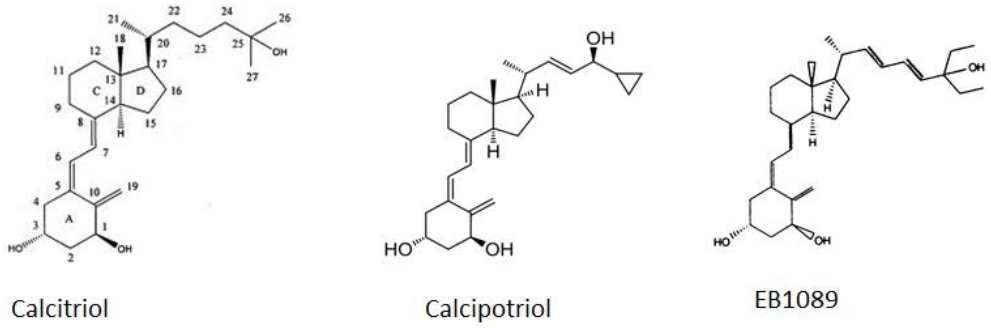


Fig. 6. **Estructura química del calcitriol, el calcipotriol y el EB1089.** A la izquierda se encuentra la estructura del calcitriol indicando la posición y número de carbonos. En el caso de los dos análogos que se encuentran enseguida se aprecian las modificaciones en la cadena lateral mencionadas en el texto.

10. PLANTEAMIENTO DEL PROBLEMA

Pacientes con tumores mamarios que sobreexpresan al EGFR y/o al HER2, entre los que se incluyen los fenotipos HER2 positivo y triple negativo, presentan pobre pronóstico y resistencia al tratamiento convencional. Moléculas inhibidoras de la actividad tirosina cinasa de estos receptores son una opción terapéutica prometedora para estos pacientes. Por otro lado, el calcitriol es otro compuesto con importantes efectos antiproliferativos, antineoplásicos y de diferenciación en cáncer de mama, que incrementa la respuesta de diferentes tratamientos antineoplásicos. Con la finalidad de incrementar las tasas de respuesta global, prolongar la supervivencia global y superar la resistencia al tratamiento, en este trabajo se propuso investigar el tratamiento combinado de inhibidores tirosina cinasa con calcitriol o EB1089 y calcipotriol.

11. HIPÓTESIS

El tratamiento combinado del gefitinib, el lapatinib o el neratinib con calcitriol o sus análogos inhibirá en mayor medida la proliferación de células de cáncer de triple negativo y HER2 positivo comparado con el tratamiento por separado de los antineoplásicos. Asimismo, las combinaciones incrementarán la apoptosis e inhibirán la activación de las vías PI3K/Akt y MEK-ERK.

12. OBJETIVO GENERAL

Evaluar el efecto y determinar el mecanismo de acción de la combinación de los inhibidores de residuos de tirosinas cinasa, gefitinib, lapatinib y neratinib, con el calcitriol o sus análogos sintéticos en células de cáncer de mama con fenotipo triple negativo y HER2 positivo.

13. OBJETIVOS ESPECÍFICOS

a) Caracterizar la presencia del EGFR, el HER2 y el VDR, en el cultivo primario MBCDF y en las líneas de cáncer de mama: SUM-229PE, SK-BR-3, HCC1937 y MDA-MB-231.

b) Determinar la concentración inhibitoria (CI) al 20 y 50% de los inhibidores de residuos de tirosina cinasa, el calcitriol, el calcipotriol y el EB1089 en células de cáncer de mama mediante estudios de proliferación.

c) Evaluar el efecto farmacológico de los inhibidores de residuos de tirosina en combinación con el calcitriol y sus análogos en la proliferación celular.

d) Investigar la expresión génica de mediadores de apoptosis y de metabolismo celular en células de cáncer de mama expuestas a la combinación del gefitinib, el lapatinib y el neratinib con el calcitriol o sus análogos.

e) Analizar la distribución de las diferentes fases del ciclo celular en los cultivos de carcinoma mamario expuestos a las diferentes combinaciones del calcitriol o sus análogos con los inhibidores de residuos de tirosinas cinasas.

f) Evaluar la inducción de la caspasa 3 activa en los cultivos de carcinoma mamario expuestos a las diferentes combinaciones de antineoplásicos.

g) Determinar el efecto de la combinación del gefitinib, el lapatinib y el neratinib con calcitriol o EB1089 en la fosforilación de la vía de señalización MAPK y Akt en las diferentes líneas de cáncer de mama.

h) Investigar si los esquemas combinados de los inhibidores de residuos de tirosina en combinación con calcitriol o su análogo EB1089 inhiben la capacidad clonogénica de células de cáncer de mama.

14. MATERIALES Y MÉTODOS

Cultivo Celular: El cultivo primario (MBCDF) y las diferentes líneas celulares, SUM-229PE, SK-BR-3, HCC1937 y MDA-MB-231 se sembraron en su medio específico y fueron mantenidas a 37ºC con una atmósfera de 5% de CO_2 y 95% de humedad.

Ensayos de proliferación: Los análisis de proliferación se llevaron a cabo sembrando 1000-2000 células por pozo dependiendo de cada línea y fueron incubadas en presencia de concentraciones crecientes del calcitriol, el calcipotriol (Sigma Aldrich), el EB1089 (Tocris Cookson), el gefitinib (donación de Astrazeneca), el lapatinib y el neratinib (Sequoia Research Products). Posteriormente, la concentración de DNA fue cuantificada por medio del kit de proliferación CyQuant (Invitrogen). Los valores de las CIs fueron calculados por un software especializado (OriginLab Corporation, Northampton, MA, versión 5.0). Los experimentos se realizaron por sextuplicado en al menos 3 experimentos independientes.

Efecto farmacológico de los estudios de combinación: Los valores del índice de combinación fueron obtenidos a través de los datos de las curvas concentración respuesta de las combinaciones de las CI_{20} y $_{50}$. Los valores fueron calculados aplicando la ecuación del efecto múltiple de fármacos de Chou Talalay [132]. Para este análisis, los parámetros son los siguientes: IC < 1 efecto sinérgico, IC > 1 efecto antagónico e IC = 1 efecto aditivo.

PCR en tiempo real: Para evaluar la regulación génica del EGFR, del factor de transcripción FOXO3a y de biomarcadores implicados en los procesos de apoptosis (BIM y Bcl-2), las células de cáncer de mama fueron tratadas con los ITC solos o en combinación con calcitriol o sus análogos. El RNA sé extrajo utilizando Trizol (Life Technology) y para la síntesis de ADN complementario (ADNc) se empleó la transcriptasa reversa y oligo-dT (Roche Diagnostics Mannheim, Germany). La reacción de amplificación en cadena de la polimerasa en tiempo real se realizó con iniciadores específicos para cada gen. Las amplificaciones se llevaron a cabo en el equipo LigthCycler® 2.0 de Roche (Roche Diagnostics), de acuerdo al siguiente protocolo: activación de la Taq DNA polimerasa y desnaturalización del DNA a 95 °C por 10 minutos, seguido de 45 ciclos de amplificación los cuales consistieron de 10 s a 95 °C, 30 s

a 60 °C y 45 s a 72 °C. La normalización de los resultados se realizó utilizando la amplificación del gene constitutivo gliceraldehído-3-fosfato deshidrogenasa (GAPDH).

Distribución del ciclo celular: Las células fueron incubadas con las combinaciones de los fármacos durante 96 horas. Posterior al tratamiento, las células fueron colectadas y lavadas con buffer de fosfatos pH 7.2, fijadas en etanol 70% v/v y almacenadas a -20 °C. Para el análisis del ciclo celular, las muestras se lavaron e incubaron en una solución con RNAsa (10mg/ml), triton X-100 0.1% v/v y yoduro de propidio (1mg/ml) en oscuridad a temperatura ambiente por 20 min. El contenido de DNA fue determinado usando un citómetro de flujo FACsCanto II. Para el análisis del ciclo celular y la detección del pico SubG1 un total de 35,000 eventos fueron adquiridos. El análisis de los resultados se realizó con el software Flow-Jo (Tree Star Inc versión 9.3.2.).

Detección de la caspasa 3 activa: Con la finalidad de evaluar el efecto de las combinaciones en la activación de la caspasa 3 se utilizaron cultivos en monocapa y tridimensionales de células de cáncer de mama.

Para el cultivo celular en monocapa, las células fueron incubadas con los antineoplásicos solos o en combinación por 72 h. Posteriormente, las células positivas para la caspasa 3 activa se marcaron con un kit comercial de apoptosis (BD Pahrmingen, CA, USA). Las células se colectaron, lavaron y resuspendieron en el buffer BD Cytofix/Cytoperm y fueron incubadas por 20 minutos a 4 °C. La suspensión celular fue centrifugada y lavada dos veces con el buffer BD Perm/Wash. Subsecuentemente, las células fueron incubadas con el anticuerpo anti-caspasa 3 marcado con isotiocianato de fluoresceína (FITC) por 30 min. Las muestras fueron lavadas, resuspendidas con buffer BD Perm/Wash y analizadas con el citómetro de flujo FACsCanto II (Becton Dickinson, San Jose CA, USA). Un total de 20,000 eventos fueron adquiridos.

Para la evaluación de la caspasa 3 activa en el cultivo celular en tres dimensiones se siguió el siguiente protocolo: En cajas de cultivo celular de 8 pozos tratadas previamente con Matrigel (Corning) fueron sembradas las células de cáncer de mama. Posterior a 7 días de cultivo, las células fueron expuestas a los diferentes tratamientos solos o en combinación por 48 hs. Posteriormente, las células fueron fijadas con formalina al 4%, lavadas con

buffer de fosfatos pH7.2/glicina, después fueron expuestas a un bloqueo primario con un buffer para inmunofluorescencia y suero de cabra al 10% durante 1 h a temperatura ambiente. Pasando este periodo, la solución de bloqueo fue aspirada y se incubó con el segundo buffer de bloqueo (buffer de inmunufluorescencia, suero de cabra al 10% y la región F(ab) 1mg/mL) (Jackson Immuno Research) y el anticuerpo primario (caspasa 3 activa, Cell Signaling, dilución 1:100) por toda la noche a 4 ºC. Pasando este periodo, las muestras fueron lavadas 3 veces por 20 min con buffer de inmunofluorescencia a temperatura ambiente. Posteriormente, las células se incubaron por 1 h a temperatura ambiente con una solución que contenía el primer buffer de bloqueo con el anticuerpo secundario. Finalmente, las muestras se lavaron 3 veces con el primer buffer de bloqueo y fueron incubadas con DAPI al 25% por 20 min a temperatura ambiente. Pasando este periodo, las muestras fueron lavadas con el primer buffer de bloqueo, se aspiró y se agregó la solución Prolong (Molecular Probes). Finalmente, las laminillas fueron cubiertas con un cubre objetos, se dejaron secar a temperatura ambiente y se guardaron a -20 ºC hasta su posterior análisis por microscopia confocal.

Western Blot: Posterior a las 72 h de tratamiento, las células fueron lavadas con PBS pH 7.3 y lisadas con buffer RIPA. Posteriormente, 20 μg de proteína fueron separados en SDS-PAGE y transferidos a una membrana de fluoruro de polivinilideno, la cual fue bloqueada con TBST con leche al 5% por 30 min. Las membranas fueron incubadas toda la noche a 4° C con diferentes anticuerpos anti-EGFR y anti-VDR (1:1000, Santa Cruz Biotechnology Inc., CA, USA), anti-HER2, anti-phospho-p44/42 MAPK (ERK1/2) y anti-Akt (serina 453) (1:1000 Cell Signaling, Boston, Massachusetts), a 4 ºC. Después de la incubación con el anticuerpo primario, las membranas fueron lavadas e incubadas con su respectivo anticuerpo secundario acoplado con peroxidasa (1:1000) por 1 h a temperatura ambiente. Para la normalización, las membranas fueron incubadas con un anticuerpo anti-GAPDH (1:1000 Santa Cruz Biotechnology Inc.). Las proteínas fueron detectadas usando ECL y visualizadas usando un revelador Kodak. La densitometría fue realizada usando el software Image J.

Ensayos de clonogenicidad: La determinación de la capacidad de las células para la formación de colonias, después de haber sido expuestas a los tratamientos se realizó por la

técnica de agar suave. Las células fueron sembradas en una concentración de 5000 células por pozo en placas de 6 pozos con una mezcla de agarosa (Bioexpress) y su respectivo medio específico (1:4). Las células fueron tratadas con los antineoplásicos y se dejaron crecer por 30 días, el medio fue reemplazado cada 7 días. Posterior a los 30 días, las colonias fueron fijadas y teñidas con una solución de metanol y cristal violeta. Imágenes representativas fueron tomadas con el microscopio Evos (Thermo Fisher Scientific) en campo claro. La cuantificación del número de colonias no se realizó debido a que no se encontró formación de estas en los tratamientos combinados, ya que se consideró como colonia una masa celular de 50 o más células las cuales miden aproximadamente 50 μm.

Análisis estadístico: Los datos se expresaron como la media ± desviación estándar. Las diferencias estadísticas de los ensayos de proliferación y de expresión génica fueron determinadas mediante un análisis de varianza (ANOVA) seguido de la prueba Holm-Sidak, utilizando un software especializado (SigmaStat, Jandel Scientific versión 3.5). Valores de $p<0.05$ y de $p<0.001$ se consideraron estadísticamente significativos.

15. RESULTADOS

15.1 Caracterización celular

La expresión proteica del VDR, el HER2 y el EGFR se determinó por western blot en el cultivo primario MBCDF y en las células SUM-229PE, SK-BR-3, HCC1937 y MDA-MB-231. Todas las líneas celulares fueron positivas al VDR y EGFR, la presencia de este último receptor en el cultivo primario no pudo ser detectada. Las células SK-BR-3 y el cultivo primario fueron positivos al HER2, **Tabla 2**.

Tabla 2. Caracterización celular de líneas de cáncer de mama

Línea Celular	VDR	EGFR	HER2
SUM-229PE	+	+	-
SK-BR-3	+	+	+
MBCDF	+	-	+
HCC1937	+	+	-
MDA-MB-231	+	+	-

Receptor de Vitamina D (VDR), receptor del factor de crecimiento epidérmico tipo 1 (EGFR) y tipo 2 (HER2).

15.2 Efecto antiproliferativo de los inhibidores de residuos de tirosinas cinasas, calcitriol y sus análogos en células de cáncer de mama

Con la finalidad de estudiar el efecto de los diferentes antineoplásicos en la proliferación de células de cáncer de mama. Las células fueron incubadas en la presencia de diferentes concentraciones de los compuestos durante siete días.

En la **Fig. 7** se muestra la actividad antineoplásica del calcitriol (A), y sus análogos, calcipotriol (B) y EB1089(C), y los ITC gefitinib (D), lapatinib (E) y neratinib (F) en las diferentes líneas de cáncer de mama.

La proliferación celular de las líneas de cáncer de mama utilizadas fue inhibida significativamente por los antineoplásicos de manera dependiente de la concentración. Los compuestos mostraron diferentes potencias a las concentraciones más alta ($1X10^{-7}$, $1X10^{-6}$ y $1X10^{-4}$) dependiendo del fenotipo molecular.

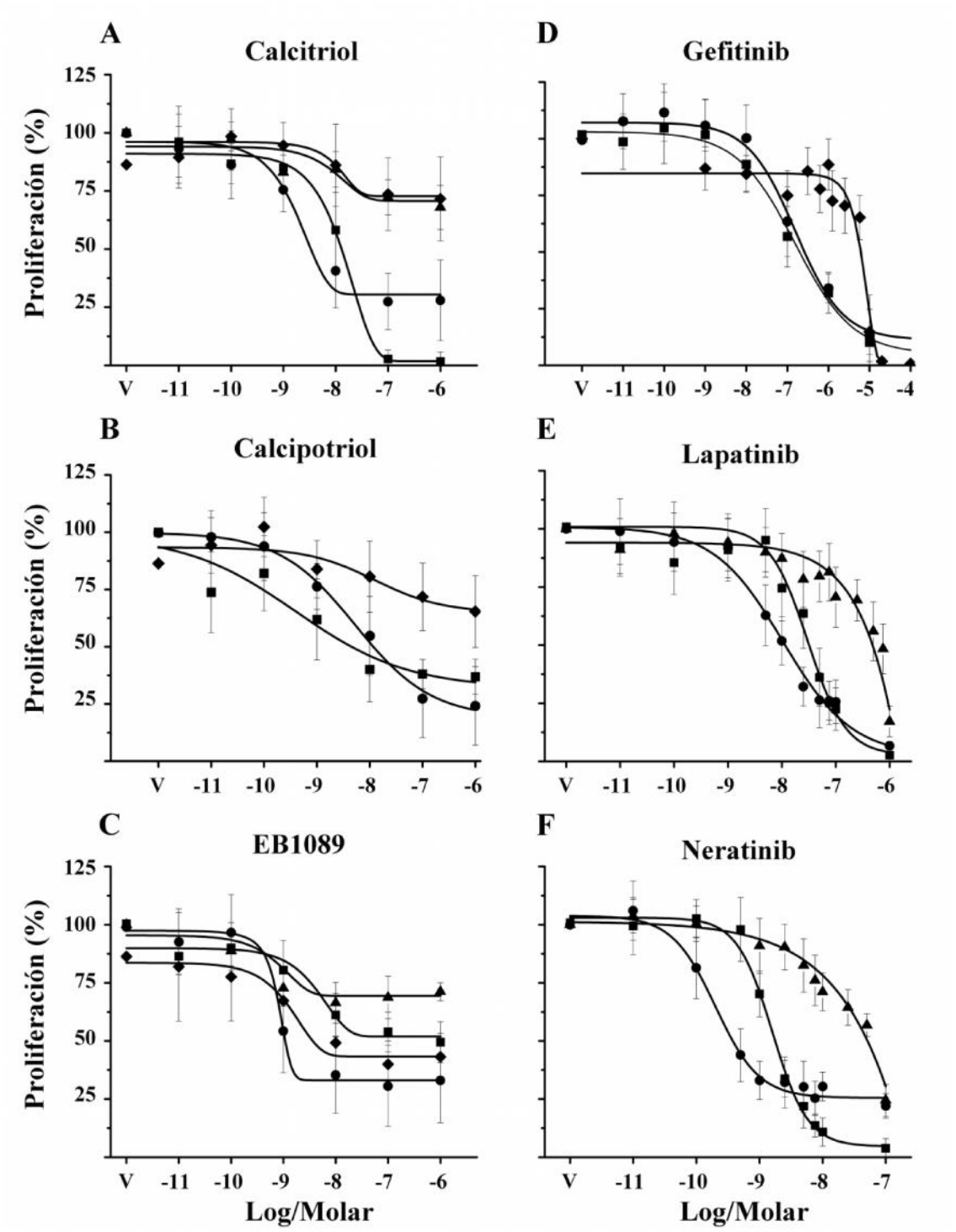


Fig. 7. **Efecto antiproliferativo del calcitriol, el calcipotriol, el EB1089, el gefitinib, el lapatinib y el neratinib en células de cáncer de mama**. Las líneas establecidas SUM-229PE (■), SK-BR-3 (●), MBCDF (♦) y HCC1937 (▲) fueron incubadas en presencia de concentraciones crecientes de los antineoplásicos durante 7 días. La proliferación celular fue evaluada por el ensayo CyQuant. El vehículo fue considerado como el 100%. Los valores representan el promedio ± DE de tres experimentos independientes por sextuplicado.

De las curvas concentración-respuesta obtenidas de cada compuesto en las diferentes líneas celulares se determinaron las CI_{20} y CI_{50} (**Tabla 3)**.

Considerando los valores de las CI_{50} de cada compuesto, las células de cáncer de mama mostraron la siguiente sensibilidad: calcitriol (SK-BR-3 < SUM-229PE < MBCDF), calcipotriol (SUM-229PE < SK-BR-3 < MBCDF), EB1089 (SK-BR-3 < MBCDF < SUM-229PE), gefitinib (SUM-229PE = SK-BR-3 < MBCDF), lapatinib y neratinib (SK-BR-3 < SUM-229PE < HCC1937). La línea celular HCC1937 no fue sensible a los efectos antiproliferativos del calcitriol o sus análogos. Ninguno de los compuestos evaluados inhibió la proliferación de las células MDA-MB-231. La actividad antiproliferativa de lapatinib y neratinib, no se determinó en la línea MBCDF.

Tabla 3. Concentraciones inhibitorias al 20 y 50% de los antineoplásicos en células de cáncer de mama

Ligando	CI	SUM-229PE (mol/L)	SK-BR-3 (mol/L)	MBCDF (mol/L)	HCC1937 (mol/L)
Calcitriol	20	4.6×10^{-9}	4.6×10^{-10}	7.4×10^{-9}	-
	50	1.4×10^{-8}	2.4×10^{-9}	1.7×10^{-8}	-
Calcipotriol	20	1.3×10^{-11}	5.3×10^{-10}	1.6×10^{-9}	-
	50	4.0×10^{-10}	5.3×10^{-9}	1.2×10^{-8}	-
EB1089	20	2.0×10^{-10}	4.3×10^{-10}	2.3×10^{-10}	-
	50	2.7×10^{-9}	7.5×10^{-10}	1.6×10^{-9}	-
Gefitinib	20	1.6×10^{-8}	2.5×10^{-8}	2.0×10^{-6}	-
	50	1.5×10^{-7}	1.5×10^{-7}	3.0×10^{-6}	-
Lapatinib	20	1.3×10^{-7}	1.6×10^{-8}	nd	4.5×10^{-8}
	50	3.5×10^{-7}	8.9×10^{-8}	nd	6.0×10^{-6}
Neratinib	20	6.7×10^{-9}	8.1×10^{-10}	nd	8.5×10^{-8}
	50	1.4×10^{-8}	2.1×10^{-9}	nd	7.5×10^{-7}

CI, concentraciones inhibitorias (CI). nd, no determinado.

15.3 Efecto farmacológico del tratamiento combinado del gefitinib, el lapatinib y el neratinib con el calcitriol y sus análogos en la proliferación celular

A partir de los valores de las CIs de cada compuesto se realizaron diferentes combinaciones. La concentración de $1X10^{-8}$ M del calcitriol y el EB1089 se utilizó para los esquemas de combinación en la línea celular HCC1937, debido a que esta concentración inhibió la proliferación celular de manera significativa comparada con el vehículo. Para el caso particular de la línea MDA-MB-231, la concentración utilizada del calcitriol o el EB1089 fue de $1X10^{-7}$ M y para el lapatinib y el neratinib se utilizaron las concentraciones de $1X10^{-5}$ M y $1X10^{-6}$ M, respectivamente.

La **Fig. 8** muestra los datos obtenidos de la línea celular SUM-229PE expuesta a las CI_{20} y CI_{50} del calcitriol, el gefitinib y sus combinaciones. La proliferación celular fue inhibida en mayor medida cuando las células fueron tratadas a las CI de calcitriol y gefitinib 20:20; 20:50 y 50:50, respectivamente.

Adicionalmente, las combinaciones del calcitriol o sus análogos con gefitinib, lapatinib y neratinib fueron evaluadas en las líneas celulares de cáncer de mama SUM-229, SK-BR-3, MBCDF, HCC1937 y MDA-MB-231. La **Tabla 4** muestra el porcentaje de inhibición de la proliferación celular (I%) ejercido por los compuestos solos o en combinación. En general, las co-incubaciones en presencia de los ITC con calcitriol, calcipotriol o EB1089 dieron como resultado una significativa y más robusta inhibición del crecimiento celular que el obtenido con cada fármaco en la mayoría de las combinaciones utilizadas. De manera interesante, las células insensibles a los efectos de los antineoplásicos (MBCDF, HCC1937 y MDA-MB-231) al tratarlas con los compuestos en conjunto, la resistencia *de novo* a los agentes fue revertida.

De los resultados obtenidos en los estudios de proliferación con las diferentes combinaciones se determinó el valor del índice de combinación de acuerdo a la ecuación de Chou Talalay. Este valor fue utilizado para determinar el efecto farmacológico resultante de las combinaciones de fármacos. El índice de combinación está dado por las siguientes condiciones IC < 1 efecto sinérgico, IC > 1 antagónico e IC = 1 aditivo.

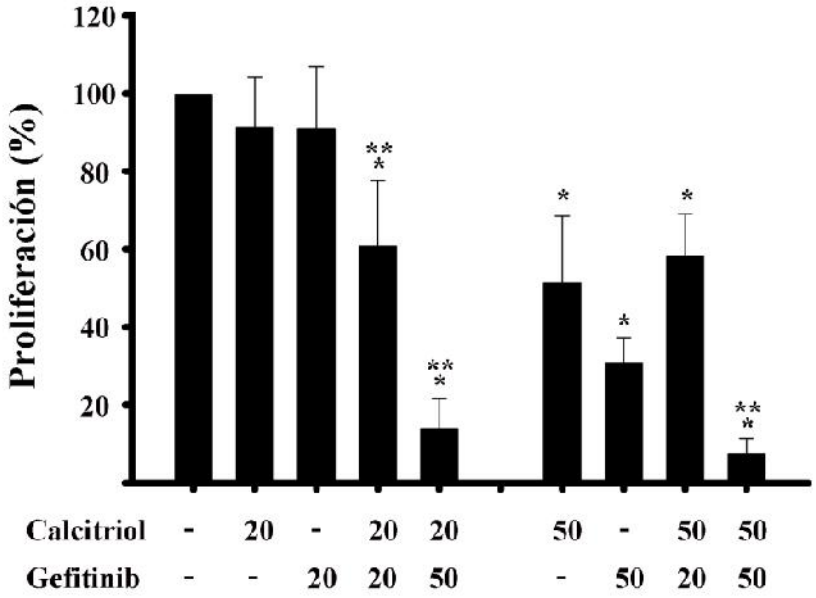


Fig. 8. **Efecto antiproliferativo de la combinación de las CIs al 20 y 50% del gefitinib y del calcitriol en la línea SUM-229PE**. Las células fueron incubadas en presencia de las diferentes combinaciones de los antineoplásicos durante 7 días. El vehículo fue considerado como el 100%. Los valores representan el promedio ± DE de tres experimentos independientes por sextuplicado. * Diferencia significativa con respecto al control ($p<0.001$). ** Diferencia significativa con respecto a la administración de los antineoplásicos de manera independiente ($p<0.001$).

Tabla 4. Porcentaje de inhibición de la proliferación celular ejercido por los compuestos solos o en combinación en células de cáncer de mama

SUM-229PE			G		L		N	
			CI_{20}	CI_{50}	CI_{20}	CI_{50}	CI_{20}	CI_{50}
Lg	**CI**	I%	8.2 ± 6.6	64.06 ± 7.8	23.0±12.9	48.0±10.0	12.6±11.7	35.6±11.6
			Combinaciones					
C	**20**	15.4±8.7	39.2 ± 16.8*	86.0 ± 7.7*	26.3±14.2	59.2±12.5	48.7±8.4*	58.0±11.7*
	50	45.7±7.8	41.9 ± 10.9	92.6 ± 4.1*	56.9±14.2	72.5±7.4*	64.7±10.3*	73.8±8.5*
E	**20**	10.8±6.0	4.9 ± 1.7	55.4 ± 7.5*	40.0±10.0*	55.7±11.5	37.3±12.6*	47.9±8.8
	50	41.6±8.3	33.2 ± 13.5	75.6 ± 6.9*	65.2±7.1*	76.5±76.6*	65.5±9.5*	73.2±8.2*
Cp	**20**	16.7±13.8	16.3 ± 16.4	63.8 ± 9.2	nd	nd	nd	nd
	50	24.0±14.6	37.3 ± 21.3	70.5 ± 8.9	nd	nd	nd	nd

SK-BR-3			G		L		N	
			CI_{20}	CI_{50}	CI_{20}	CI_{50}	CI_{20}	CI_{50}
Lg	**CI**	**I%**	15.23 ± 12.0	51.5 ± 13.4	24.4±10.2	60.4±10.8	52.3±9.9	77.7±4.6
			Combinaciones					
C	**20**	10.6±7.7	44.8 ± 13.6*	33.9 ± 5.1*	33.4±7.1	71.9±4.5*	78.0±5.4*	58.1±7.1
	50	22.0±9.4	55.1 ± 14.4*	49.9 ± 6.6	36.8±10.8	70.3±5.0*	78.5±4.5*	63.1±11.7
E	**20**	14.6±7.7	49.2 ± 13.7*	46.2 ± 7.0	28.6±5.9	70.6±6.7*	79.5±3.7*	59.5±7.0
	50	31.6±11.2	57.5 ± 12.6*	59.4 ± 5.8*	43.0±11.1	71.1±4.6*	78.4±4.8*	63.9±7.9*
Cp	**20**	7.0 ± 7.7	52.1 ± 12.0*	43.1 ± 9.8*	nd	nd	nd	nd
	50	26.0 ±8.9	62.4 ± 13.6*	59.9 ± 5.8*	nd	nd	nd	nd

MBCDF			G	
			CI_{20}	CI_{50}
Lg	**CI**	**I%**	9.4 ± 12.0	23.5 ± 11.8
			Combinaciones	
C	**20**	18.5±10.6	32.2±13.2*	41.9±12.5*
	50	11.5±16.0	53.8±13.6*	56.4±11.9*
E	**20**	16.0±10.3	32.0±11.1*	24.9 ± 7.5
	50	27.1 ± 9.9	39.5 ± 12.0	46.6 ± 9.2*
Cp	**20**	13.9 ± 9.9	33.2±13.5 *	38.7±11.9*
	50	24.6 ± 6.3	39.0±9.7*	44.6±11.2*

HCC1937			L		N	
			CI_{20}	CI_{50}	CI_{20}	CI_{50}
Lg	**CI mol/L**	**I%**	11.6±9.4	39.7±9.8	22.5±8.3	64.9±6.1
			Combinaciones			
C	**1X10-8**	7.3±1.5	67.1±3.1*	39.8±6.8	42.9±13.1 *	63.3±8.4
E	**1X10-8**	8.9±6.9	53.3±6.9*	57.1±8.8*	33.9±5.6*	61.1±5.9

MDA-MB-231			L	N
			$1X10^{-5}$ mol/L	$1X10^{-6}$ M mol/L
Lg	CI mol/L	I%	18.5±2.2	24.2±3.5
			Combinaciones	
C	$1X10^{-7}$	-4.0±9.2	31.7±10.9*	21.2±3.8
E	$1X10^{-7}$	-1.7±9.7	42.5±8.7*	34.2±8.8*

Ligando (Lg), concentración inhibitoria (CI), porcentaje de inhibición de la proliferación celular (I%), calcitriol (C), EB1089 (E), calcipotriol (Cp), gefitinib (G), lapatinib (L), neratinib (N), no determinado (nd). Los valores representan el promedio ± DE de tres experimentos independientes por sextuplicado. * Diferencia significativa con respecto la administración de los antineoplásicos de manera independiente ($p<0.001$).

La **Fig. 9** muestra el valor del índice de combinación de los diferentes esquemas combinados del gefitinib A), el lapatinib B) y el neratinib C) con calcitriol y sus análogos en el cultivo primario MBCDF (solo en **Fig. 9 A**) y en las líneas SUM-229PE y SK-BR-3. De manera general, se observa que la mayoría de las combinaciones tienen un valor de índice de combinación menor a 1. Sin embargo, los esquemas combinados con el lapatinib o el neratinib (B, C), mostraron más combinaciones que tuvieron efecto sinérgico comparados con los esquemas combinados del gefitinib (A).

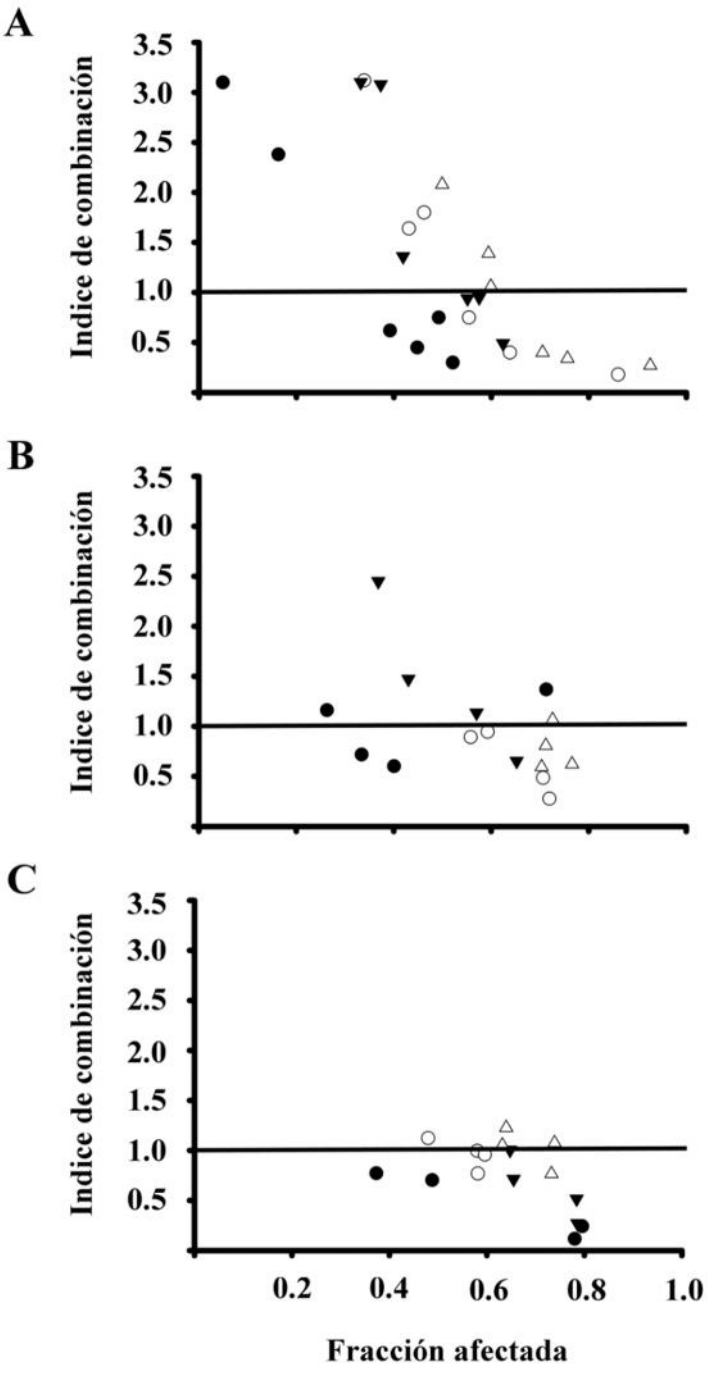


Fig. 9. Efecto farmacológico en diferentes líneas celulares de cáncer de mama expuestas a diferentes esquemas de combinación de fármacos. El cultivo primario MBCDF (solo en el panel A) y las células SUM-229PE y SK-BR-3 fueron incubadas con las siguientes combinaciones de fármacos; compuestos de vitamina D: gefitinib, lapatinib y neratinib, 20:20 ●; 50:20 ▼; 20:50 ○; 50:50 Δ. El valor del índice de combinación fue calculado con un modelo matemático desarrollado por Chou Talalay. Los puntos debajo del índice de combinación igual a 1 denotado por una línea horizontal son indicativos de interacción sinérgica.

15.4 El tratamiento combinado del gefitinib con el calcitriol o su análogo EB1089 moduló la expresión génica y proteica de marcadores de apoptosis y proliferación

Con el objetivo de evaluar si el tratamiento combinado regula la expresión génica del blanco molecular del gefitinib se llevaron a cabo estudios de qPCR en tiempo real. La **Fig. 10** muestra la regulación génica del EGFR por el EB1089 y el gefitinib solos y en combinación. La mRNA del EGFR disminuyó con la combinación de los compuestos en ambas líneas celulares SUM-229PE (barras negras) y SK-BR-3 (barras blancas). La combinación del gefitinib con calcitriol mostró una tendencia a disminuir la expresión génica del EGFR tanto en SUM-229PE como en SKBR3 (datos no mostrados). Además, la expresión del gen HER2 también fue evaluada, sin embargo, su expresión no se vio afectada (datos no mostrados).

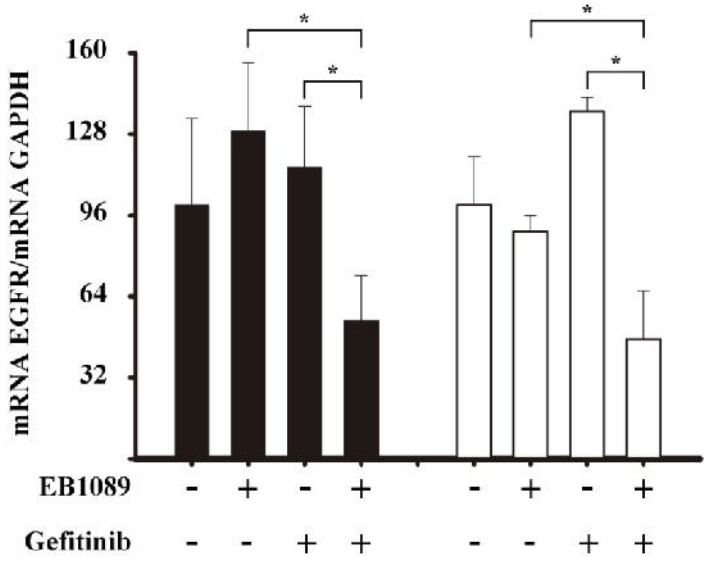


Fig. 10. La combinación del gefitinib con el EB1089 inhibió la expresión génica del EGFR en células de cáncer de mama. Las células SUM-229PE (barras negras) fueron incubadas con los valores correspondientes CI_{50} del EB1089, el gefitinib o su combinación durante 24 h. Las células SK-BR-3 (barras blancas) fueron incubadas con los correspondientes valores de CI_{20} de los compuestos. Subsecuentemente, el mRNA se extrajo y los análisis se llevaron a cabo por la técnica de qPCR para la expresión génica. Los resultados se muestran como la media ± D. E. de tres experimentos realizados de manera independiente. A las células tratadas con el vehículo arbitrariamente se les dio un valor de 100% *$p \leq 0.05$ vs cada compuesto solo.

Considerando que la combinación de fármacos inhibió la proliferación celular de manera sinérgica, se decidió evaluar la modulación de marcadores de apoptosis, regulados por la familia EGFR, como BIM y Bcl-2.

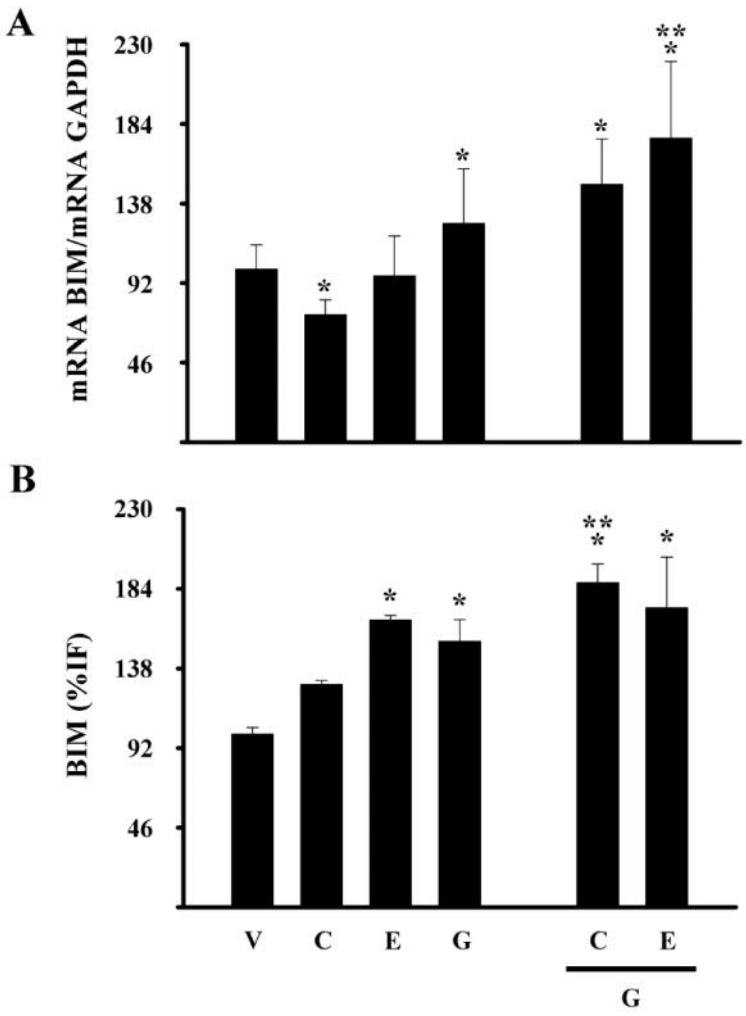


Fig. 11. La combinación del gefitinib con el calcitriol o el EB1089 indujo la expresión del marcador pro-apoptótico BIM en células de cáncer de mama. Las células SUM-229PE fueron incubadas con su correspondiente valor de CI_{50} del gefitinib, del calcitriol o del EB1089 solos o en combinación durante 24 h (A) o durante 48 h (B). Los análisis de expresión génica se llevaron a cabo por las técnicas de qPCR y estudios de citometría de flujo se realizaron para la cuantificación de BIM, representada por el porcentaje de intensidad de fluorescencia (IF). Los resultados se muestran como la media ± D. E. de tres experimentos realizados de manera independiente. Las células tratadas con el vehículo arbitrariamente se les dio un valor de 100%. *p≤0.05 vs el grupo control, **p≤0.05 vs cada compuesto solo.

La combinación del gefitinib con el calcitriol o el EB1089 indujo significativamente la expresión génica (A) y proteica (B) del marcador pro-apoptótico BIM comparado contra el control, en las células SUM-229PE, **Fig. 11.**

De manera inversa al efecto de las combinaciones encontrado en BIM, la expresión génica del marcador anti-apoptótico, Bcl-2, disminuyó con la combinación de los compuestos en ambas líneas SUM-229PE (barras negras) y SK-BR-3 (barras blancas), **Fig.12**. Todos los tratamientos fueron significativos con respecto al grupo control, y únicamente las combinaciones del gefitinib con el EB1089 fueron significativas con respecto a los tratamientos de manera independiente.

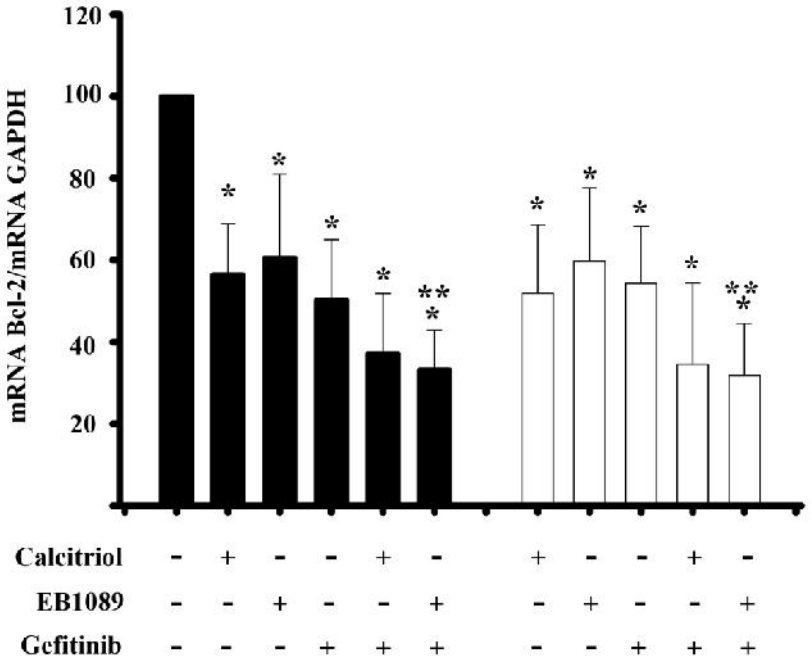


Fig. 12. La combinación del gefitinib con el calcitriol o el EB1089 redujo la expresión génica del marcador anti-apoptótico Bcl-2 en células de cáncer de mama. Las células SUM-229PE (barras negras) fueron incubadas con los valores correspondientes CI_{50} del EB1089, el gefitinib o su combinación durante 24 h. Las células SK-BR-3 (barras blancas) fueron incubadas con los correspondientes valores de CI_{20} de los compuestos. Subsecuentemente, el mRNA se extrajo y los análisis se llevaron a cabo por la técnica de qPCR para la expresión génica. Los resultados se muestran como la media ± D.E. de tres experimentos realizados de manera independiente. Las células tratadas con el vehículo en ambas líneas celulares arbitrariamente se les dio un valor de 100% *p≤0.05 vs el grupo control, **p≤0.05 vs cada compuesto solo.

15.5 El tratamiento combinado del calcitriol o su análogo EB1089 con el lapatinib o el neratinib disminuyó la expresión génica de FOXO3a

Debido a que el factor de transcripción FOXO3a modula genes involucrados en el control de la proliferación y el ciclo celular, la expresión de este gen fue investigada en células de cáncer de mama expuestas a los fármacos.

Los tratamientos de manera independiente, excepto el tratamiento con el calcitriol, o en combinación indujeron significativamente la expresión génica de FOXO3a comparado contra el grupo control en las células SK-BR-3, **Fig. 13.**

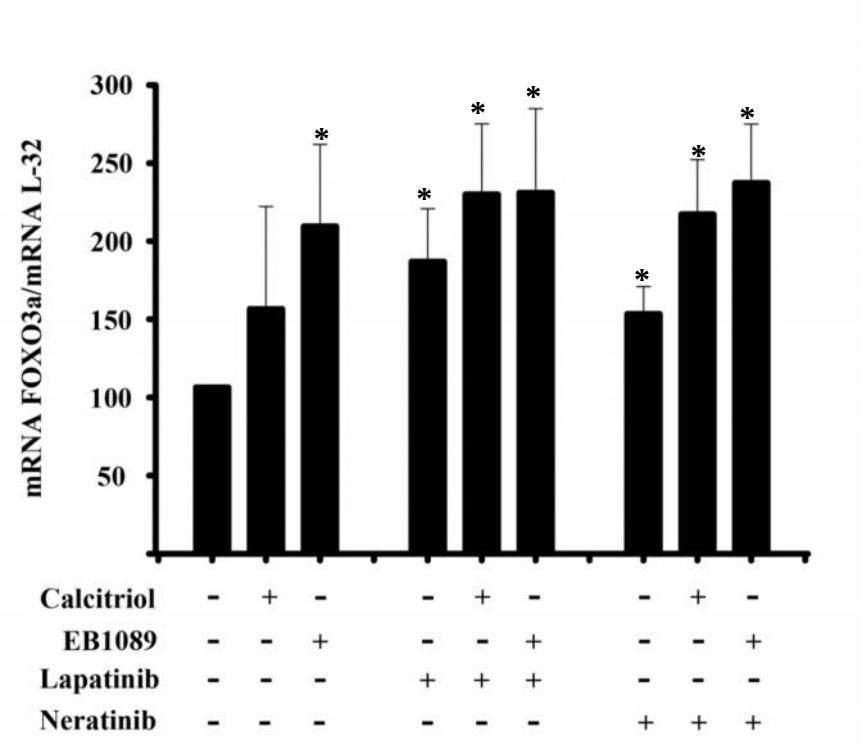


Fig. 13. La combinación del lapatinib o el neratinib con el calcitriol o el EB1089 indujo la expresión del factor de transcripción FOXO3a en células de cáncer de mama. Las células SK-BR-3 fueron incubadas con su correspondiente valor de CI_{20} del calcitriol, del EB1089, del lapatinib, del neratinib o de su combinación durante 24 horas. Subsecuentemente, el mRNA se extrajo y los análisis para evaluar la expresión génica se llevaron a cabo por la técnica de qPCR. Los resultados se muestran como la media ± D.E. de dos experimentos realizados de manera independiente. Las células tratadas con el vehículo arbitrariamente se les dio un valor de 100%. *$p \leq 0.05$ vs el grupo control.

15.6 La combinación de los antineoplásicos incrementó el porcentaje de células en el pico SubG1 y disminuyó la fase G2/M del ciclo celular en células de cáncer de mama.

Considerando que el efecto sinérgico de los compuestos es a través de regular la expresión de genes implicados en el control de la proliferación y apoptosis se decidió evaluar los cambios inducidos en las fases del ciclo celular por los tratamientos en cáncer de mama.

La **Fig. 14** muestra los histogramas del perfil del ciclo celular de las células SUM-229PE tratadas con la combinación de los compuestos posterior a las 96 horas de tratamiento. La combinación del calcitriol o el EB1089 con el gefitinib, el lapatinib o el neratinib incrementó el porcentaje de células en la región o pico SubG1, mientras que el porcentaje de células en la fase S y G2/M disminuyó significativamente en comparación con los compuestos de manera independiente y con el control (**Tabla 5)**. Notablemente, las combinaciones con el lapatinib y el neratinib indujeron mayor muerte celular, en comparación con las combinaciones con el gefitinib, como se juzga por la inducción de la región celular SubG1. Estos datos sugieren que el aumento del pico subG1 deriva de la reducción en el porcentaje de células a partir de las fases S y G2 M.

Para determinar si el porcentaje de células en el pico SubG1 inducido por las combinaciones se debía a la activación de apoptosis, la presencia de la caspasa 3 activa fue evaluada en las células expuestas a los tratamientos. La combinación del calcitriol o el EB1089 con los ITC, incrementó significativamente el porcentaje de células positivas a la caspasa 3 en comparación con cada compuesto por separado, **Fig. 15 (A)**. Estos resultados indican que la combinación de los compuestos inhibe la proliferación a través de activar la vía apoptótica intrínseca en las células SUM-229PE.

Debido a que los modelos tridimensionales de células epiteliales mantienen la organización estructural y la complejidad multicelular del epitelio mamario y son útiles para evaluar terapias experimentales se decidió evaluar la presencia de la caspasa 3 inducida por los compuestos en un modelo celular de tres dimensiones. La combinación del lapatinib o el neratinib con el calcitriol o el EB1089, de manera similar a lo observado en los cultivos tradicionales en monocapa, incrementó la presencia de esta proteína en su forma activa en las células SUM-229PE, **Fig. 15 (B).**

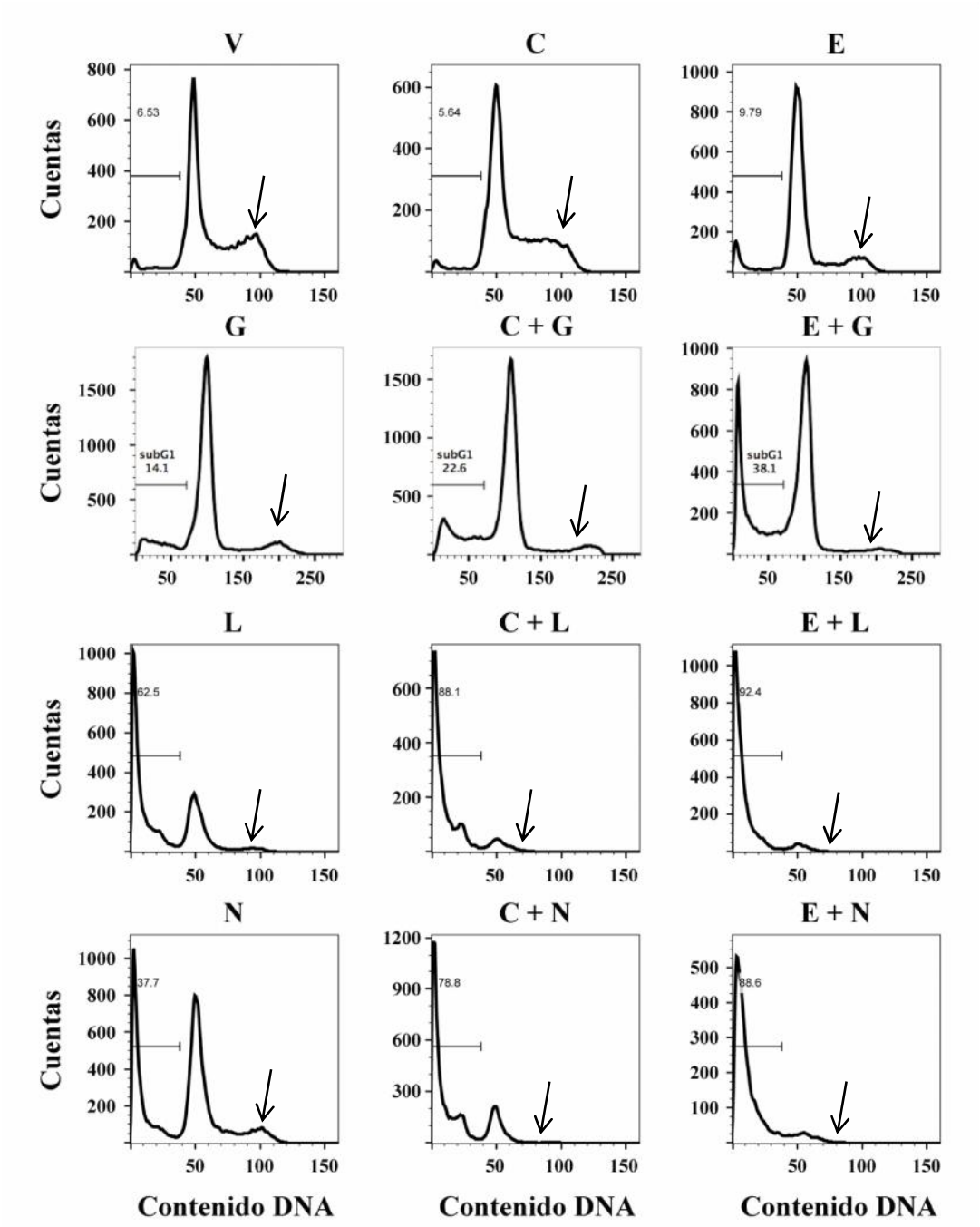


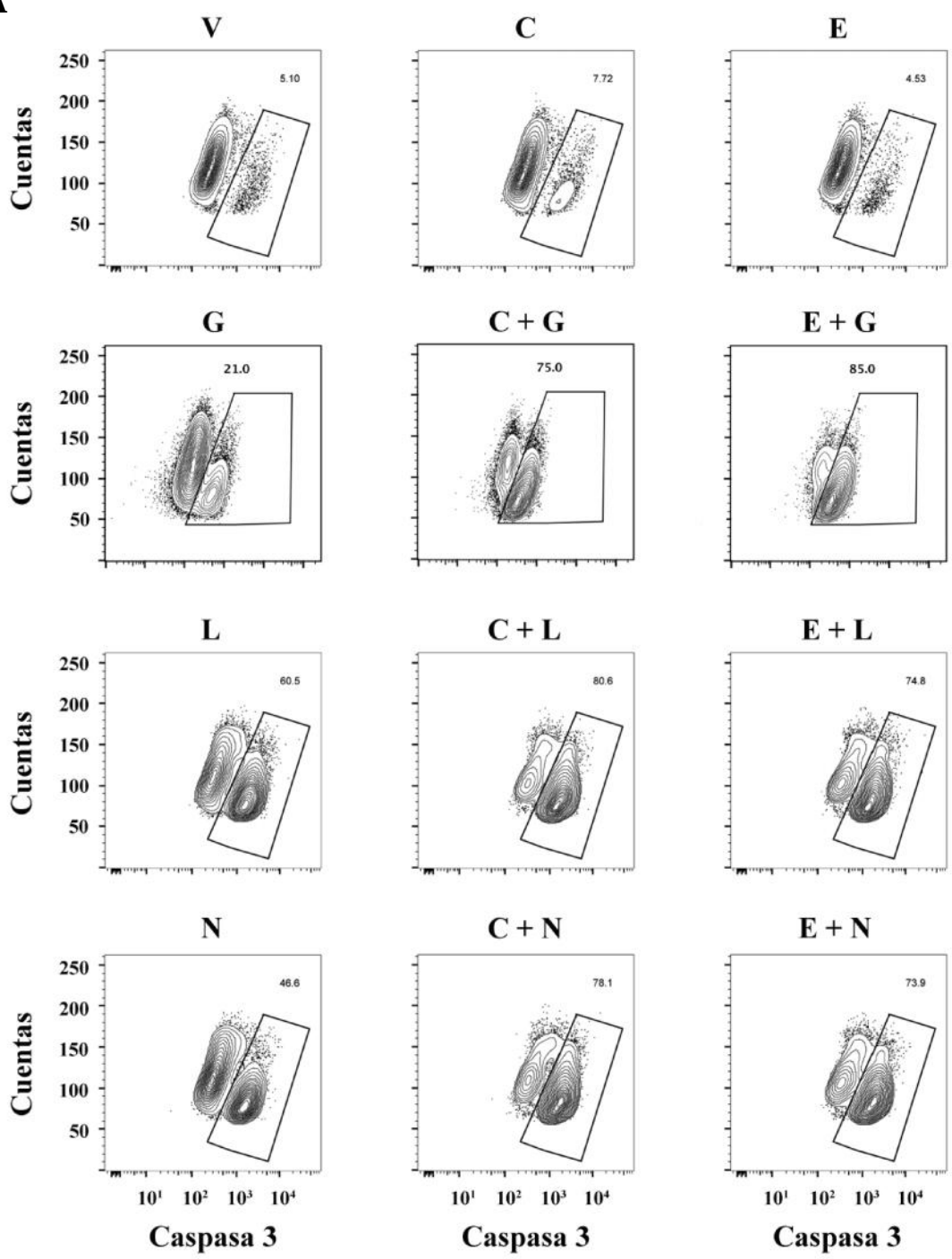
Fig. 14. La combinación del calcitriol o el EB1089 con los ITC indujo cambios en el perfil del ciclo celular de células de cáncer de mama. Las células SUM-229PE fueron incubadas en ausencia (V) o presencia de su correspondiente valor de CI_{50} del calcitriol (C), el EB1089 (E), el gefitinib (G), el lapatinib (L), el neratinib (N) o la combinación de los compuestos por 96 h. El contenido de DNA del ciclo celular fue analizado. Los histogramas muestran el perfil del ciclo celular de las células tratadas con los compuestos solos o en combinación. El pico SubG1 está indicado con una línea horizontal y la reducción de la fase G2/M está señalada con una flecha. Los histogramas son representativos de 3 experimentos de manera independiente.

Tabla 5. Porcentaje de las células SUM-229PE en las diferentes fases del ciclo celular posterior a los tratamientos del gefitinib, el lapatinib o el neratinib con el calcitriol o el EB1089 solos o en combinación

Tratamiento	Fases del ciclo celular			
	SubG1	**G1**	**S**	**G2/M**
V	10.7±6.1	34.0±2.6	39.7±1.9	15.0±1.6
C	9.7±4.4 [a]	42.7±5.5	35.1±2.9	13.2±3.0
E	14.6±7.2 [a]	59.5±10.8	18.5±3.8 [a]	7.9±1.2 [a]
G	14.3±0.2 [a]	60.2±6.0 [a]	15.3±3.2a	10.0±2.0 [a]
C+G	27.8±7.3a,b	61.6±7.4 [a]	19.1±4.4 [a]	2.7±3.2 [a,b]
E+G	38.7±0.8 [a,b]	67.0±5.7[a]	14.1±4.2[a]	0.3±0.1[a,b]
L	62.5±0.0 [a]	29.4±2.3	10.6±0.3 [a]	2.0±2.8 [a]
C+L	86.2±3.3 [a,b]	13.4±3.9 [a,b]	0.2±0.0 [a,b]	0.0±0.0 [a]
E+L	98.5±0.2 [a,b]	1.4±0.1 [a,b]	0.0±0.0 [a,b]	0.0±0.0 [a]
N	31.8±9.8 [a]	36.8±0.1	23.6±5.0 [a]	7.1±4.7 [a]
C+N	98.7±0.2 [a,b]	11.3±3.8 [a,b]	3.9±5.0 [a,b]	0.3±0.0 [a,b]
E+N	95.3±5.0 [a,b]	0.6±0.5 [a,b]	3.9±5.0 [a,b]	0.0±0.0 [a,b]

V, vehículo; C, calcitriol; E, EB1089; G, gefitinib; L, lapatinib; N, neratinib. Los resultados están expresados como la media ± D. E. de 3 experimentos de manera independiente. [a] $p < 0.001$ vs. V, [b] $p < 0.001$ vs. cada compuesto solo.

A


V
C
E
G
C + G
E + G
L
C + L
E + L
N
C + N
E + N
5.10
7.72
4.53
21.0
75.0
85.0
60.5
80.6
74.8
46.6
78.1
73.9
Cuentas
250
200
150
100
50
10^1 10^2 10^3 10^4
Caspasa 3

B

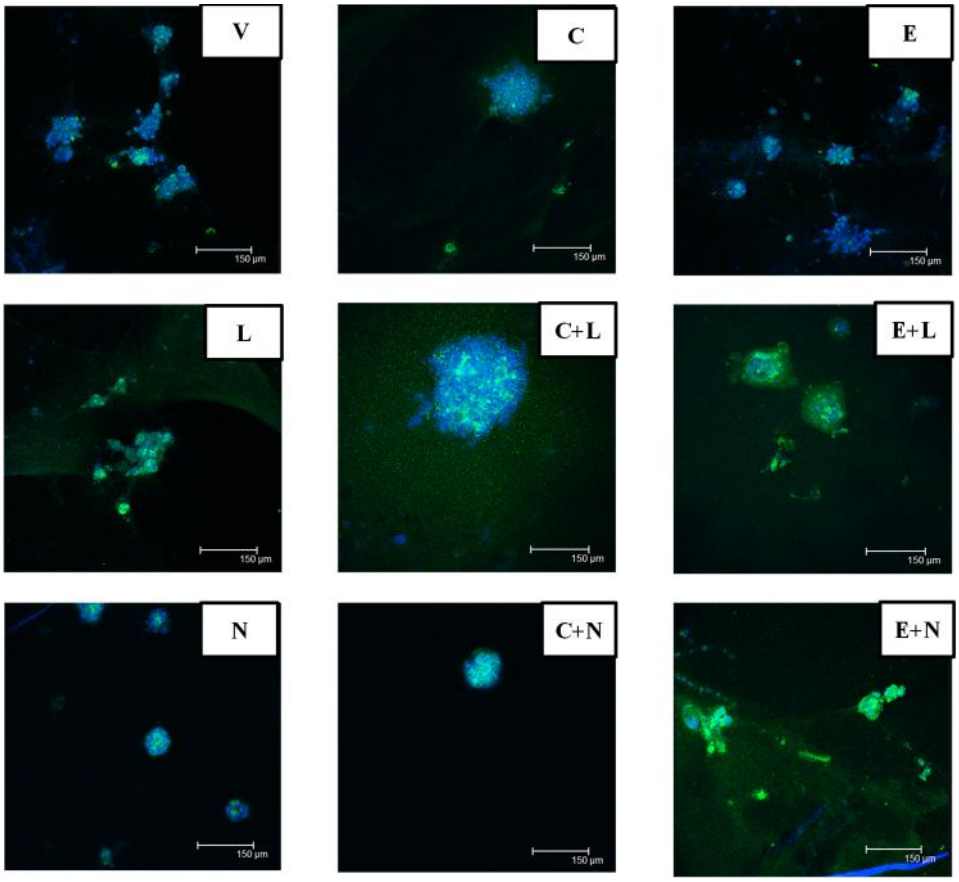


Fig. 15. La combinación del calcitriol o el EB1089 con el gefitinib, el lapatinib y el neratinib incrementó la caspasa 3 activa en células de cáncer de mama. A) Las células SUM-229PE fueron incubadas en la ausencia (V) o presencia del calcitriol (C), el EB1089 (E), el gefitinib (G), el lapatinib (L), y el neratinib (N) solos o en la combinación por 72 h con su correspondiente valor de CI_{50}. Las células fueron permeadas, fijadas y teñidas con un anticuerpo anti-caspasa 3 activa. Posteriormente, las células fueron analizadas por citometría de flujo y las células positivas a la caspasa 3 son mostradas en el recuadro. Las imágenes son representativas de 3 experimentos de manera independiente. **B)** Las células SUM-229PE fueron crecidas en presencia de matrigel, posterior a 7 días de cultivo fueron tratadas con los compuestos por 48 h. Las células fueron fijadas, permeadas y teñidas con DAPI y con un anticuerpo anti-caspasa 3. Posteriormente, se adquirieron imágenes con un microscopio confocal. Las imágenes son representativas de 2 experimentos de manera independiente.

15.7 La combinación del calcitriol o el EB1089 con el gefitinib, el lapatinib o el neratinib disminuyó en mayor medida la fosforilación de las proteínas ERK y Akt

Con el fin de dilucidar los posibles mecanismos implicados en los efectos antiproliferativos de las combinaciones de fármacos se evaluaron dos vías de señalización activadas por la familia EGFR, ERK y Akt y que están implicadas en procesos de proliferación y metabolismo celular.

Los tratamientos combinados del calcitriol o el EB1089 con el gefitinib disminuyeron la fosforilación de la vía de señalización ERK 1 y 2 en la línea celular SUM-229PE, **Fig. 16.** Adicionalmente, las combinaciones del calcitriol o el EB1089 con el lapatinib y el neratinib inhibieron la fosforilación de las proteínas Akt y ERK en esta misma línea celular, sin embargo, el efecto inhibitorio de estos dos últimos inhibidores de residuos de tirosina fue más marcado que el efecto inhibitorio del gefitinib, **Fig. 17.**

De manera notable, la fosforilación de estas proteínas fue disminuida con los tratamientos combinados del calcitriol o el EB1089 con el lapatinib o el neratinib en la línea celular MDA-MB-231, la cual resultó ser menos sensible a los compuestos de manera independiente, **Fig. 17**.

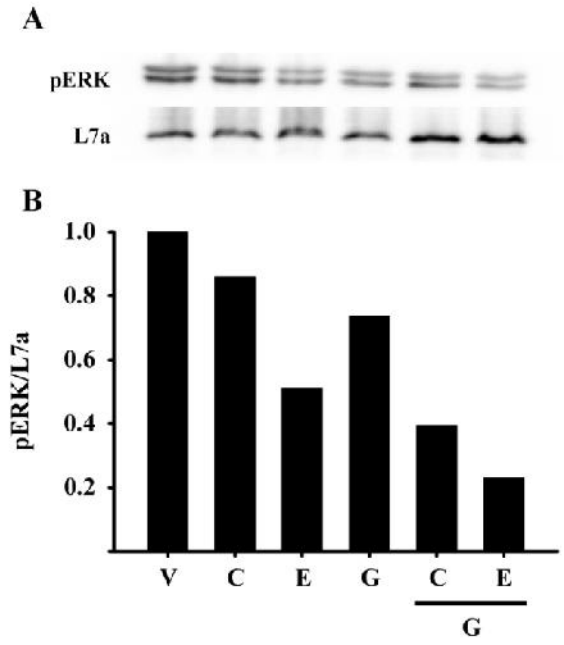


Fig. 16. La combinación del calcitriol con el gefitinib inhibió la fosforilación de ERK 1/2 en células de cáncer de mama. Las células SUM-229PE fueron incubadas en ausencia (V) o presencia del calcitriol (C), el EB1089 (E), el gefitinib (G), solos o en combinación durante 48 horas. Posteriormente, los análisis de Western blot fueron realizados para detectar la forma fosforilada de la proteína ERK 1/2 (Thr202/Tyr204) y la proteína ribosomal L7 fue utilizada como control de carga (A). Los valores de las células tratadas con el vehículo fueron llevados a 1. La normalización de los valores de cada tratamiento se realizó con respecto a la proteína L7 (B). Se muestra la imagen representativa y la densitometría de dos experimentos de manera independiente.

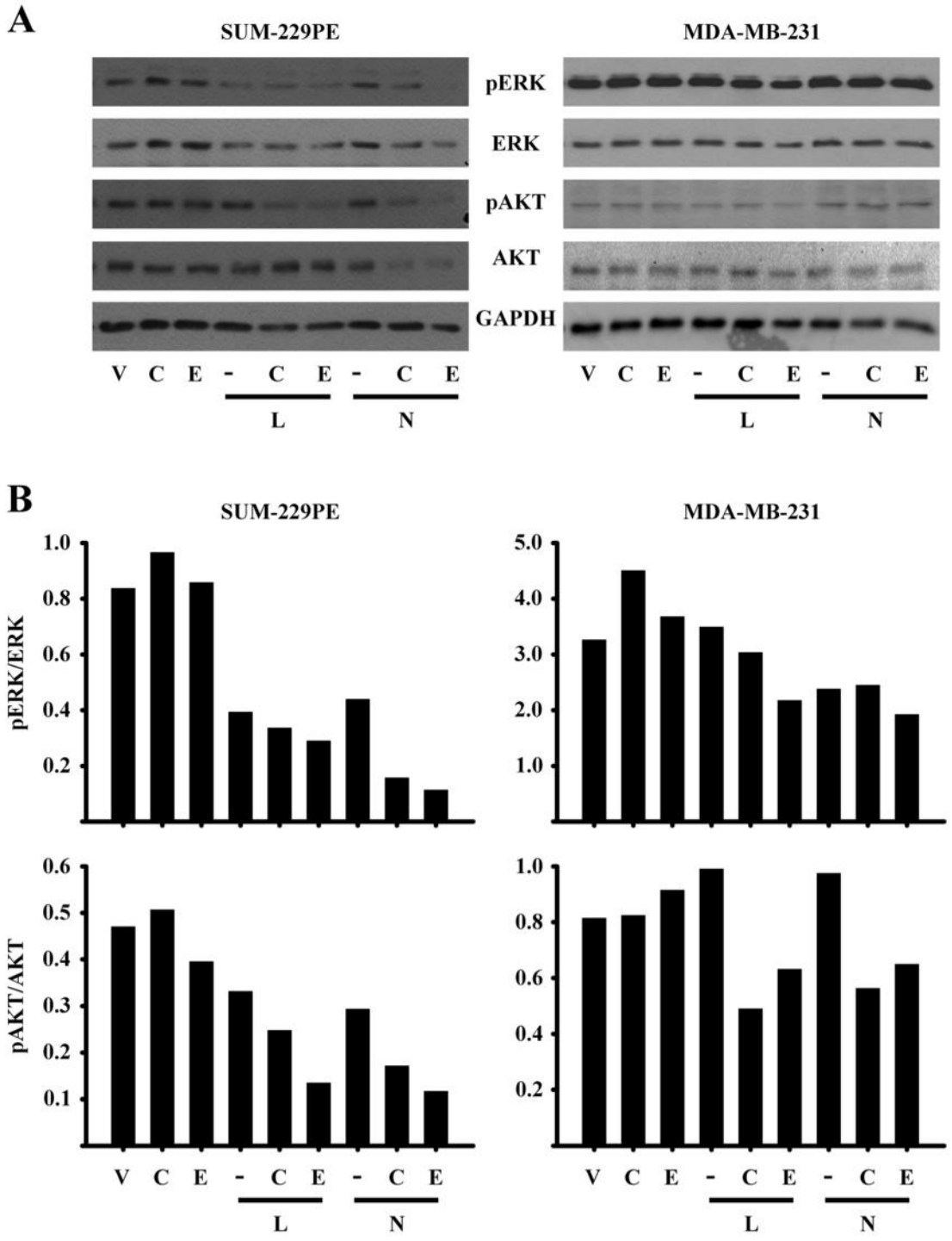


Fig. 17. La combinación del calcitriol o el EB1089 con el lapatinib o el neratinib inhibió la fosforilación de las proteínas ERK ½ y Akt en células de cáncer de mama. (**A**) Las células SUM-229PE y las células MDA-MB-231 fueron incubadas en ausencia (V) o presencia del calcitriol (C), el EB1089 (E), el lapatinib (L), el neratinib (N) solos o en combinación durante 72 h. Posteriormente, los análisis de Western blot fueron realizados para detectar la forma fosforilada de las proteínas ERK 1/2 y Akt, la proteína gliceraldehído 3-fosfato deshidrogenasa (GAPDH) fue

utilizada como control de carga. Los valores de las células tratadas con el vehículo fueron llevados a 1. (**B**) La normalización de los valores de cada tratamiento se realizó con respecto a la proteína total de ERK o Akt. Se muestra la imagen representativa y la densitometría de tres (SUM-229PE) y dos (MDA-MB-231) experimentos de manera independiente.

15.8 El tratamiento combinado del calcitriol o el EB1089 con el lapatinib o el neratinib inhibió la capacidad clonogénica de células de cáncer de mama

Para evaluar la capacidad de las células SUM-229PE para dividirse y formar colonias posterior al tratamiento con los antineoplásicos se llevaron a cabo ensayos de clonogenicidad celular en agar suave.

La **Fig. 18** muestra a las células SUM-229PE expuestas a los diferentes tratamientos solos o en combinación. La formación de colonias no se vio afectada en las células tratadas con el vehículo. En contraste, en las células tratadas con los compuestos individuales (calcitriol, EB1089, lapatinib y neratinib) la clonogenicidad se vio inhibida drásticamente. De manera interesantemente, la formación de colonias en las células expuestas a los tratamientos combinados fue abatida completamente.

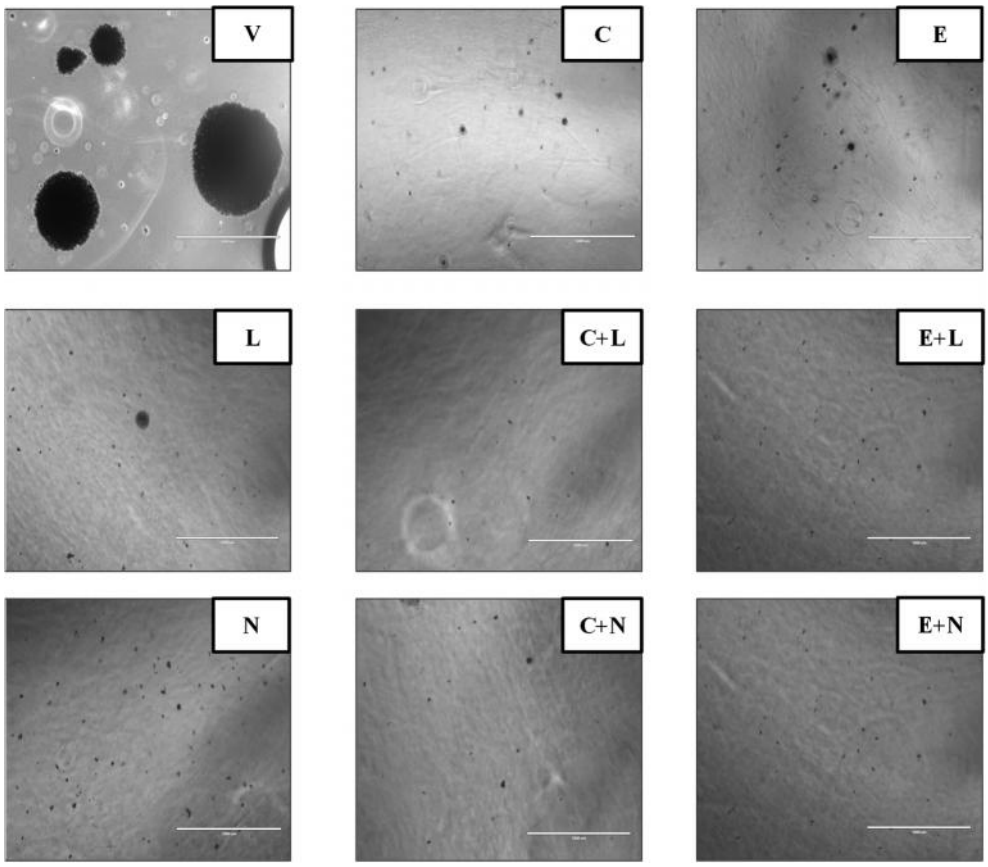


Fig. 18. **La combinación del calcitriol o el EB1089 con el lapatinib o el neratinib inhibió la capacidad clonogénica de células de cáncer de mama.** Las células SUM-229PE fueron sembradas en agar suave, posteriormente, las células fueron incubadas con los tratamientos solos o en combinación por 30 días, y cada 7 días se realizó un recambio de medio con su respectivo tratamiento. Las colonias fueron teñidas con cristal violeta. Se muestran imágenes representativas de dos experimentos de manera independiente.

16. DISCUSIÓN

Pacientes con tumores que sobreexpresan al EGFR y/o al HER2 tienen peor pronóstico, comportamiento clínico agresivo y resistencia a los tratamientos convencionales [133-135]. Por ello, la inhibición de los miembros de la familia EGFR resulta en una estrategia terapéutica importante en cáncer de mama y en otros tipos de tumores [136-138]. Un ejemplo de ello es el uso del lapatinib en pacientes con cáncer de mama HER2 positivo, el cual actualmente está aprobado como terapia de segunda línea [139]. A este respecto, el gefitinib y el neratinib también han mostrado reducir la proliferación de células de cáncer de mama que expresan diferentes niveles de EGFR o HER2, a través de inhibir la actividad cinasa de estos receptores [43, 74, 77, 140].

Por otra parte, el calcitriol ha sido considerado como un potente inhibidor del crecimiento de células de cáncer de mama independientemente de su fenotipo molecular en modelos *in vitro* e *in vivo* [141-144]. Además, los efectos antiproliferativos de este compuesto se han evaluado en varios tratamientos combinados con múltiples agentes antineoplásicos en fases preclínicas y clínicas, dando como resultado un aumento en la actividad antitumoral de los mismos [145].

El principal interés de esta investigación fue evaluar el efecto antiproliferativo e inductor de apoptosis de los principales inhibidores de residuos de tirosina cinasa (gefitinib, lapatinib y neratinib) mayormente estudiados en cáncer de mama EGFR o HER2 positivo en combinación con el metabolito activo de la vitamina D (calcitriol) y dos análogos sintéticos (EB1089 y calcipotriol). En este trabajo se demostró que la combinación del calcitriol o dos análogos sintéticos con el gefitinib, el lapatinib o el neratinib resultó en un mejor efecto antineoplásico en comparación con los compuestos de manera independiente en diversos fenotipos tumorales de cáncer de mama [146], lo cual sustenta trabajos previos en donde la adición del calcitriol en esquemas de terapia combinada potencia la actividad antineoplásica de varios compuestos [145].

En lo referente a los efectos inhibitorios del gefitinib en los cultivos celulares, los resultados mostraron que células de cáncer de mama con diferentes fenotipos, es decir, la línea SUM-229PE, la cual tiene sobreexpresión del EGFR, pero no del HER2 y la línea SK-BR-3, con sobreexpresión del HER2; fueron igualmente sensibles a este compuesto basado en su valor de

CI_{50}, el cual fue el mismo para ambas líneas celulares. Estos resultados sugieren que el potencial clínico del gefitinib no se limita a tumores con sobreexpresión del EGFR, como es el caso de cáncer de pulmón no microcítico donde este fármaco ya es terapia aprobada [147]. Así mismo, estos resultados podrían representar una nueva modalidad terapéutica en el tratamiento de pacientes con tumores EGFR y HER2 positivos, lo cual es consistente con reportes previos [77, 78].

En este trabajo, también se comparó el efecto del lapatinib y el neratinib en la proliferación de células de cáncer de mama con diferentes fenotipos. De manera general, los resultados mostraron que el neratinib fue más potente que el lapatinib y el gefitinib para inhibir la proliferación celular, lo cual concuerda con trabajos previos. Así mismo, la relación de potencia entre el neratinib y el lapatinib ya ha sido probada en células de cáncer de mama que sobreexpresan al HER2, donde se concluyó que la presencia de esta proteína determina la sensibilidad de células de cáncer de mama a estos dos compuestos [148]. Este efecto se vio reflejado en los resultados mostrados en este trabajo ya que las células SK-BR-3 fueron mayormente sensibles a ambos compuestos. Adicionalmente, los valores de las CI_{50} del gefitinib, el lapatinib y el neratinib obtenidos en este trabajo en las diferentes líneas celulares evaluadas son similares a los reportados en otros estudios, lo que asegura la reproducibilidad de los datos [78, 148].

En lo que respecta a los efectos del calcitriol y sus análogos en la proliferación celular, este estudio mostró que las líneas de cáncer de mama estudiadas presentaron diferencias en la sensibilidad al calcitriol o a sus análogos. Las líneas más sensibles al calcitriol y al EB1089 fueron SUM-229PE y SK-BR-3. Es importante mencionar que los valores de las CI_{50} de los compuestos obtenidos en las células de cáncer de mama con fenotipo EGFR o HER2 positivo fueron muy similares a los reportados en trabajos previos en células de cáncer de mama con fenotipo hormonal, sugiriendo que el efecto antiproliferativo del calcitriol y sus análogos es independiente de la presencia de los receptores hormonales; estrógeno y progesterona [149].

Por otro lado, las líneas menos sensibles al calcitriol y sus análogos fueron HCC1937 y MDA-MB-231, las cuales presentan fenotipo triple negativo, el cual es pobremente diferenciado y que se ha reportado que la expresión del VDR puede perderse más que en otros fenotipos [150, 151]. A este respecto, muy pocos trabajos han evaluado el efecto del calcitriol en este

fenotipo molecular de cáncer de mama. De hecho, nuestro datos concuerdan con Peng, et al., quienes demostraron que la línea MDA-MB-231 no es sensible a los efectos antiproliferativos del calcitriol [152].

En lo que respecta a las combinaciones del calcitriol con los inhibidores de residuos de tirosina, existen únicamente dos estudios fase I que han evaluado el efecto del calcitriol en combinación con el gefitinib. Estos estudios fueron enfocados a determinar las dosis máximas toleradas de la combinación de estos fármacos por vía intravenosa en paciente con tumores sólidos, incluyendo únicamente un paciente con cáncer de mama. La conclusión de estos estudios fue que la combinación del calcitriol con el gefitinib no estaba asociada con el incremento en la sobrevida del paciente, sin embargo, el fenotipo molecular de los tumores no fue caracterizado [115, 153]. Considerando que los blancos moleculares del gefitinib son el EGFR y el HER2 [43, 77] en este trabajo se utilizaron líneas celulares que tuvieran la expresión de estos receptores para estudiar los efectos de dicha combinación.

Por otro lado, trabajos anteriores han reportado que la administración independiente del calcitriol, del EB1089 o de diversos inhibidores de residuos de tirosina cinasa inhiben la proliferación e inducen apoptosis en células de cáncer de mama [43, 78, 154-156], sin embargo, cuando son co-administrados con otros tipo de compuestos como antineoplásicos, inhiben mayormente la proliferación celular [78, 141, 157, 158], indicando que la terapia combinada tiene mejores resultados que la monoterapia. A este respecto, la co-administración del calcitriol o el EB1089 con el gefitinib, el lapatinib o el neratinib inhibió mayormente la proliferación celular en comparación con los compuestos administrados de manera independientemente en todas las líneas de cáncer de mama utilizadas.

Cabe destacar que a pesar de que el cultivo primario, así como la línea MDA-MB-231 mostraron resistencia *de novo* a los compuestos, cuando las células fueron expuestas a las diferentes combinaciones de fármacos la resistencia a los agentes fue revertida. Este evento podría deberse a la capacidad del calcitriol para inducir diferenciación celular [159] y así las células se sensibilizan a los efectos inhibitorios de los ITC.

La mayoría de las combinaciones probadas en este trabajo mostraron tener un efecto sinérgico para inhibir la proliferación celular. De manera notable, la línea celular SUM-229PE fue la

más sensible a la combinación de los ITC con el calcitriol o el EB1089, con respecto a las otras líneas celulares utilizadas. Es importante destacar que las concentraciones utilizadas de todos los compuestos probados son alcanzables terapéuticamente en los niveles séricos en sangre humana, como se menciona en estudios previos [115, 153, 160-163]. Los esquemas de combinación probados en esta investigación podrían participar en la reducción de los efectos secundarios de los inhibidores de tirosina, debido a que la adición del calcitriol o su análogo al esquema de terapia combinada permite el uso de concentraciones bajas de los inhibidores de residuos de tirosina en el ámbito terapéutico, pero con potenciación de su efecto antineoplásico.

Adicionalmente, los resultados mostraron que el tratamiento combinado del calcitriol o el EB1089 con el gefitinib disminuyó significativamente la expresión génica del EGFR en la línea celular SUM-229PE. A este respecto, se ha reportado que la región promotora del EGFR posee elementos de respuesta a vitamina D [38] y que el calcitriol decrece las proteínas de unión del ligando (EGF) del EGFR en células de cáncer de mama [26]. Los resultados obtenidos sugieren que la combinación del calcitriol o el EB1089 con el gefitinib tiene como blanco molecular al EGFR, a través de disminuir su expresión génica y de bloquear su señalización. Con respecto a la inhibición de la señalización del EGFR y HER2, se ha descrito que el gefitinib, el lapatinib o el neratinb, son capaces de inhibir la fosforilación de las proteínas ERK y Akt [43, 52, 65, 78, 140, 164].

En este trabajo se observó que el tratamiento combinado de los ITC con el calcitriol o su análogo EB1089 disminuyó en mayor medida la fosforilación de las proteínas ERK y Akt en comparación con los tratamientos individuales en las líneas SUM-229PE y MDA-MB-231, que se caracterizan por sobreexpresar al EGFR. Los resultados anteriores notablemente sugieren que cualquiera de los esquemas combinados del calcitriol o el EB1089 con el gefitinib, el lapatinib o el neratinib podría ser útil para la terapia de pacientes con cáncer de mama que tenga sobreexpresión del EGFR.

El bloqueo de la señalización del EGFR y el HER2 por gefitinib y el lapatinib induce al mediador de apoptosis BIM [53, 165, 166]. Además, el incremento de este marcador se ha asociado con la prevención de procesos como metástasis y quimioresistencia [167]. La administración conjunta del calcitriol o el EB1089 con el gefitinib indujo en mayor medida la

expresión génica y proteica de BIM en comparación con la administración del gefitinib de manera independiente en la línea celular SUM-229PE. Además, el marcador anti-apoptótico Bcl-2 esta sobreexpresado en muchos tipos de cáncer y contribuye a la iniciación tumoral, progresión y resistencia a la terapia [168, 169]. En este sentido, el tratamiento combinado del gefitinib con el calcitriol o el EB1089 inhibió la expresión de este marcador en la línea SUM-229PE. Estos resultados sugieren que el tratamiento combinado podría participar en la prevención de procesos metastásicos y de resistencia a la terapia.

Debido a la participación de Bcl-2 y de BIM en la activación de la apoptosis intrínseca, la inducción de la forma activa de la caspasa 3 y las modificaciones en el perfil del ciclo celular fueron evaluadas en la línea celular SUM-229PE [170, 171]..

El gefitinib, el lapatinib y el neratinib *per se* incrementaron el pico SubG1 y disminuyeron el porcentaje de células en la fase de síntesis y mitosis, efectos que ya han sido reportados en estudios previos [77, 172]. Sin embargo, la adición del calcitriol o su análogo a estos inhibidores incrementaron notablemente el porcentaje de fragmentos celulares correlacionado con el aumento del porcentaje de células positivas a la caspasa 3. Cabe resaltar que en los esquemas de combinación donde se utiliza el EB1089 se encontró el mayor porcentaje de muerte celular. Esto podría deberse a que el análogo por si solo es más potente que el calcitriol para inducir apoptosis en células de cáncer de mama, como previamente se ha reportado [173]. Este resultado sustenta el uso de compuestos análogos de la vitamina D como adyuvantes en el tratamiento del cáncer debido a sus efectos antineoplásicos y bajos efectos calcémicos. Posteriores investigaciones en modelos *in vivo* serían de gran relevancia para corroborar este efecto.

En lo que respecta a los resultados en la línea celular SK-BR-3, la cual caracteriza al fenotipo HER2 positivo, los resultados mostraron que el tratamiento independiente con el gefitinib, el lapatinib o el neratinib tuvo una buena respuesta para inhibir la proliferación celular, además, cualquiera de los esquemas de combinación inhibió mayormente la proliferación celular en comparación con los tratamientos de manera independiente. Adicionalmente, las combinaciones inhibieron la expresión del gen Bcl-2 e incrementaron la expresión del gen FOXO3a, lo cual se ha atribuido a una mejor respuesta en la terapia [169, 174].

Es la primera vez que se reporta que el calcitriol o el EB1089 disminuyeron drásticamente la capacidad clonogénica celular en cáncer de mama EGFR o HER2 positivo. Estos resultados permiten generar nuevas hipótesis de investigación de estos compuestos para frenar la invasividad de células tumorales. Los tratamientos del lapatinib y el neratinib también disminuyeron la formación de colonias y sobresalientemente, la capacidad clonogénica fue inhibida completamente en la administración conjunta de los antineoplásicos. De acuerdo a reportes previamente publicados podría sugerirse que los tratamientos combinados probados en este trabajo inhiben la carcinogénesis celular, una característica conocida dentro de las marcas del cáncer [175]. Así mismo, la capacidad de una célula para formar colonias en este tipo de ensayos se ha asociado importantemente con el potencial de las células cancerosas para causar una recaída posterior al tratamiento en modelos *in vivo* [176]. Por lo que, posteriores investigaciones en modelos *in vivo* serían de gran relevancia para corroborar este efecto. Así mismo, la búsqueda de diversos mecanismos moleculares de la actividad antineoplásica del calcitriol o su análogo en combinación con los inhibidores de residuos de tirosina cinasa es necesaria para optimizar las aplicaciones clínicas de estos compuestos en cáncer de mama con sobreexpresión en el EGFR.

Adicionalmente, de este trabajo surgen nuevas propuestas de investigación sobre los mecanismos implicados del calcitriol o su análogo EB1089 en combinación con los ITC para entender las diferentes vías involucradas en la inhibición sinérgica de la proliferación celular y también para prevenir la resistencia adquirida a estos fármacos. En este sentido, el calcitriol ha mostrado inhibir la expresión proteica de la subunidad alfa del factor inducido por hipoxia (HIF-1α) [177], la cual está relacionada con la adaptación metabólica, la progresión celular y la resistencia al lapatinib [178]. El calcitriol también puede inhibir la expresión génica de diversos receptores, incluidos aquellos con actividad tirosina cinasa, así como sus ligandos, los cuales están relacionados con los procesos de generación de resistencia adquirida a los ITC [179-181]. Algunos ejemplos de blancos moleculares inhibidos por el calcitriol son; el receptor del factor de crecimiento de hepatocitos (c-MET), el cual está asociado en procesos de migración, invasión y proliferación celular [182, 183], los receptores Notch, los cuales promueven la progresión y procesos de metástasis en cáncer [184], el ligando del IGFR-1, el cual participa en el crecimiento celular de tumores y la inhibición de apoptosis [185]. Además, el calcitriol inhibe la acción de las metaloproteínas, las cuales son enzimas que favorecen la

invasión celular y metástasis, a través de degradar la matriz extracelular [186]. Adicionalmente, el calcitriol tiene importantes acciones como inmunomodulador en diversas estirpes celulares del sistema inmune y también inhibe la expresión de interleucinas inflamatorias las cuales favorecen procesos patológicos como el cáncer [187]. Futuros estudios sobre estos y otros mecanismos serían de relevancia terapéutica para dar soporte al uso del calcitriol en esquemas combinados con los ITC en cáncer de mama.

17. CONCLUSIONES

El tratamiento simultáneo del calcitriol o el EB1089 con el gefitinib, el lapatinib o el neratinib en células de cáncer de mama con EGFR y/o HER2 positivo mostró ser significativamente mejor para inhibir la proliferación e inducir apoptosis. Estos efectos fueron mediados por la modulación de las vías PI3K/Akt y MAPK, así como el incremento de la proteína BIM y la inhibición de Bcl-2, lo cual resultó en la activación de la vía apoptótica intrínseca regulada por la caspasa 3 activa, **Fig 19**.

El tratamiento combinado del lapatinib o el neratinib con el calcitriol o el EB1089 inhibió por completo la formación de colonias celulares.

Los diferentes esquemas de combinación probados en este trabajo podrían ser una alternativa terapéutica para pacientes con cáncer de mama EGFR y/o HER2 positivo, los cuales tienen pocas opciones de tratamiento.

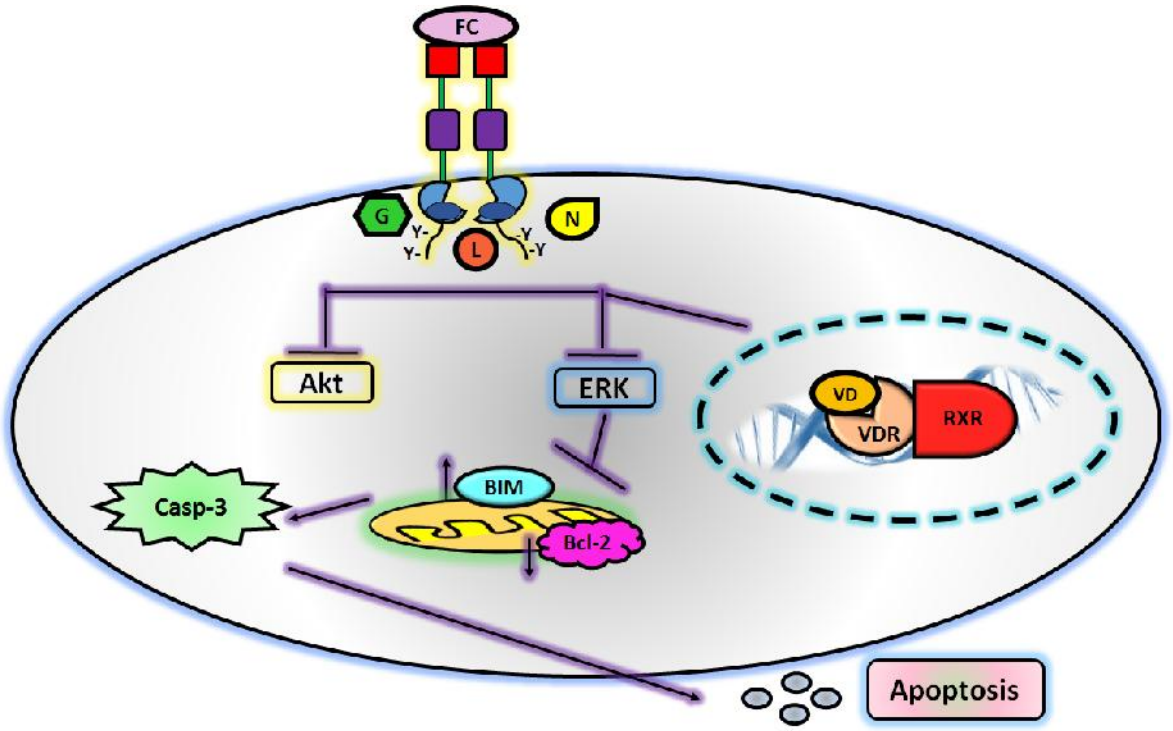


Fig. 19. **Mecanismo de acción de la combinación del calcitriol o el EB1089 con el gefitinib, el lapatinib o el neratinib en células de cáncer de mama.** Posterior a la unión de sus ligandos, los factores de crecimiento (FC), los miembros de la familia EGFR forman heterodímeros y se fosforilan en sus residuos de tirosina, lo que resulta en la activación de vías de señalización rio abajo. Sin embargo, debido a su pequeño tamaño los inhibidores de residuos de tirosina cinasa (gefitinib (G), lapatinib (L) o neratinib (N)) atraviesan la membrana celular y se une a su correspondiente blanco molecular. El calcitriol o el EB1089 (VD), por su carácter lipofílico, difunden la membrana celular y se unen a su receptor nuclear (VDR). La acción del tratamiento combinado bloquea la fosforilación de las vías MAPK y AKT lo que resulta en el incremento de la expresión del marcador pro-apoptótico BIM y la inhibición de la expresión del marcador anti-apoptótico, Bcl-2. Estos efectos incrementan la caspasa 3 activa, con la consecuente inducción de la apoptosis e inhibición de la capacidad clonogénica celular.

18. PESPECTIVAS

* Indagar si el efecto del calcitriol y el EB1089 es a través de su receptor específico usando un antagonista del VDR.

*Determinar el efecto del calcitriol o su análogo EB1089 en un modelo de tratamiento neoadyuvante y adyuvante en cáncer de mama.

* Investigar si el calcitriol o el EB1089 pueden frenar o revertir la generación de resistencia adquirida a los inhibidores de residuos de tirosina cinasa en cáncer de mama.

* Evaluar el efecto de estas combinaciones en modelos *in vivo*.

19. LITERATURA CITADA

1. http://globocan.iarc.fr/factsheets/cancers/all.asp, (Globocan 2011).
2. Society, A.C., *Cancer Facts and Figures 2016.* Atlanta: American Cancer Society, 2016.
3. www.inegi.org.mx/inegi/contenidos/espanol/estadisticas/2011/cancer.doc). *Instituto Nacional de Estadística y Geografía (INEGI). Estadísticas a propósito del día mundial contra el cáncer. Datos nacionales. .* 2011.
4. Imtiaz, S., et al., *Vitamin D deficiency in newly diagnosed breast cancer patients.* Indian J Endocrinol Metab, 2012. **16**(3): p. 409-13.
5. Bauer, S.R., et al., *Plasma vitamin D levels, menopause, and risk of breast cancer: dose-response meta-analysis of prospective studies.* Medicine (Baltimore), 2013. **92**(3): p. 123-31.
6. Kim, Y., et al., *Plasma 25-hydroxyvitamin D3 is associated with decreased risk of postmenopausal breast cancer in whites: a nested case-control study in the multiethnic cohort study.* BMC Cancer, 2014. **14**: p. 29.
7. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
8. Berger, U., et al., *Immunocytochemical determination of estrogen receptor, progesterone receptor, and 1,25-dihydroxyvitamin D3 receptor in breast cancer and relationship to prognosis.* Cancer Res, 1991. **51**(1): p. 239-44.
9. Edwards, B.J., et al., *Elevated incidence of fractures in women with invasive breast cancer.* Osteoporos Int, 2016. **27**(2): p. 499-507.
10. Garcia-Becerra, R., et al., *Calcitriol inhibits Ether-a go-go potassium channel expression and cell proliferation in human breast cancer cells.* Exp Cell Res, 2010. **316**(3): p. 433-42.
11. Santagata, S., et al., *Taxonomy of breast cancer based on normal cell phenotype predicts outcome.* J Clin Invest, 2014. **124**(2): p. 859-70.
12. Hussain, E.A., *Efficacy and mechanism of action of 1alpha-hydroxy-24-ethyl-cholecalciferol (1alpha[OH]D5) in breast cancer prevention and therapy.* Recent Results Cancer Res., 2003. **164**: p. 393–411.
13. Kulkarni, A.D., et al., *Use of combination therapy with cisplatin and calcitriol in the treatment of Y-79 human retinoblastoma xenograft model.* Br J Ophthalmol, 2009. **93**(8): p. 1105-8.
14. Christensen, G.L., et al., *Sequential versus combined treatment of human breast cancer cells with antiestrogens and the vitamin D analogue EB1089 and evaluation of predictive markers for vitamin D treatment.* Breast Cancer Res Treat, 2004. **85**(1): p. 53-63.
15. Apetoh, L., et al., *Cancer is not just a disease of a tissue: it is a host disease. How to reactivate host defense against tumors using conventional therapies of cancer?* Ann Endocrinol (Paris), 2008. **69**(2): p. 151-2.
16. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
17. (OMS)., O.M.d.l.S., *Cáncer. Nota descriptiva No. 297.* (http://www.who.int/mediacentre/factsheets/fs297/es/index.html), 2011. .
18. INEGI, www.inegi.org.mx/inegi/contenidos/espanol/.../2010/cancer10.doc, 2010.
19. Jha, K., M. Shukla, and M. Pandey, *Survivin expression and targeting in breast cancer.* Surg Oncol, 2012. **21**(2): p. 125-31.
20. Bosch, A., et al., *Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research.* Cancer Treat Rev, 2010. **36**(3): p. 206-15.

21. Haibe-Kains, B., et al., *Comparison of prognostic gene expression signatures for breast cancer.* BMC Genomics, 2008. **9**: p. 394.
22. Wolff, A.C., et al., *American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer.* J Clin Oncol, 2007. **25**(1): p. 118-45.
23. Gassmann, M., et al., *Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor.* Nature, 1995. **378**(6555): p. 390-4.
24. Sibilia, M. and E.F. Wagner, *Strain-dependent epithelial defects in mice lacking the EGF receptor.* Science, 1995. **269**(5221): p. 234-8.
25. Roepstorff, K., et al., *Endocytic downregulation of ErbB receptors: mechanisms and relevance in cancer.* Histochem Cell Biol, 2008. **129**(5): p. 563-78.
26. Shi, F., et al., *ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation.* Proc Natl Acad Sci U S A, 2010. **107**(17): p. 7692-7.
27. Yarden, Y., *The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities.* Eur J Cancer, 2001. **37 Suppl 4**: p. S3-8.
28. Yarden, Y. and M.X. Sliwkowski, *Untangling the ErbB signalling network.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 127-37.
29. Wada, T. and J.M. Penninger, *Mitogen-activated protein kinases in apoptosis regulation.* Oncogene, 2004. **23**(16): p. 2838-49.
30. Kim, E.K. and E.J. Choi, *Pathological roles of MAPK signaling pathways in human diseases.* Biochim Biophys Acta, 2010. **1802**(4): p. 396-405.
31. Broek, R.V., et al., *The PI3K/Akt/mTOR axis in head and neck cancer: functions, aberrations, crosstalk, and therapies.* Oral Dis, 2013.
32. McCubrey, J.A., et al., *Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance.* Adv Enzyme Regul, 2006. **46**: p. 249-79.
33. Meche, A., A.M. Cimpean, and M. Raica, *Immunohistochemical expression and significance of epidermal growth factor receptor (EGFR) in breast cancer.* Rom J Morphol Embryol, 2009. **50**(2): p. 217-21.
34. Rimawi, M.F., et al., *Epidermal growth factor receptor expression in breast cancer association with biologic phenotype and clinical outcomes.* Cancer, 2010. **116**(5): p. 1234-42.
35. Massarweh, S. and R. Schiff, *Resistance to endocrine therapy in breast cancer: exploiting estrogen receptor/growth factor signaling crosstalk.* Endocr Relat Cancer, 2006. **13 Suppl 1**: p. S15-24.
36. Park, H.S., et al., *High EGFR gene copy number predicts poor outcome in triple-negative breast cancer.* Mod Pathol, 2014. **27**(9): p. 1212-22.
37. Changavi, A.A., A. Shashikala, and A.S. Ramji, *Epidermal Growth Factor Receptor Expression in Triple Negative and Nontriple Negative Breast Carcinomas.* J Lab Physicians, 2015. **7**(2): p. 79-83.
38. Di Fiore, P.P., et al., *Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells.* Cell, 1987. **51**(6): p. 1063-70.
39. Corkery, B., et al., *Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer.* Ann Oncol, 2009. **20**(5): p. 862-7.
40. Ueno, N.T. and D. Zhang, *Targeting EGFR in Triple Negative Breast Cancer.* J Cancer, 2011. **2**: p. 324-8.
41. Crown, J., J. O'Shaughnessy, and G. Gullo, *Emerging targeted therapies in triple-negative breast cancer.* Ann Oncol, 2012. **23 Suppl 6**: p. vi56-65.

42. Ross, J.S. and J.A. Fletcher, *The HER-2/neu oncogene: prognostic factor, predictive factor and target for therapy.* Semin Cancer Biol, 1999. **9**(2): p. 125-38.
43. Anido, J., et al., *ZD1839, a specific epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, induces the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers and prevents heregulin signaling in HER2-overexpressing breast cancer cells.* Clin Cancer Res, 2003. **9**(4): p. 1274-83.
44. Zhan, L., B. Xiang, and S.K. Muthuswamy, *Controlled activation of ErbB1/ErbB2 heterodimers promote invasion of three-dimensional organized epithelia in an ErbB1-dependent manner: implications for progression of ErbB2-overexpressing tumors.* Cancer Res, 2006. **66**(10): p. 5201-8.
45. Brand, T.M., et al., *The nuclear epidermal growth factor receptor signaling network and its role in cancer.* Discov Med, 2011. **12**(66): p. 419-32.
46. Graus-Porta, D., et al., *ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling.* EMBO J, 1997. **16**(7): p. 1647-55.
47. Traxler, P., *Tyrosine kinases as targets in cancer therapy - successes and failures.* Expert Opin Ther Targets, 2003. **7**(2): p. 215-34.
48. Segovia-Mendoza, M., et al., *Efficacy and mechanism of action of the tyrosine kinase inhibitors gefitinib, lapatinib and neratinib in the treatment of HER2-positive breast cancer: preclinical and clinical evidence.* Am J Cancer Res, 2015. **5**(9): p. 2531-61.
49. Tevaarwerk, A.J. and J.M. Kolesar, *Lapatinib: a small-molecule inhibitor of epidermal growth factor receptor and human epidermal growth factor receptor-2 tyrosine kinases used in the treatment of breast cancer.* Clin Ther, 2009. **31 Pt 2**: p. 2332-48.
50. Spector, N.L., et al., *Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies.* J Clin Oncol, 2005. **23**(11): p. 2502-12.
51. Xia, W., et al., *Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways.* Oncogene, 2002. **21**(41): p. 6255-63.
52. Gril, B., et al., *Effect of lapatinib on the outgrowth of metastatic breast cancer cells to the brain.* J Natl Cancer Inst, 2008. **100**(15): p. 1092-103.
53. Tanizaki, J., et al., *Roles of BIM induction and survivin downregulation in lapatinib-induced apoptosis in breast cancer cells with HER2 amplification.* Oncogene, 2011. **30**(39): p. 4097-106.
54. Nahta, R., et al., *Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling.* Mol Cancer Ther, 2007. **6**(2): p. 667-74.
55. Scaltriti, M., et al., *Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer.* J Natl Cancer Inst, 2007. **99**(8): p. 628-38.
56. Press, M.F., et al., *HER-2 gene amplification, HER-2 and epidermal growth factor receptor mRNA and protein expression, and lapatinib efficacy in women with metastatic breast cancer.* Clin Cancer Res, 2008. **14**(23): p. 7861-70.
57. Zhou, X., et al., *Lapatinib plus capecitabine versus capecitabine alone for HER2+ (ErbB2+) metastatic breast cancer: quality-of-life assessment.* Breast Cancer Res Treat, 2009. **117**(3): p. 577-89.
58. Blackwell, K.L., et al., *Single-agent lapatinib for HER2-overexpressing advanced or metastatic breast cancer that progressed on first- or second-line trastuzumab-containing regimens.* Ann Oncol, 2009. **20**(6): p. 1026-31.

59. Baselga, J., et al., *Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial.* Lancet, 2012. **379**(9816): p. 633-40.
60. Eichhorn, P.J., et al., *Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235.* Cancer Res, 2008. **68**(22): p. 9221-30.
61. Jegg, A.M., et al., *PI3K independent activation of mTORC1 as a target in lapatinib-resistant ERBB2+ breast cancer cells.* Breast Cancer Res Treat, 2012. **136**(3): p. 683-92.
62. Tseng, J.T., *Understanding acquired resistance to Lapatinib in breast cancer cells. Thesis.* Graduate School of Biomedical Sciences at Digital Commons., 2010.
63. Xia, W., et al., *A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer.* Proc Natl Acad Sci U S A, 2006. **103**(20): p. 7795-800.
64. Mueller, K.L., et al., *Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells.* Cancer Res, 2008. **68**(9): p. 3314-22.
65. Rabindran, S.K., et al., *Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase.* Cancer Res, 2004. **64**(11): p. 3958-65.
66. Ito, Y., et al., *Safety, efficacy and pharmacokinetics of neratinib (HKI-272) in Japanese patients with advanced solid tumors: a Phase 1 dose-escalation study.* Jpn J Clin Oncol, 2012. **42**(4): p. 278-86.
67. Chow, L.W., et al., *Combination neratinib (HKI-272) and paclitaxel therapy in patients with HER2-positive metastatic breast cancer.* Br J Cancer, 2013. **108**(10): p. 1985-93.
68. Burstein, H.J., et al., *Neratinib, an irreversible ErbB receptor tyrosine kinase inhibitor, in patients with advanced ErbB2-positive breast cancer.* J Clin Oncol, 2010. **28**(8): p. 1301-7.
69. Wong, K.K., et al., *A phase I study with neratinib (HKI-272), an irreversible pan ErbB receptor tyrosine kinase inhibitor, in patients with solid tumors.* Clin Cancer Res, 2009. **15**(7): p. 2552-8.
70. Abbas, R., et al., *A double-blind, randomized, multiple-dose, parallel-group study to characterize the occurrence of diarrhea following two different dosing regimens of neratinib, an irreversible pan-ErbB receptor tyrosine kinase inhibitor.* Cancer Chemother Pharmacol, 2012. **70**(1): p. 191-9.
71. Berardi, R., et al., *Novel small molecule EGFR inhibitors as candidate drugs in non-small cell lung cancer.* Onco Targets Ther, 2013. **6**: p. 563-76.
72. Esteva, F.J., *Monoclonal antibodies, small molecules, and vaccines in the treatment of breast cancer.* Oncologist, 2004. **9 Suppl 3**: p. 4-9.
73. Chan, K.C., et al., *Effect of epidermal growth factor receptor tyrosine kinase inhibition on epithelial proliferation in normal and premalignant breast.* Cancer Res, 2002. **62**(1): p. 122-8.
74. Campiglio, M., et al., *Inhibition of proliferation and induction of apoptosis in breast cancer cells by the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor ZD1839 ('Iressa') is independent of EGFR expression level.* J Cell Physiol, 2004. **198**(2): p. 259-68.
75. Bunn, P.A., Jr. and W. Franklin, *Epidermal growth factor receptor expression, signal pathway, and inhibitors in non-small cell lung cancer.* Semin Oncol, 2002. **29**(5 Suppl 14): p. 38-44.
76. Bachawal, S.V., V.B. Wali, and P.W. Sylvester, *Combined gamma-tocotrienol and erlotinib/gefitinib treatment suppresses Stat and Akt signaling in murine mammary tumor cells.* Anticancer Res, 2010. **30**(2): p. 429-37.

77. Moulder, S.L., et al., *Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo.* Cancer Res, 2001. **61**(24): p. 8887-95.
78. Moasser, M.M., et al., *The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells.* Cancer Res, 2001. **61**(19): p. 7184-8.
79. Pao, W., et al., *EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib.* Proc Natl Acad Sci U S A, 2004. **101**(36): p. 13306-11.
80. Bhargava, R., et al., *EGFR gene amplification in breast cancer: correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations.* Mod Pathol, 2005. **18**(8): p. 1027-33.
81. Han, C., et al., *[Comparison of EGFR and KRAS status between primary non-small cell lung cancer and corresponding metastases: a systematic review and meta-analysis].* Zhongguo Fei Ai Za Zhi, 2010. **13**(9): p. 882-91.
82. Han, C.B., et al., *Concordant KRAS mutations in primary and metastatic colorectal cancer tissue specimens: a meta-analysis and systematic review.* Cancer Invest, 2012. **30**(10): p. 741-7.
83. Santarpia, L., et al., *Mutation profiling identifies numerous rare drug targets and distinct mutation patterns in different clinical subtypes of breast cancers.* Breast Cancer Res Treat, 2012. **134**(1): p. 333-43.
84. Sartore-Bianchi, A., et al., *Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, KRAS codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof-of-concept, multicentre, open-label, phase 2 trial.* Lancet Oncol, 2016. **17**(6): p. 738-46.
85. Pautier, P., et al., *Phase II study of gefitinib in combination with paclitaxel (P) and carboplatin (C) as second-line therapy for ovarian, tubal or peritoneal adenocarcinoma (1839IL/0074).* Gynecol Oncol, 2010. **116**(2): p. 157-62.
86. Jankowitz, R.C., et al., *Safety and efficacy of neratinib in combination with weekly paclitaxel and trastuzumab in women with metastatic HER2positive breast cancer: an NSABP Foundation Research Program phase I study.* Cancer Chemother Pharmacol, 2013. **72**(6): p. 1205-12.
87. DeLuca, H.F., *The control of calcium and phosphorus metabolism by the vitamin D endocrine system.* Ann N Y Acad Sci, 1980. **355**: p. 1-17.
88. Chakraborti, C.K., *Vitamin D as a promising anticancer agent.* Indian J Pharmacol, 2011. **43**(2): p. 113-20.
89. Swami, S., et al., *Inhibitory effects of calcitriol on the growth of MCF-7 breast cancer xenografts in nude mice: selective modulation of aromatase expression in vivo.* Horm Cancer, 2011. **2**(3): p. 190-202.
90. Bilinski, K. and J. Boyages, *Association between 25-hydroxyvitamin D concentration and breast cancer risk in an Australian population: an observational case-control study.* Breast Cancer Res Treat, 2013. **137**(2): p. 599-607.
91. Wang, D., et al., *Serum 25-hydroxyvitamin D and breast cancer risk: a meta-analysis of prospective studies.* Tumour Biol, 2013. **34**(6): p. 3509-17.
92. Mohr, S.B., et al., *Meta-analysis of vitamin D sufficiency for improving survival of patients with breast cancer.* Anticancer Res, 2014. **34**(3): p. 1163-6.

93. Yao, S. and C.B. Ambrosone, *Associations between vitamin D deficiency and risk of aggressive breast cancer in African-American women.* J Steroid Biochem Mol Biol, 2013. **136**: p. 337-41.
94. Peppone, L.J., et al., *The association between breast cancer prognostic indicators and serum 25-OH vitamin D levels.* Ann Surg Oncol, 2012. **19**(8): p. 2590-9.
95. Rainville, C., Y. Khan, and G. Tisman, *Triple negative breast cancer patients presenting with low serum vitamin D levels: a case series.* Cases J, 2009. **2**: p. 8390.
96. Narvaez, C.J., et al., *The impact of vitamin D in breast cancer: genomics, pathways, metabolism.* Front Physiol, 2014. **5**: p. 213.
97. Deeb, K.K., D.L. Trump, and C.S. Johnson, *Vitamin D signalling pathways in cancer: potential for anticancer therapeutics.* Nat Rev Cancer, 2007. **7**(9): p. 684-700.
98. Wang, Q.M., J.B. Jones, and G.P. Studzinski, *Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells.* Cancer Res, 1996. **56**(2): p. 264-7.
99. Liu, M., et al., *Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937.* Genes Dev, 1996. **10**(2): p. 142-53.
100. Matsumoto, K., et al., *Growth-inhibitory effects of 1,25-dihydroxyvitamin D3 on normal human keratinocytes cultured in serum-free medium.* Biochem Biophys Res Commun, 1990. **166**(2): p. 916-23.
101. Schwartz, G.G., et al., *1 alpha,25-Dihydroxyvitamin D (calcitriol) inhibits the invasiveness of human prostate cancer cells.* Cancer Epidemiol Biomarkers Prev, 1997. **6**(9): p. 727-32.
102. Campbell, M.J., et al., *Inhibition of proliferation of prostate cancer cells by a 19-nor-hexafluoride vitamin D3 analogue involves the induction of p21waf1, p27kip1 and E-cadherin.* J Mol Endocrinol, 1997. **19**(1): p. 15-27.
103. Beer, T.M. and A. Myrthue, *Calcitriol in cancer treatment: from the lab to the clinic.* Mol Cancer Ther, 2004. **3**(3): p. 373-81.
104. Rossi, A.M., et al., *MAPK inhibition by 1alpha,25(OH)2-Vitamin D3 in breast cancer cells. Evidence on the participation of the VDR and Src.* J Steroid Biochem Mol Biol, 2004. **89-90**(1-5): p. 287-90.
105. Tong, W.M., et al., *Mechanism of antimitogenic action of vitamin D in human colon carcinoma cells: relevance for suppression of epidermal growth factor-stimulated cell growth.* Oncol Res, 1999. **11**(2): p. 77-84.
106. Shen, Z., et al., *The coupling of epidermal growth factor receptor down regulation by 1alpha,25-dihydroxyvitamin D3 to the hormone-induced cell cycle arrest at the G1-S checkpoint in ovarian cancer cells.* Mol Cell Endocrinol, 2011. **338**(1-2): p. 58-67.
107. Feldman, D., et al., *The role of vitamin D in reducing cancer risk and progression.* Nat Rev Cancer, 2014. **14**(5): p. 342-57.
108. Mawer, E.B., et al., *Serum 1,25-dihydroxyvitamin D may be related inversely to disease activity in breast cancer patients with bone metastases.* J Clin Endocrinol Metab, 1997. **82**(1): p. 118-22.
109. Janowsky, E.C., et al., *Association between low levels of 1,25-dihydroxyvitamin D and breast cancer risk.* Public Health Nutr, 1999. **2**(3): p. 283-91.
110. Welsh, J., *Vitamin D and breast cancer: insights from animal models.* Am J Clin Nutr, 2004. **80**(6 Suppl): p. 1721S-4S.
111. Al-Azhri, J., et al., *Tumor Expression of Vitamin D Receptor and Breast Cancer Histopathological Characteristics and Prognosis.* Clin Cancer Res, 2016.

112. Johnson, C.S., et al., *Vitamin D receptor: a potential target for intervention.* Urology, 2002. **60**(3 Suppl 1): p. 123-30; discussion 130-1.
113. Urata, Y.N., et al., *Calcitriol supplementation effects on Ki67 expression and transcriptional profile of breast cancer specimens from post-menopausal patients.* Clin Nutr, 2013.
114. Knight, J.A., et al., *Vitamin D and reduced risk of breast cancer: a population-based case-control study.* Cancer Epidemiol Biomarkers Prev, 2007. **16**(3): p. 422-9.
115. Fakih, M.G., et al., *A phase I pharmacokinetic and pharmacodynamic study of intravenous calcitriol in combination with oral gefitinib in patients with advanced solid tumors.* Clin Cancer Res, 2007. **13**(4): p. 1216-23.
116. Muindi, J.R., et al., *A phase I and pharmacokinetics study of intravenous calcitriol in combination with oral dexamethasone and gefitinib in patients with advanced solid tumors.* Cancer Chemother Pharmacol, 2009. **65**(1): p. 33-40.
117. Smith, D.C., et al., *A Phase I trial of calcitriol (1,25-dihydroxycholecalciferol) in patients with advanced malignancy.* Clin Cancer Res, 1999. **5**(6): p. 1339-45.
118. Gulliford, T., et al., *A phase I study of the vitamin D analogue EB 1089 in patients with advanced breast and colorectal cancer.* Br J Cancer, 1998. **78**(1): p. 6-13.
119. Hansen, C.M., et al., *Vitamin D and cancer: effects of 1,25(OH)2D3 and its analogs on growth control and tumorigenesis.* Front Biosci, 2001. **6**: p. D820-48.
120. de Los Angeles Rey, M., et al., *New Synthetic Strategies to Vitamin D Analogues Modified at the Side Chain and D Ring. Synthesis of 1alpha,25-Dihydroxy-16-ene-vitamin D(3) and C-20 Analogues(1).* J Org Chem, 1999. **64**(9): p. 3196-3206.
121. Marcinkowska, E., A. Kutner, and C. Radzikowski, *Cell differentiating and anti-proliferative activity of side-chain modified analogues of 1,25-dihydroxyvitamin D3.* J Steroid Biochem Mol Biol, 1998. **67**(1): p. 71-8.
122. Vakirlis, E., A. Kastanis, and D. Ioannides, *Calcipotriol/betamethasone dipropionate in the treatment of psoriasis vulgaris.* Ther Clin Risk Manag, 2008. **4**(1): p. 141-8.
123. Colston, K.W., et al., *Effects of synthetic vitamin D analogues on breast cancer cell proliferation in vivo and in vitro.* Biochem Pharmacol, 1992. **44**(4): p. 693-702.
124. Bower, M., et al., *Topical calcipotriol treatment in advanced breast cancer.* Lancet, 1991. **337**(8743): p. 701-2.
125. Kissmeyer, A., et al., *Pharmacokinetic studies of vitamin D analogues: relationship to vitamin D binding protein (DBP).* Endocrine, 1995. **3**(4): p. 263-6.
126. Simboli-Campbell, M., et al., *Comparative effects of 1,25(OH)2D3 and EB1089 on cell cycle kinetics and apoptosis in MCF-7 breast cancer cells.* Breast Cancer Res Treat, 1997. **42**(1): p. 31-41.
127. Wu, G., et al., *Modulation of cell cycle control by vitamin D3 and its analogue, EB1089, in human breast cancer cells.* Oncogene, 1997. **15**(13): p. 1555-63.
128. Colston, K.W., et al., *EB1089: a new vitamin D analogue that inhibits the growth of breast cancer cells in vivo and in vitro.* Biochem Pharmacol, 1992. **44**(12): p. 2273-80.
129. James, S.Y., et al., *Effects of a new synthetic vitamin D analogue, EB1089, on the oestrogen-responsive growth of human breast cancer cells.* J Endocrinol, 1994. **141**(3): p. 555-63.
130. Sundaram, S., et al., *The vitamin D3 analog EB 1089 enhances the antiproliferative and apoptotic effects of adriamycin in MCF-7 breast tumor cells.* Breast Cancer Res Treat, 2000. **63**(1): p. 1-10.
131. Sundaram, S. and D.A. Gewirtz, *The vitamin D3 analog EB 1089 enhances the response of human breast tumor cells to radiation.* Radiat Res, 1999. **152**(5): p. 479-86.

132. Chou, T.C., *Drug combination studies and their synergy quantification using the Chou-Talalay method.* Cancer Res, 2010. **70**(2): p. 440-6.
133. Ross, J.S., et al., *The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine.* Oncologist, 2009. **14**(4): p. 320-68.
134. Harris, A.L., et al., *Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu.* J Steroid Biochem, 1989. **34**(1-6): p. 123-31.
135. Osaki, A., et al., *Prognostic significance of co-expression of c-erbB-2 oncoprotein and epidermal growth factor receptor in breast cancer patients.* Am J Surg, 1992. **164**(4): p. 323-6.
136. Singh, J.C., K. Jhaveri, and F.J. Esteva, *HER2-positive advanced breast cancer: optimizing patient outcomes and opportunities for drug development.* Br J Cancer, 2014. **111**(10): p. 1888-98.
137. Sellmann, L., K. Fenchel, and W.C. Dempke, *Improved overall survival following tyrosine kinase inhibitor treatment in advanced or metastatic non-small-cell lung cancer-the Holy Grail in cancer treatment?* Transl Lung Cancer Res, 2015. **4**(3): p. 223-7.
138. Li, S.G. and L. Li, *Targeted therapy in HER2-positive breast cancer.* Biomed Rep, 2013. **1**(4): p. 499-505.
139. Wang, H., *Lapatinib for the treatment of breast cancer in the People's Republic of China.* Onco Targets Ther, 2014. **7**: p. 1367-73.
140. Canonici, A., et al., *Neratinib overcomes trastuzumab resistance in HER2 amplified breast cancer.* Oncotarget, 2013. **4**(10): p. 1592-605.
141. Garcia-Quiroz, J., et al., *Astemizole synergizes calcitriol antiproliferative activity by inhibiting CYP24A1 and upregulating VDR: a novel approach for breast cancer therapy.* PLoS One, 2012. **7**(9): p. e45063.
142. Santos-Martinez, N., et al., *Calcitriol restores antiestrogen responsiveness in estrogen receptor negative breast cancer cells: a potential new therapeutic approach.* BMC Cancer, 2014. **14**: p. 230.
143. Trump, D.L., K.K. Deeb, and C.S. Johnson, *Vitamin D: considerations in the continued development as an agent for cancer prevention and therapy.* Cancer J, 2010. **16**(1): p. 1-9.
144. Swami, S., A.V. Krishnan, and D. Feldman, *1alpha,25-Dihydroxyvitamin D3 down-regulates estrogen receptor abundance and suppresses estrogen actions in MCF-7 human breast cancer cells.* Clin Cancer Res, 2000. **6**(8): p. 3371-9.
145. Ma, Y., D.L. Trump, and C.S. Johnson, *Vitamin D in combination cancer treatment.* J Cancer, 2010. **1**: p. 101-7.
146. Segovia-Mendoza, M., et al., *Calcitriol and its analogues enhance the antiproliferative activity of gefitinib in breast cancer cells.* J Steroid Biochem Mol Biol, 2015. **148**: p. 122-31.
147. Birnbaum, A. and N. Ready, *Gefitinib therapy for non-small cell lung cancer.* Curr Treat Options Oncol, 2005. **6**(1): p. 75-81.
148. O'Neill, F., et al., *A gene expression profile indicative of early stage HER2 targeted therapy response.* Mol Cancer, 2013. **12**: p. 69.
149. Fieldman, D., *Vitamin D.* Academic Press, 1997.: p. 1039.
150. Ovcaricek, T., et al., *Triple negative breast cancer - prognostic factors and survival.* Radiol Oncol, 2011. **45**(1): p. 46-52.
151. Rehman, A., *Triple-negative phenotype of poorly-differentiated metaplastic breast carcinoma in a male: an oncological rarity.* J Coll Physicians Surg Pak, 2013. **23**(5): p. 370-2.

152. Peng, X., et al., *Overexpression of ER and VDR is not sufficient to make ER-negative MDA-MB231 breast cancer cells responsive to 1alpha-hydroxyvitamin D5.* Carcinogenesis, 2007. **28**(5): p. 1000-7.
153. Fakih, M.G., et al., *A Phase I and pharmacokinetic study of selenomethionine in combination with a fixed dose of irinotecan in solid tumors.* Cancer Chemother Pharmacol, 2008. **62**(3): p. 499-508.
154. Fife, R.S., G.W. Sledge, Jr., and C. Proctor, *Effects of vitamin D3 on proliferation of cancer cells in vitro.* Cancer Lett, 1997. **120**(1): p. 65-9.
155. Wang, W., et al., *Vitamin D analog EB1089 induces apoptosis in a subpopulation of SGC-7901 gastric cancer cells through a mitochondrial-dependent apoptotic pathway.* Nutr Cancer, 2013. **65**(7): p. 1067-75.
156. Kizildag, S., H. Ates, and S. Kizildag, *Treatment of K562 cells with 1,25-dihydroxyvitamin D3 induces distinct alterations in the expression of apoptosis-related genes BCL2, BAX, BCLXL, and p21.* Ann Hematol, 2010. **89**(1): p. 1-7.
157. Clavarezza, M., et al., *Dual Block with Lapatinib and Trastuzumab Versus Single-Agent Trastuzumab Combined with Chemotherapy as Neoadjuvant Treatment of HER2-Positive Breast Cancer: A Meta-analysis of Randomized Trials.* Clin Cancer Res, 2016.
158. Awada, A., et al., *Neratinib Plus Paclitaxel vs Trastuzumab Plus Paclitaxel in Previously Untreated Metastatic ERBB2-Positive Breast Cancer: The NEfERT-T Randomized Clinical Trial.* JAMA Oncol, 2016.
159. Lazzaro, G., et al., *Induction of differentiation by 1alpha-hydroxyvitamin D(5) in T47D human breast cancer cells and its interaction with vitamin D receptors.* Eur J Cancer, 2000. **36**(6): p. 780-6.
160. Cohen, M.H., et al., *United States Food and Drug Administration Drug Approval summary: Gefitinib (ZD1839; Iressa) tablets.* Clin Cancer Res, 2004. **10**(4): p. 1212-8.
161. Beer, T.M., *Development of weekly high-dose calcitriol based therapy for prostate cancer.* Urol Oncol, 2003. **21**(5): p. 399-405.
162. Cizkova, M., et al., *High lapatinib plasma levels in breast cancer patients: risk or benefit?* Tumori, 2012. **98**(1): p. 162-5.
163. Hug, B., et al., *A single-dose, crossover, placebo- and moxifloxacin-controlled study to assess the effects of neratinib (HKI-272) on cardiac repolarization in healthy adult subjects.* Clin Cancer Res, 2010. **16**(15): p. 4016-23.
164. Konecny, G.E., et al., *Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells.* Cancer Res, 2006. **66**(3): p. 1630-9.
165. Costa, D.B., et al., *BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations.* PLoS Med, 2007. **4**(10): p. 1669-79; discussion 1680.
166. Cragg, M.S., et al., *Gefitinib-induced killing of NSCLC cell lines expressing mutant EGFR requires BIM and can be enhanced by BH3 mimetics.* PLoS Med, 2007. **4**(10): p. 1681-89; discussion 1690.
167. Akiyama, T., C.R. Dass, and P.F. Choong, *Bim-targeted cancer therapy: a link between drug action and underlying molecular changes.* Mol Cancer Ther, 2009. **8**(12): p. 3173-80.
168. Tsujimoto, Y., et al., *Involvement of the bcl-2 gene in human follicular lymphoma.* Science, 1985. **228**(4706): p. 1440-3.
169. Oakes, S.R., et al., *Sensitization of BCL-2-expressing breast tumors to chemotherapy by the BH3 mimetic ABT-737.* Proc Natl Acad Sci U S A, 2012. **109**(8): p. 2766-71.

170. Bean, G.R., et al., *PUMA and BIM are required for oncogene inactivation-induced apoptosis.* Sci Signal, 2013. **6**(268): p. ra20.
171. Hasegawa, H., et al., *Induction of apoptosis by HBI-8000 in adult T-cell leukemia/lymphoma is associated with activation of Bim and NLRP3.* Cancer Sci, 2016. **107**(8): p. 1124-33.
172. Tang, L., et al., *Lapatinib induces p27(Kip1)-dependent G(1) arrest through both transcriptional and post-translational mechanisms.* Cell Cycle, 2013. **12**(16): p. 2665-74.
173. Mathiasen, I.S., U. Lademann, and M. Jaattela, *Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53.* Cancer Res, 1999. **59**(19): p. 4848-56.
174. Taylor, S., et al., *Evaluating the evidence for targeting FOXO3a in breast cancer: a systematic review.* Cancer Cell Int, 2015. **15**(1): p. 1.
175. Borowicz, S., et al., *The soft agar colony formation assay.* J Vis Exp, 2014(92): p. e51998.
176. Crowley, L.C. and N.J. Waterhouse, *Measuring Survival of Hematopoietic Cancer Cells with the Colony-Forming Assay in Soft Agar.* Cold Spring Harb Protoc, 2016. **2016**(8): p. pdb prot087189.
177. Ben-Shoshan, M., et al., *1alpha,25-dihydroxyvitamin D3 (Calcitriol) inhibits hypoxia-inducible factor-1/vascular endothelial growth factor pathway in human cancer cells.* Mol Cancer Ther, 2007. **6**(4): p. 1433-9.
178. Karakashev, S.V. and M.J. Reginato, *Hypoxia/HIF1alpha induces lapatinib resistance in ERBB2-positive breast cancer cells via regulation of DUSP2.* Oncotarget, 2015. **6**(4): p. 1967-80.
179. Chattopadhyay, N., et al., *1alpha,25(OH)2-vitamin D3 inhibits HGF synthesis and secretion from MG-63 human osteosarcoma cells.* Am J Physiol Endocrinol Metab, 2003. **284**(1): p. E219-27.
180. Kovalenko, P.L., et al., *1,25 dihydroxyvitamin D-mediated orchestration of anticancer, transcript-level effects in the immortalized, non-transformed prostate epithelial cell line, RWPE1.* BMC Genomics, 2010. **11**: p. 26.
181. Vink-van Wijngaarden, T., et al., *Inhibition of insulin- and insulin-like growth factor-I-stimulated growth of human breast cancer cells by 1,25-dihydroxyvitamin D3 and the vitamin D3 analogue EB1089.* Eur J Cancer, 1996. **32A**(5): p. 842-8.
182. Organ, S.L. and M.S. Tsao, *An overview of the c-MET signaling pathway.* Ther Adv Med Oncol, 2011. **3**(1 Suppl): p. S7-S19.
183. Huang, L. and L. Fu, *Mechanisms of resistance to EGFR tyrosine kinase inhibitors.* Acta Pharm Sin B, 2015. **5**(5): p. 390-401.
184. Yuan, X., et al., *Notch signaling: an emerging therapeutic target for cancer treatment.* Cancer Lett, 2015. **369**(1): p. 20-7.
185. Kaaks, R., *Nutrition, insulin, IGF-1 metabolism and cancer risk: a summary of epidemiological evidence.* Novartis Found Symp, 2004. **262**: p. 247-60; discussion 260-68.
186. Gialeli, C., A.D. Theocharis, and N.K. Karamanos, *Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting.* FEBS J, 2011. **278**(1): p. 16-27.
187. Di Rosa, M., et al., *Vitamin D3: a helpful immuno-modulator.* Immunology, 2011. **134**(2): p. 123-39.

20. ANEXO 1: ARTÍCULO REQUISITO

1.- Calcitriol and its analogues enhance the antiproliferative activity of gefitinib in breast cancer cells.

Segovia-Mendoza M, Díaz L, González-González ME, Martínez-Reza I, García-Quiroz J, Prado-Garcia H, Ibarra-Sánchez MJ, Esparza-López J, Larrea F, García-Becerra R.

J Steroid Biochem Mol Biol. 2015 Dec 12. doi: 10.1016/j.jsbmb.2014.12.006.

Journal of Steroid Biochemistry & Molecular Biology 148 (2015) 122–131

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Calcitriol and its analogues enhance the antiproliferative activity of gefitinib in breast cancer cells

Mariana Segovia-Mendoza [a,b], Lorenza Díaz [a], María Elena González-González [a], Isela Martínez-Reza [a], Janice García-Quiroz [a], Heriberto Prado-Garcia [c], María J. Ibarra-Sánchez [d], José Esparza-López [d], Fernando Larrea [a], Rocío García-Becerra [a,*]

[a] Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Sección XVI, Tlalpan 14000, México, D.F., Mexico
[b] Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Av. Cuidad Universitaria 3000, Coyoacán 04360, México, D.F, Mexico
[c] Departamento de Enfermedades Crónico-Degenerativas, Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas", Calzada de Tlalpan 4502, Sección XVI, Tlalpan 14080, México, D.F., Mexico
[d] Unidad de Bioquímica, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Sección XVI, Tlalpan 14000, México, D.F., Mexico

ARTICLE INFO

Article history:
Received 15 August 2014
Received in revised form 6 December 2014
Accepted 10 December 2014
Available online 12 December 2014

Keywords:
Gefitinib
Calcitriol
Vitamin D analogs
Breast cancer
HER2
EGFR

ABSTRACT

Coexpression of EGFR and HER2 has been associated with poor disease outcome, high rates of metastasis and resistance to conventional treatments in breast cancer. Gefitinib, a tyrosine kinase inhibitor, reduces both cell proliferation and tumor growth of breast cancer cells expressing EGFR and/or HER2. On the other hand, calcitriol and some of its synthetic analogs are important antineoplastic agents in different breast cancer subtypes. Herein, we evaluated the effects of the combined treatment of gefitinib with calcitriol or its analogs on cell proliferation in breast cancer cells.

The presence of EGFR, HER2 and vitamin D receptor were evaluated by Western blot in two established breast cancer cell lines: SUM-229PE, SKBR3 and a primary breast cancer-derived cell line. The antiproliferative effects of gefitinib alone or in combination with calcitriol and its analogs, calcipotriol and EB1089, were assessed by growth assay using a DNA content-based method. Inhibitory concentrations on cell proliferation were calculated by non-linear regression analysis using sigmoidal fitting of dose-response curves. Pharmacological effects of the drug combinations were calculated by the Chou–Talalay method. Phosphorylation of ERK1/2 MAPK was evaluated by Western blot. Gene expression of EGFR, HER2 and BIM was assessed by real time PCR. BIM protein levels were analyzed in cells by flow cytometry. The effects of the drugs alone or combinated on cell cycle phases were determined using propidium iodide. Apoptosis was evaluated by detection of subG1 peak and determination of active caspase 3 by flow cytometry.

Gefitinib, calcitriol, calcipotriol and EB1089 inhibited cell proliferation in a dose dependent manner. The combinations of gefitinib with calcitriol or its analogs were more effective to inhibit cell growth than each compound alone in all breast cancer cells studied. The gene expression of EGFR and HER2 was downregulated and not affected, respectively, by the combined treatment. Furthermore, phosphorylation of ERK 1/2 was inhibited a greater extent in co-treated cells than in the cells treated with alone compounds. The combination of gefitinib with calcitriol or their synthetic analogs induced apoptosis in SUM-229PE cells, this was shown by the significant upregulation of BIM protein levels, higher percentages of cells in subG1 peak and increase of caspase 3-positive cells.

The combination of gefitinib with calcitriol or their synthetic analogs resulted in a greater antiproliferative effect than with either of the agents alone in EGFR and HER2 positive breast cancer

Abbreviations: CK-7, cytokeratin 7; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FSC-A, forward scatter area; FSC-H, forward scatter height; GI, growth inhibitory; HER2, epidermal growth factor receptor type II; IC, inhibitory concentration; MFI, mean fluorescence intensity; TKI, tyrosine kinase inhibitors; VDR, vitamin D receptor.

* Corresponding author at. Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Vasco de Quiroga No. 15, Sección XVI, Tlalpan 14000, México, D.F, Mexico. Tel.: +525 54 87 09 00x2417.
E-mail address: rocio.garciab@quetzal.innsz.mx (R. García-Becerra).

http://dx.doi.org/10.1016/j.jsbmb.2014.12.006
0960-0760/

cells. The mechanistic explanation for these results includes downregulation of MAPK signaling pathway, decrease of cells in G2/M phase and induction of apoptosis mediated by upregulation of BIM and activation of caspase 3.

This article is part of a Special Issue entitled '17th Vitamin D Workshop'.

Contents

1. Introduction

The ErbB or epidermal growth factor receptor (EGFR) family consists of four members: EGFR/HER1/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. Ligand binding to the receptors induces the formation of homo- and heterodimers and activation of the kinase domain, resulting in phosphorylation of specific tyrosine residues. Phosphorylation triggers several signaling pathways such as the PI3K/Akt and the Ras/Raf/MEK/MAPK that promotes cell proliferation, survival, adhesion, migration and differentiation [1,2]. Although HER2 has no ligands, this receptor is activated via heterodimerization with ligand-activated ErbB receptors. HER2 overexpression occurs in about 20–30% of patients with breast cancer [3,4]. Notably, EGFR and HER3 are the major partners of HER2 [5,6]. Coexpression of EGFR/HER2 has been observed in 10–36% of all primary human breast carcinomas, and it has been associated with a more aggressive clinical behavior when compared with those tumors that express a single receptor [7–9]. Therefore, drugs that selectively inhibit these targets represent good therapeutic alternatives for HER2 and EGFR-positive breast cancer tumors. In this regard, small molecule tyrosine kinase inhibitors (TKIs) have been developed. Gefitinib, a TKI, has been approved by the FDA for treatment of advanced non-small cell lung carcinoma with activating EGFR mutations [10]. In addition, gefitinib has been shown to reduce cell proliferation and tumor growth in breast cancer cell lines or under *in vivo* conditions in xenografted animals with different levels of EGFR or HER2 expression [11–13]. Interestingly, gefitinib is more potent in inhibiting proliferation of breast cancer cells with a high and low levels of HER2 and EGFR respectively, compared to those cells with high levels of EGFR [11,13]. Gefitinib effects on HER2 and EGFR-expressing breast cancer cells are mediated by the inhibition of Akt and MAPK signaling pathways [11–14]. In addition, in these cells, gefitinib induces cell cycle arrest in G1 phase and an increase of pro-apoptotic BIM protein expression [2,11,15].

Calcitriol, through its nuclear vitamin D receptor (VDR), exerts an important antitumor activity [16]. In this regard, epidemiological studies have demonstrated an association between low levels of calcidiol, the precursor of calcitriol, with an increased risk of developing breast cancer and tumor progression [17,18]. VDR expression is found in 90% of all human breast tumors, which correlates with a longer disease-free survival compared with VDR-negative tumors [19]. Among the mechanism by which calcitriol exerts its antiproliferative activity are those related with cell cycle arrest, stimulation of cell differentiation and regulation of anti-apoptotic proteins [20–22]. Recent observations from this laboratory have shown the important antiproliferative effects of calcitriol alone or combined with other antineoplastic agents [23,24]. The main drawback for calcitriol clinical use is that high doses of calcitriol induces disturbances in calcium homeostasis [25]. To overcome this unwanted secondary effect of calcitriol, numerous synthetic vitamin D analogs have been developed. EB1089 and calcipotriol are vitamin D synthetic analogs that retain the ability to inhibit cell proliferation and induce cell differentiation while display reduced calcemic activity. Interestingly, calcitriol and its analog EB1089 downregulate EGFR levels in both ovarian and breast cancer cell lines [26,27].

Taking into account all these observations, the aim of this study was to investigate the effects of a combination of gefitinib

with calcitriol or its analogs upon cell proliferation in breast cancer cells.

2. Material and methods

2.1. Reagents

Cell culture medium was obtained from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Hyclone Laboratories Inc., Gefitinib (Iressa, ZD1839) was kindly donated by AstraZeneca (Wilmington, DE, USA). calcitriol (1α,25-Dihidroxivitamina D_3) and calcipotriol (MC 903) were purchased from Sigma (St. Louis, MO, USA). EB1089 (seocalcitol) was obtained from Tocris Bioscience (Bristol, United Kingdom). TRIzol and the oligonucleotides for real time polymerase chain reaction (qPCR) were from Invitrogen (CA, USA). The TaqMan master reaction, probes, capillaries and transcriptor first strand cDNA kit were purchased from Roche (Roche Applied Science, IN, USA). RNase A solution was from Promega (Madison, WI, USA). Propidium iodide was obtained from Sigma.

2.2. Cell culture

The primary breast cancer cell culture (MBCDF) was generated from a biopsy obtained from a radical mastectomy performed on a patient with an infiltrating ductal carcinoma stage IV. The protocol was approved by the Committee of Ethics and Research from the National Institute for Medical Sciences and Nutrition "Salvador Zubirán" (INCMNSZ) Ref 1549, BQO-008-06/9-1) [28]. Cells were maintained in humidified atmosphere with 5% CO_2 at 37 °C in RPMI-1640 medium supplemented with 100 units/mL penicillin plus 100 μg/mL streptomycin and 5% heat-inactivated fetal bovine serum. The EGFR-overexpressing SUM-229PE (Asterand, San Francisco, CA) and the HER2-overexpressing SKBR3 (ATCC, Manassas, VA, USA) established cell lines were cultured and maintained following indications from the supplier.

2.3. Western blot

Cells were lysed in a buffer containing HEPES 50 mM pH 7.4, NaCl 250 mM, EDTA 5 mM, Nonidet P-40 0.1%, NaF 10 mM, β-glycerophosphate 50 mM, Na_3VO_4 1 mM and complete EDTA-free protease inhibitors cocktail (Sigma, St. Louis, MO, USA). 25 μg of protein were separated in SDS-PAGE, transferred to polyvinylidene difluoride membranes Immobilon-P (Sigma) and blocked with milk 5% in PBS-tween for 1 h. Membranes were incubated overnight at 4 °C with anti-EGFR (1:1000, Santa Cruz Biotechnology Inc., CA, USA), anti-HER2 (1:1000 Santa Cruz), anti-VDR (1:500, Santa Cruz), or anti-phospho-p44/42 MAPK (ERK1/2, 1:1000, Cell Signaling, Danvers, MA). Then membranes were washed and incubated with HRP-conjugated secondary antibody (1:5000) for 1 h at room temperature. For normalization, blots were stripped in boiling stripping buffer (2% w/v SDS, 62.5 mM Tris–HCl pH 6.8, 100 mM 2-mercapto-ethanol) for 30 min at 50 °C and sequentially incubated with mouse anti-β actin (1:5000, Santa Cruz Biotechnology Inc.), anti-ribosomal protein L7a (1:800, Cell Signaling), anti-rabbit-HRP (1:4000, Jackson ImmunoResearch Laboratories) and anti-mouse-HRP (1:10000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Proteins were detected with ECL-Plus western blotting detection system (GE Healthcare, UK) and visualized using the Bio-Rad ChemiDoc XRS detection system (Bio-Rad Hercules, CA). Densitometry was performed with Image Lab software (Bio-Rad).

2.4. Proliferation and drug combination treatment

The cells were seeded in 96-well culture plates by sextuplicate at a density of 200–2000 cells/well depending on the cell line. After 24 h, cells were incubated in the presence of different concentrations of gefitinib, calcitriol, calcipotriol and EB1089 or vehicle alone (0.1% v/v ethanol or dimethyl sulfoxide) for 7 days. Cell proliferation was determined by using a DNA content-based method (CyQUANT kit, Invitrogen) according to manufacturer's instruction. Inhibitory concentrations 20% and 50% (IC_{20} and IC_{50}) values were obtained by non-linear regression analysis using sigmoidal fitting with a dose-response curve by means of a scientific graphing software (Origin 5, OriginLab Corporation, Northampton MA). Combination of gefitinib with calcitriol or its synthetic analogs were performed using the IC_{20} and IC_{50} values. In order to determine the pharmacologic effect of the different drug combinations we used the combination index equation described by Chou–Talalay [29]. For this analysis, synergy is defined as combination index values <1.0, antagonism as values >1.0, and additivity as a value = 1.0.

2.5. Reverse transcriptase-qPCR amplifications

For BIM and EGFR gene expression analysis, the SUM-229PE and SKBR3 cells were incubated in the presence of different combinations of gefitinib with calcitriol or EB1089 during 24 h. RNA was extracted with TRIzol reagent and then subjected to reverse transcription. Probes and primers for qPCR amplification were designed with the Universal Probe Library Assay Design Center from Roche. Real-time PCR was carried out using the LightCycler 2.0 from Roche (Roche Diagnostics, Mannheim, Germany), according to the following protocol: activation of Taq DNA polymerase and DNA denaturation at 95 °C for 10 min, proceeded by 45 amplification cycles consisting of 10 s at 95 °C, 30 s at 60 °C, and 1 s at 72 °C. The following oligonucleotides were used: BIM-L, 5′-GCTGTGGAGGCTGAATCC-3′; BIMR, 5′-TCGGCTGCTTGGTAATTATTC-3′; EGFR-L, 5′-GCCTTGACTGAGGACAGCA-3′; EGFR-R, 5′-TTTGGGAACGGACTGGTTTA-3′; HER2-L, 5′-GGGAAACCTGGAACTCACCTA-3′; HER2-R, 5′-CCCTGCACCTCCTGGATA-3′. The gene expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) GAPDH-L, 5′-AGCCACATCGCTCAGACAC-3′; GAPDH-R, 5′-GCCCAATACGACCAAATCC-3′ was used as an internal control. Data obtained from qPCR were analyzed using the ΔΔCt method.

2.6. Analysis of BIM protein levels

SUM-229 PE cells were incubated with the combination of gefitinib plus calcitriol or EB1089 for 48 h. Then, BIM protein levels were analyzed by flow cytometry. After treatment, cells were harvested, fixed with formaldehyde 16% (v/v) and permeabilized with methanol 90% (v/v). Next, cells were incubated with unlabeled rabbit anti-BIM polyclonal antibody (Cell Signaling), or corresponding isotype control antibody at room temperature for 40 min. For detection of BIM, cells were washed and incubated with alexa 488 mouse anti-rabbit (Molecular Probes, Eugene OR) mAb at room temperature for 40 min. After washing, cells were analyzed by flow cytometry.

Forward scatter area (FSC-A) vs. forward scatter height (FSC-H) contour-plot graph was used to gate out cell aggregates. Next, FSC-A vs. SSC-A contour-plot graphs were done for cellular debris, necrotic cells exclusion and selection of tumor cells. Then, mean fluorescence intensity (MFI) for BIM molecule was obtained by histograms.

2.7. Detection of active caspase 3

SUM-229PE cells were incubated with the combination of gefitinib plus calcitriol or EB1089 for 72 h. Then, FITC Active Caspase-3 Apoptosis Kit (BD Pharmingen, CA, USA) was used to determine the presence of active caspase 3-positive cells, according to the manufacturer's instructions. Briefly, cells were collected, washed with PBS, resuspended in BD Cytofix/Cytoperm buffer and incubated for 20 min at 4 °C. The suspension was centrifuged and washed twice with BD Perm/Wash buffer. Afterwards, cells were incubated with fluorescein isothiocyanate (FITC) anti-active caspase 3 antibody for 30 min. The samples were washed and resuspended with BD Perm/Wash buffer and analyzed using a FACsCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA). Forward scatter area (FSC-A) vs. forward scatter height (FSC-H) contour-plot graph was used to gate out cell aggregates. Next, FSC-A vs. SSC-A contour-plot graphs were done for cellular debris exclusion and selection of tumor cells. A total of 20,000 events were acquired from the gate of tumor cells. Then, percentage of active caspase 3-positive cells was obtained from FSC-A vs. FITC active caspase 3 contour-plot.

2.8. Cell cycle distribution

SUM-229PE cells were incubated with the combination of gefitinib plus calcitriol or EB1089 during 96 h. After treatment, the cells were collected, washed with PBS, fixed in ethanol 70% v/v and kept at −20 °C. For cell cycle analysis, samples were washed twice with PBS pH 7.2, and incubated in a solution containing RNAse (10 μg/mL), Triton X-100 0.1% (v/v) and propidium iodide (PI) 1 μg/mL in the dark at room temperature for 20 min. DNA content was determined using a FACsCanto II flow cytometer. A total of 35,000 events from PI-area vs. PI-wide gate were acquired. For cell cycle analysis and subG1 peak detection Flow-Jo (Tree Star Inc., version 9.3.2) software was used.

2.9. Statistical analyses

Data are expressed as the mean ± standard deviation (S.D.). Statistical analyses were determined by one-way ANOVA followed by the Holm–Sidak method, using a specialized software package (SigmaStat, Jandel Scientific).

3. Results

3.1. Cell characterization

SUM-229PE, SKBR3 and MBCDF cells were characterized by western blot to corroborate the presence of gefitinib and calcitriol molecular targets. All cell lines were VDR and HER2 positive. Only in the MBCDF cell line the presence of EGFR could not be detected (data not shown).

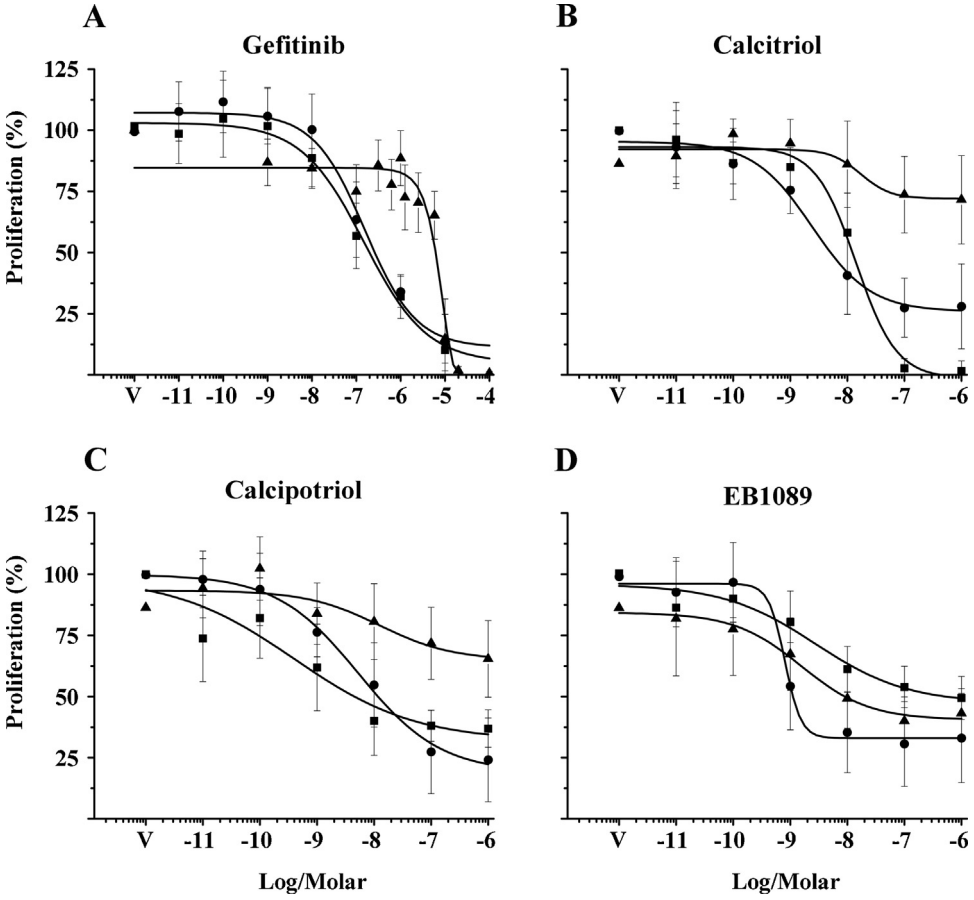


Fig. 1. Gefitinib, calcitriol and its synthetic analogs antiproliferative effects in cultured breast cancer cells. SUM-229PE (■), SKBR3 (●) and MBCDF (▲) cells were incubated in the presence of different concentrations of gefitinib (A), calcitriol (B), calcipotriol (C), or EB1089 (D) during 7 days. Cell proliferation was evaluated by determination of DNA using the CyQUANT kit. Results are the mean ± S.D. of sextuplicate determinations and represent at least three different experiments. Data from vehicle-treated cells were normalized to 100%.

Table 1
Inhibitory concentrations (IC) $_{20}$ and $_{50}$ values of gefitinib, calcitriol and its synthetic analogs on breast cancer cells proliferation.

Compound	IC	SUM-229PE (mol/L)	SKBR3 (mol/L)	MBCDF (mol/L)
Gefitinib	20	1.6×10^{-8}	2.5×10^{-8}	2.0×10^{-6}
	50	1.5×10^{-7}	1.5×10^{-7}	3.0×10^{-6}
Calcitriol	20	4.6×10^{-9}	4.6×10^{-10}	7.4×10^{-9}
	50	1.4×10^{-8}	2.4×10^{-9}	1.7×10^{-8}
Calcipotriol	20	1.3×10^{-11}	5.3×10^{-10}	1.6×10^{-9}
	50	4.0×10^{-10}	5.3×10^{-9}	1.2×10^{-8}
EB1089	20	2.0×10^{-10}	4.3×10^{-10}	2.3×10^{-10}
	50	2.7×10^{-9}	7.5×10^{-10}	1.6×10^{-9}

3.2. Antiproliferative effects of gefitinib, calcitriol and its analogs in breast cancer cells

Cells were incubated in the presence of different concentrations of gefitinib, calcitriol or its analogs during 7 days and the antiproliferative response was evaluated. As shown in Fig. 1, the compounds inhibited cell proliferation in a dose dependent manner in all breast cancer cell lines, but the sensitivity of the cells to the compounds varied among cell lines (Fig. 1A–D). Based on the calculated IC_{50} values (Table 1), SUM-229PE and SKBR3 cell lines showed similar sensitivity to gefitinib. Nevertheless, MBCDF cells showed an IC_{50} value around 10-fold higher than that obtained with the established cell lines. The sensitivity to calcitriol considering IC_{50} values was: SKBR3 > SUM-229PE > MBCDF, while for calcipotriol and EB1089: SUM-229PE > SKBR3 > MBCDF; and SKBR3 > MBCDF > SUM-229PE, respectively.

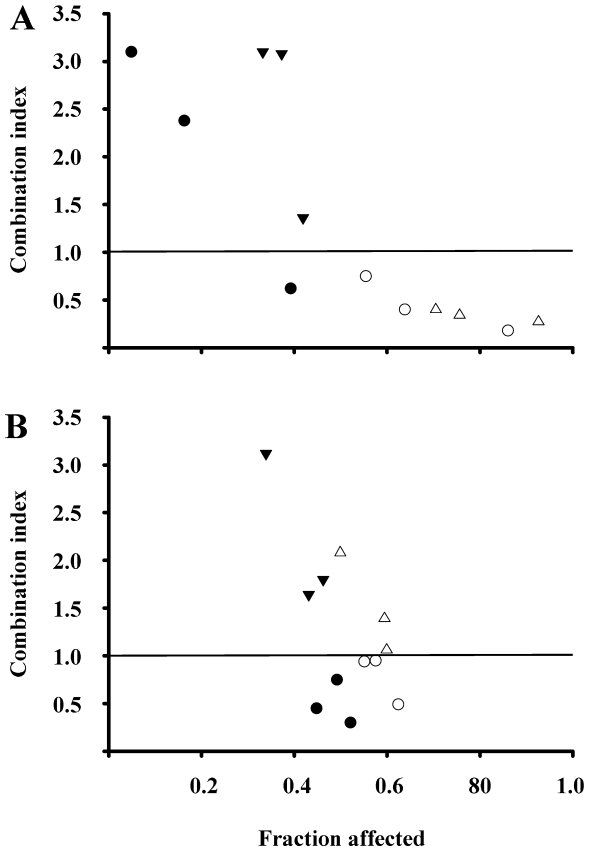


Fig. 2. Combination index values obtained in the established breast cancer cell lines exposed to different schemes of drug combinations. SUM-229PE (A) and SKBR3 (B) cells were incubated with the next drug combinations; gefitinib: vitamin D compounds; 20:20 (●); 20:50 (▼); 50:20 (○); and 50:50 (Δ). The combination index was calculated with a mathematical model developed by Chou and Talalay. The data points below combination index values of 1 denoted by a horizontal line on each plot are indicative of synergistic interactions.

3.3. Effects of gefitinib in combination with calcitriol or its synthetic analogs on cell growth

The breast cancer cell lines were treated with gefitinib, calcitriol or vitamin D analogs at their corresponding IC_{20} and IC_{50} values for each cell line. Treatment was done individually or in combination and the cell growth was then analyzed. As depicted in Table 2, incubations in the presence of gefitinib in combination with calcitriol, calcipotriol or EB1089 resulted in a significant and more robust inhibition of cell growth than that obtained with each drug alone in most of the combinations used (Table 2). A combination index equation, as described under material and methods, was used to determine the pharmacological effects resulting from drug combinations. As depicted in Fig. 2, in SUM-22PE cells combination of gefitinib (IC_{50}) with any of the vitamin D compounds (IC_{20} or IC_{50}) resulted in a synergistic interaction as shown by combination

Table 2
Growth inhibitory effects (GI%) exerted by gefitinib, calcitriol and its analogs alone or in combination in breast cancer cells.

SUM-299PE				
Compound	IC	GI%	Gefitinib IC_{20} GI% = 8.2 ± 6.6 Combinations GI%	Gefitinib IC_{50} GI% = 64.06 ± 7.8 Combinations GI%
Calcitriol	20	8.2 ± 12.7	39.2 ± 16.8[a]	86.0 ± 7.7[a]
	50	48.6 ± 17.1	41.9 ± 10.9	92.6 ± 4.1[a]
Calcipotriol	20	16.7 ± 13.8	16.3 ± 16.4	63.8 ± 9.2
	50	24.0 ± 14.6	37.3 ± 21.3	70.5 ± 8.9
EB1089	20	5.7 ± 11.5	4.9 ± 1.7	55.4 ± 7.5[a]
	50	29.3 ± 16.0	33.2 ± 13.5	75.6 ± 6.9[a]
SKBR3				
Compound	IC	GI%	Gefitinib IC_{20} GI% = 15.23 ± 12.0 Combinations GI%	Gefitinib IC_{50} GI% = 51.5 ± 13.4 Combinations GI%
Calcitriol	20	5.1 ± 8.2	44.8 ± 13.6[a]	33.9 ± 5.1[a]
	50	14.4 ± 8.6	55.1 ± 14.4[a]	49.9 ± 6.6
Calcipotriol	20	7.0 ± 7.7	52.1 ± 12.0[a]	43.1 ± 9.8[a]
	50	26.0 ± 8.9	62.4 ± 13.6[a]	59.9 ± 5.8[a]
EB1089	20	8.3 ± 8.4	49.2 ± 13.7[a]	46.2 ± 7.0
	50	32.8 ± 11.6	57.5 ± 12.6[a]	59.4 ± 5.8[a]
MBCDF				
Compound	IC	GI (%)	Gefitinib IC_{20} GI% = 9.4 ± 12.0 Combinations GI%	Gefitinib IC_{50} GI% = 23.5 ± 11.8 Combinations GI%
Calcitriol	20	18.5 ± 10.6	32.2 ± 13.2[a]	41.9 ± 12.5[a]
	50	11.5 ± 16.0	53.8 ± 13.6[a]	56.4 ± 11.9[a]
Calcipotriol	20	13.9 ± 9.9	33.2 ± 13.5[a]	38.7 ± 11.9[a]
	50	24.6 ± 6.3	39.0 ± 9.7[a]	44.6 ± 11.2[a]
EB1089	20	16.0 ± 10.3	32.0 ± 11.1[a]	24.9 ± 7.5
	50	27.1 ± 9.9	39.5 ± 12.0	46.6 ± 9.2[a]

Results are expressed as the mean ± S.D. percent growth inhibition of sextuplicate determinations and represent at least three different experiments.
[a] $p < 0.001$ vs. each drug alone.

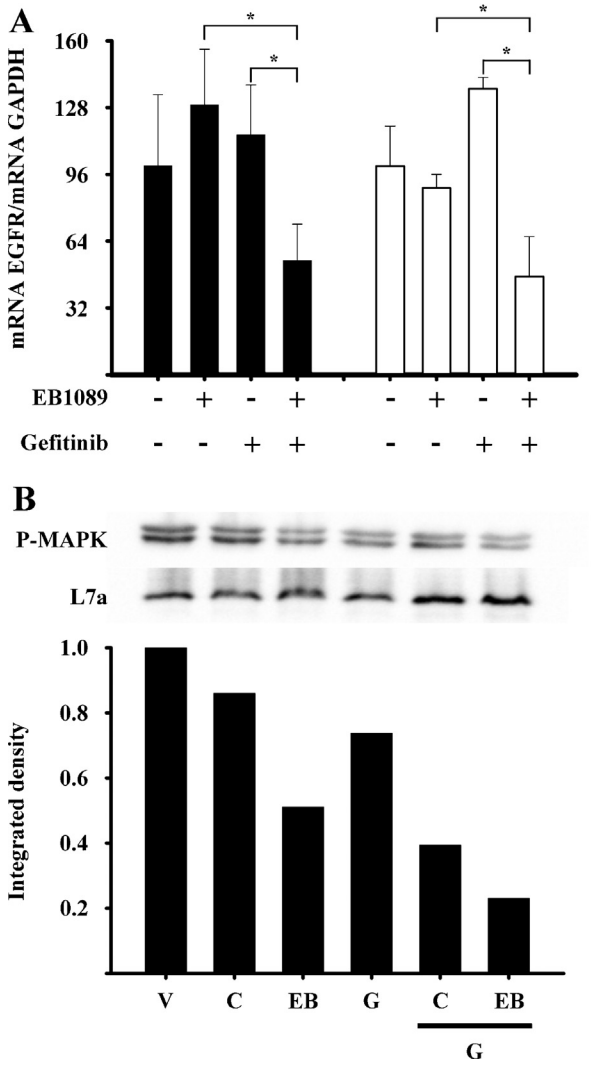


Fig. 3. Inhibition of EGFR gene expression and ERK1/2 phosphorylation by the combinations of the compounds in breast cancer cells. (A) SUM-229PE cells (black bars) were incubated with the corresponding IC_{50} values of EB1089, gefitinib and their combination during 24 h. SKBR3 cells (white bars) were incubated with the corresponding IC_{20} values of compounds. Subsequently, mRNA was extracted and qPCR was performed. Results shown are the mean ± S.D. of EGFR/GAPDH mRNA normalized ratio of three independent experiments per triplicate. Vehicle-treated cells were arbitrarily given a value of 100. $^{*}p \leq 0.05$ vs. each compound alone. (B) SUM-229PE cells were incubated in absence (V) or presence of gefitinib (G), calcitriol (C), EB1089 (EB) and drug combinations during 48 h. Western blot analysis was done to detect ERK1/2 (Thr202/Tyr204) phosphorylation and ribosomal protein L7a was used as a loading control. The value of vehicle-treated cells was set to one. Representative image from two independent experiments is shown.

index values <1 (Fig. 2A). In SKBR3 cells the synergistic effect was obtained with combinations of gefitinib (IC_{20} or IC_{50}) plus vitamin D compounds (IC_{20}) (Fig. 2B). In an EGFR negative cell line such as MBCDF the drug combinations, although inhibitory, did not result in a synergistic effect upon cell growth.

3.4. Treatment of breast cancer cells with gefitinib in combination with calcitriol or EB1089 inhibited the MAPK signaling pathway

In order to elucidate the possible mechanisms implicated in the antiproliferative effects of drug combinations, we evaluated EGFR and HER2 gene expression and the effect in MAPK signaling pathway, in the breast cancer cell lines where synergistic effect was observed. For these experiments, SUM-229PE (Fig. 3A, black bars) and SKBR3 (Fig. 3A, white bars) cell lines were cultured in the absence or presence of different drugs alone or in a combined fashion. The results demonstrated that combined treatment of gefitinib with EB1089 significantly downregulated EGFR gene expression when compared to each drug alone in both cell types. In contrast, HER2 gene expression was not affected (data not shown). The combination of gefitinib with calcitriol showed a tendency to decrease EGFR mRNA levels in both SUM-229PE and SKBR3 cell lines (data not shown).

Given that drug combinations downregulated EGFR gene expression, we decided to examine two of the members of the growth/survival transduction pathways downstream of EGFR and HER2; namely, ERK1/2 in the SUM-229PE cell line. Our results showed that the combination of gefitinib with calcitriol or EB1089 decreased the amounts of the phosphorylated form of ERK1 and ERK2 MAPK when compared to each drug alone (Fig. 3B).

3.5. Gefitinib combined with calcitriol or EB1089 induced apoptosis and diminished the percentage of cells in G2/M-phase

Based in our results regarding inhibition of cell proliferation with the drug combinations, we decided to perform cell cycle analysis and evaluate the involvement of BIM and caspase 3, as mediators of apoptosis in breast cancer cells.

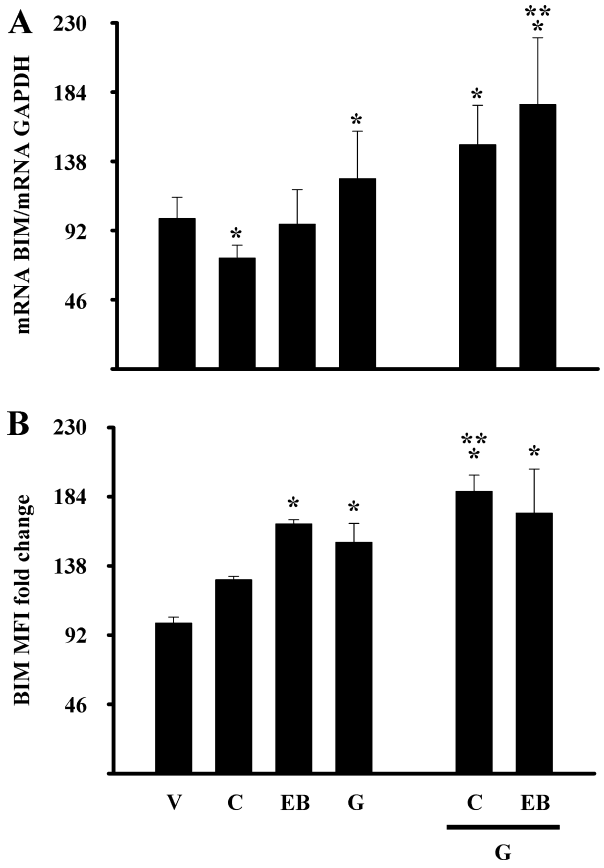


Fig. 4. Gefitinib combined with calcitriol or EB1089 increased BIM expression. (A) SUM-229PE cells were incubated with the corresponding IC_{50} values of calcitriol (C) EB1089 (EB), gefitinib (G) and their combination during 24 h. Subsequently, mRNA was extracted and qPCR was performed. Results shown are the mean ± S.D. of BIM/GAPDH mRNA normalized ratio of three independent experiments per triplicate. Vehicle-treated cells (V) were arbitrarily given a value of 100. (B) Flow cytometric analysis of BIM protein was performed after treatment of cells during 48 h. Mean fluorescence intensity (MFI) was obtained by histograms. Vehicle-treated cells were arbitrarily given a value of 100. Results are the mean ± S.D. of three different experiments. $^{**}p \leq 0.05$ vs. each compound alone. $^{*}p \leq 0.05$ vs. vehicle-treated cells.

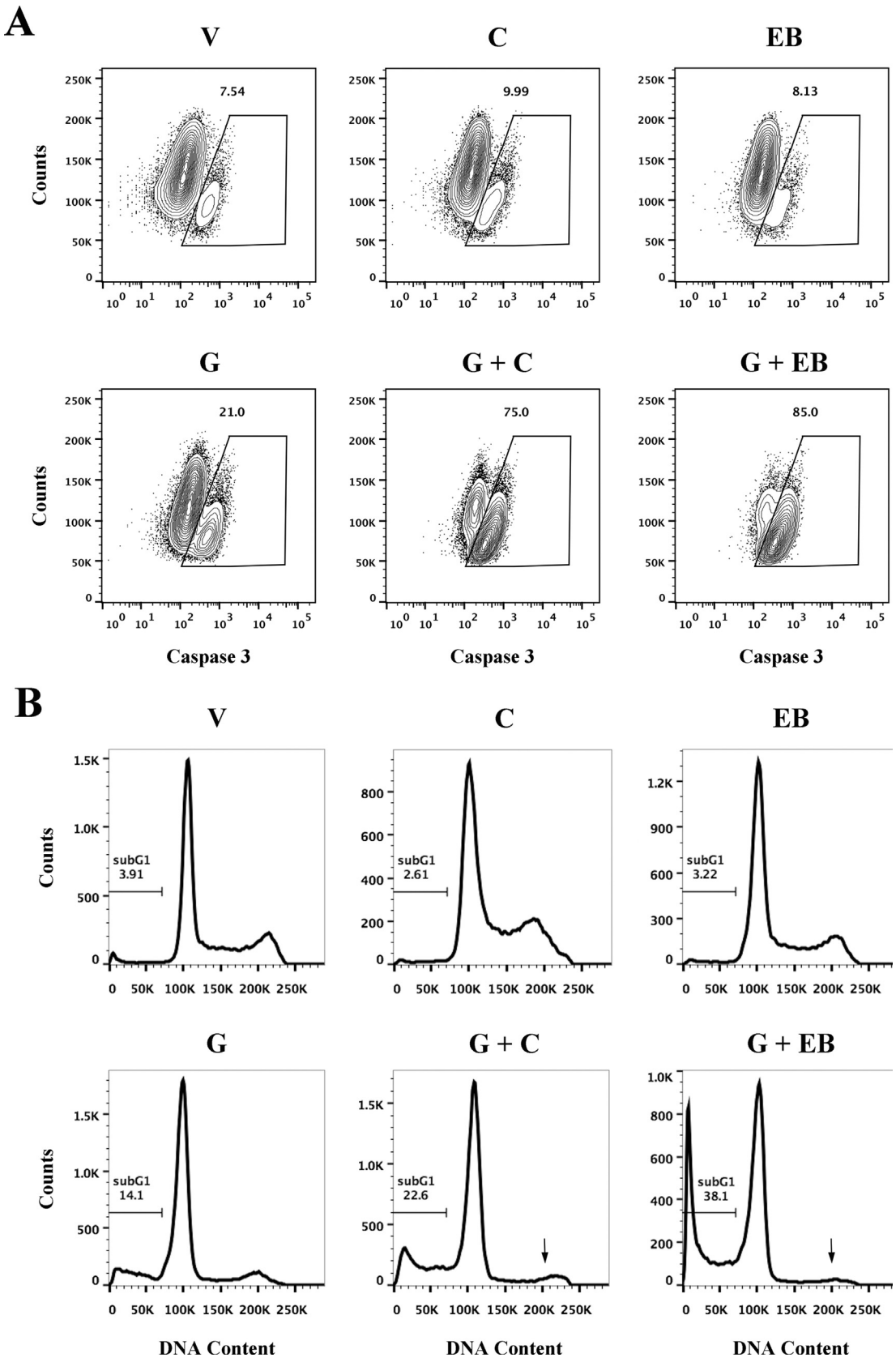


Fig. 5. Gefnitib combined with calcitriol or EB1089 induced apoptosis. SUM-229PE cells were incubated in absence (V) or presence of gefitinib (G), calcitriol (C), EB1089 (EB) and drug combinations during 72 (A) and 96 h (B). (A) Active caspase 3 analysis. Cells were permeabilized, fixed, and stained for active caspase 3. Then, cells were analyzed by flow cytometry and caspase 3-positive cells are shown in the gate. (B) DNA content on the cell cycle was analyzed. The histograms show representative DNA profiles of cells treated with the agents. SubG1 phase is indicated with a marker and reduction of G2/M-phase by the drug combinations with arrows. Images are representative from two (A) and three (B) independent experiments.

Table 3
Percentage of SUM-229PE cells in cell cycle phases after treatment with IC_{50} values of gefitinib, calcitriol, EB1089 or their combination in the cell cycle phases.

Phase	Treatment					
	V	*C*	EB	*G*	*C + G*	EB + *G*
SubG1	5.6 ± 2.6	4.6 ± 2.9	4.46 ± 1.1	14.3 ± 0.2[a]	27.8 ± 7.3[a,b]	38.7 ± 0.8[a,b]
G1	45.6 ± 5.9	38.7 ± 9.0	47.6 ± 1.3	60.2 ± 6.0[a]	61.6 ± 7.4[a]	67.0 ± 5.7[a]
S	29.2 ± 3.0	27.5 ± 5.9	24.4 ± 3.0	15.3 ± 3.2[a]	19.1 ± 4.4[a]	14.1 ± 4.2[a]
G2/M	18.8 ± 0.6	23.1 ± 2.8	18.3 ± 3.6	10.0 ± 2.0[a]	2.7 ± 3.2[a,b]	0.3 ± 0.1[a,b]

V: vehicle; C: calcitriol; EB: EB1089; G: gefitinib. Results are the mean ± S.D. from three independent assays.
[a] $p < 0.05$ vs. vehicle-treated cells.
[b] $p < 0.05$ vs. each drug alone.

Gefitinib in combination with calcitriol or EB1089 increased significantly BIM gene expression when compared to vehicle-treated cells in both SUM-229PE (Fig. 4A) and SKBR3 cells (data not shown). In SUM-229PE, the combination of gefitinib with EB1089 significantly upregulated BIM mRNA levels when compared to each drug alone. Similar results were obtained with gefitinib plus calcitriol in BIM protein levels (Fig. 4B).

Active caspase 3 was evaluated in treated cells with drug combinations by flow cytometry. Most of the active caspase 3-positive cells were smaller than unstained live cells (Fig. 5A). The combined treatments of gefitinib with calcitriol (64.4% ± 15.0) or with EB1089 (75.5% ± 13.4) significantly increased the percentages of active caspase 3-positive cells when compared to each drug alone (gefitinib, calcitriol and EB1089 17.2% ± 5.4, 9.4% ± 0.8, and 9.65 ± 2.0, respectively), in SUM-229PE cells. These results indicated that combinations of the compounds induced the activation of apoptotic pathway.

Fig. 5B shows the histograms of DNA profiles of cells treated with the agents. The combined treatments significantly increased the percentage of G1-phase cells, whereas the percentages of cells in S-phase and G2/M-phase were significantly diminished. Cell death was induced by the drug combinations as judged by the induction of subG1 cell region. This effect was statistically significant compared to each compound alone (Table 3). These data suggest that this increase of subG1 peak derived of reduction in the percentage of cells from S and G2/M-phases.

4. Discussion

HER2 is overexpressed in approximately 20% of all breast tumors. Coexpression of this receptor with EGFR has been associated with a more aggressive clinical behavior, tumor progression and resistance to treatment [3,7–9]. Concomitant inhibition of HER2 and EGFR signaling is a promising strategy in breast cancer therapy. In this regard, gefitinib has been shown to reduce proliferation of breast cancer cells expressing different levels of EGFR or HER2 by inhibiting their tyrosine kinase activity [11–13]. On the other hand, calcitriol is considered a potent inhibitor of breast cancer cells growth independently of their molecular phenotype *in vivo* and *in vitro* [23,24,30–32]. In addition, calcitriol antineoplastic effects have been studied in various combination treatments with multiple anticancer agents resulting in increased antitumor activity [33]. In this study, we demonstrated that the combination of gefitinib with calcitriol or two synthetic analogs (calcipotriol and EB1089) resulted in a greater antiproliferative effect than either drug alone in all breast cancer cells evaluated.

In addition, herein we observed that the sensitivity to gefitinib of the EGFR-overexpressing SUM-229PE cells was similar to HER2-overexpressing SKBR3 cells. In both cell lines, cell growth was inhibited by gefitinib with a similar IC_{50} of 1.5×10^{-7} M. These results indicate that the clinical potential of this drug is not limited to tumors with EGFR overexpression, and suggest that it may represent a novel modality in the treatment of patients with HER2 and EGFR-expressing tumors, which is consistent with previous reports [13,14]. We also compared the effects of calcitriol and its analogs on the proliferation of cultured breast cancer cell lines. The established cell lines and primary breast cancer cell culture included in this study had differences in the sensitivity to calcitriol and its analogs. In particular, MBCDF cells, which were EGFR-negative and HER2-positive, were less sensitive to gefitinib, calcitriol and calcipotriol, compared to the established breast cancer cells. The low sensitivity to gefitinib could be due to lack of EGFR.

Two studies have evaluated the effect of gefitinib in combination with calcitriol in phase I clinical trials. These studies were conducted to determine the maximum tolerated dose of intravenously administrated calcitriol in combination with gefitinib in patients with refractory solid tumors, including one patient with breast cancer. In these studies, drug combination was not associated with an improved clinical outcome; however, phenotype of tumors was neither included nor characterized [34,35]. Considering that EGFR/HER2 are targets of gefitinib [11,13]; in this study, we used breast cancer cells which are known to express both of them and a primary cell culture lacking EGFR. In all cell lines studies a better antiproliferative effect was observed with the combined treatment than that obtained with compounds alone. Notably, the primary cell culture, which was less sensitive to gefitinib, calcitriol or calcipotriol separately, showed a significant reduction in cell proliferation when exposed to drug combinations. Remarkably, SUM-229PE cells were more sensitive to the combination of gefitinib with any of the vitamin D compounds to inhibit cell proliferation with respect to the other two breast cancer lines. Noteworthy, the assessed combinations of IC values on cell growth using gefitinib and calcitriol or its analogs include therapeutically relevant drug concentrations that can be achieved in human blood and were previously reported (~1 μM and 3–16 nM for gefitinib and calcitriol, respectively) [34,36,37].

The presence of vitamin D response elements in EGFR promoter has been previously identified [38]. In addition, calcitriol decreased EGF binding proteins in human breast cancer cells [26]. Herein, the observation that combining gefitinib with calcitriol or EB1089 treatment significantly diminished EGFR gene expression over that obtained with the compounds alone, suggested that this treatment targets EGFR through both signaling disruption and inhibition of gene expression. Therefore, the effect of the combined treatment in phosphorylation of ERK1/2, members of the growth/survival transduction pathway downstream of EGFR and HER2, were examined. The MAPK signaling pathway was more strongly diminished by the combination of gefitinib with calcitriol or EB1089 compared to the effect of each drug alone.

Inhibition of ERK1/2 phosphorylation could induce caspase-3-dependent apoptosis via upregulation of pro-apoptotic proteins such as BIM [39]. In addition, it has been described that BIM gene expression is induced by gefitinib [15,40]. Interestingly, in this

study we showed that BIM expression was increased by the combination of gefitinib with calcitriol or EB1089. This suggests that the combined treatments activate the intrinsic pro-apoptotic pathway through to regulation of BIM, contributing to the induction of apoptosis, which is supported by the observation in this study of induction of active caspase 3 and cell death detected by subG1 region. Interestingly, these effects were greater with the treatment of gefitinib plus EB1089 compared to that of gefitinib plus calcitriol. This might be because EB1089 by itself is more potent than calcitriol to induce cell death in breast cancer cells, as previously reported [20]. This result is important given the potential use of vitamin D synthetic analogs as coadjuvants in cancer treatment, particularly because of their low calcemic effects. Further *in vivo* studies should be conducted in order to ascertain these assumptions.

In contrast to other similar studies on the isolated use of tyrosine kinase inhibitors or vitamin D [13,41], in this study we have clearly shown that combinations of these drugs, increased significantly the ability of gefitinib to inhibit cell growth and induce cellular death in breast cancer cells. These observations may have important clinical applications, since lower doses of each of these compounds could be employed for therapeutic options in a subset of EGFR and HER2 positive breast cancer.

5. Conclusion

The simultaneous treatment with gefitinib and calcitriol or EB1089 in EGFR and HER2 positive-breast cancer cells proved to be significantly better to inhibit proliferation and induce apoptosis. These effects may be mediated by the modulation of MAPKs and the upregulation of BIM expression resulting in caspase 3 dependent apoptosis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RGB was involved in the conception, design and coordination of the study as well as in data analysis, interpretation of results, actively participated in experimental procedures, and was involved in drafting the manuscript. MSM was in charge of all experimental procedures, participated in coordination, analysis and interpretation of data, as well as in drafting of manuscript. LD participated in experimental procedures, interpretation of results and made a significant contribution to the study and drafting the manuscript. MEGG, IMR, JGQ, MJIS, HPG and JEL participated in the experimental procedures and revised critically the content of the manuscript. MJIS and JEL participated in experimental procedures, provided breast biopsies, carried out the clinical data collection, retrieved patients signed informed-consent forms and revised critically the content of the manuscript. FL participated in the interpretation of data, made substantive intellectual contribution to the study and drafting the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by grant 129315 to RGB from the Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico. The authors state that there are non-financial competing interests. We thank the financial support from CONACyT (MOD-ORD-18-2014, PCI-006-06-14) granted to JGQ. We acknowledge with thanks to AstraZeneca for gefitinib donation. MSM is a Ph.D, student from the Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Mexico, and recipient of a fellowship from CONACyT (324486). This study was conducted as part of her doctoral thesis.

References

[1] N.E. Hynes, H.A. Lane, ERBB receptors and cancer: the complexity of targeted inhibitors, Nat. Rev. Cancer 5 (2005) 341–354.
[2] Y. Yarden, The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities, Eur. J. Cancer 4 (Suppl. 37) (2001) S3–8.
[3] J.S. Ross, E.A. Slodkowska, W.F. Symmans, L. Pusztai, P.M. Ravdin, G.N. Hortobagyi, The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine, Oncologist 14 (2009) 320–368.
[4] J. Baselga, S.M. Swain, Novel anticancer targets: revisiting ERBB2 and discovering ERBB3, Nat. Rev. Cancer 9 (2009) 463–475.
[5] S.T. Lee-Hoeflich, L. Crocker, E. Yao, T. Pham, X. Munroe, K.P. Hoeflich, M.X. Sliwkowski, H.M. Stern, A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy, Cancer Res. 68 (2008) 5878–5887.
[6] D. Graus-Porta, R.R. Beerli, J.M. Daly, N.E. Hynes, ErbB-2 the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling, EMBO J. 16 (1997) 1647–1655.
[7] A.L. Harris, S. Nicholson, J.R. Sainsbury, J. Farndon, C. Wright, Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu, J. Steroid Biochem. 34 (1989) 123–131.
[8] A. Osaki, M. Toi, H. Yamada, H. Kawami, K. Kuroi, T. Toge, Prognostic significance of co-expression of c-erbB-2 oncoprotein and epidermal growth factor receptor in breast cancer patients, Am. J. Surg. 164 (1992) 323–326.
[9] A. Harlozinska, J.K. Bar, R. Wenderski, M. Bebenek, Relationship between c-erbB-2 oncoprotein, epidermal growth factor receptor, and estrogen receptor expression in patients with ductal breast carcinoma. Association with tumor phenotypes, In Vivo 10 (1996) 217–222.
[10] T.S. Mok, Y.L. Wu, S. Thongprasert, C.H. Yang, D.T. Chu, N. Saijo, P. Sunpaweravong, B. Han, B. Margono, Y. Ichinose, Y. Nishiwaki, Y. Ohe, J.J. Yang, B. Chewaskulyong, H. Jiang, E.L. Duffield, C.L. Watkins, A.A. Armour, M. Fukuoka, Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma, N. Engl. J. Med. 361 (2009) 947–957.
[11] J. Anido, P. Matar, J. Albanell, M. Guzman, F. Rojo, J. Arribas, S. Averbuch, J. Baselga, ZD1839 a specific epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, induces the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers and prevents heregulin signaling in HER2-overexpressing breast cancer cells, Clin. Cancer Res. 9 (2003) 1274–1283.
[12] M. Campiglio, A. Locatelli, C. Olgiati, N. Normanno, G. Somenzi, L. Vigano, M. Fumagalli, S. Menard, L. Gianni, Inhibition of proliferation and induction of apoptosis in breast cancer cells by the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor ZD1839 ('Iressa') is independent of EGFR expression level, J. Cell Physiol. 198 (2004) 259–268.
[13] S.L. Moulder, F.M. Yakes, S.K. Muthuswamy, R. Bianco, J.F. Simpson, C.L. Arteaga, Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo, Cancer Res. 61 (2001) 8887–8895.
[14] M.M. Moasser, A. Basso, S.D. Averbuch, N. Rosen, The tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells, Cancer Res. 61 (2001) 7184–7188.
[15] J. Krol, R.E. Francis, A. Albergaria, A. Sunters, A. Polychronis, R.C. Coombes, E.W. Lam, The transcription factor FOXO3a is a crucial cellular target of gefitinib (Iressa) in breast cancer cells, Mol. Cancer Ther. 6 (2007) 3169–3179.
[16] D. Feldman, A.V. Krishnan, S. Swami, E. Giovannucci, B.J. Feldman, The role of vitamin D in reducing cancer risk and progression, Nat. Rev. Cancer 14 (2014) 342–357.
[17] E.C. Janowsky, G.E. Lester, C.R. Weinberg, R.C. Millikan, J.M. Schildkraut, P.A. Garrett, B.S. Hulka, Association between low levels of 1,25-dihydroxyvitamin D and breast cancer risk, Public Health Nutr. 2 (1999) 283–291.
[18] E.B. Mawer, J. Walls, A. Howell, M. Davies, W.A. Ratcliffe, N.J. Bundred, Serum 1,25-dihydroxyvitamin D may be related inversely to disease activity in breast cancer patients with bone metastases, J. Clin. Endocrinol. Metab. 82 (1997) 118–122.
[19] U. Berger, R.A. McClelland, P. Wilson, G.L. Greene, M.R. Haussler, J.W. Pike, K. Colston, D. Easton, R.C. Coombes, Immunocytochemical determination of estrogen receptor, progesterone receptor, and 1,25-dihydroxyvitamin D3 receptor in breast cancer and relationship to prognosis, Cancer Res. 51 (1991) 239–244.
[20] I.S. Mathiasen, U. Lademann, M. Jaattela, Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53, Cancer Res. 59 (1999) 4848–4856.
[21] M. Simboli-Campbell, C.J. Narvaez, M. Tenniswood, J. Welsh, 1,25-Dihydroxyvitamin D3 induces morphological and biochemical markers of apoptosis in MCF-7 breast cancer cells, J. Steroid Biochem. Mol. Biol. 58 (1996) 367–376.
[22] L. Lowe, C.M. Hansen, S. Senaratne, K.W. Colston, Mechanisms implicated in the growth regulatory effects of vitamin D compounds in breast cancer cells, Recent Results Cancer Res. 164 (2003) 99–110.

[23] J. Garcia-Quiroz, R. Garcia-Becerra, D. Barrera, N. Santos, E. Avila, D. Ordaz-Rosado, M. Rivas-Suarez, A. Halhali, P. Rodriguez, A. Gamboa-Dominguez, H. Medina-Franco, J. Camacho, F. Larrea, L. Diaz, Astemizole synergizes calcitriol antiproliferative activity by inhibiting CYP24A1 and upregulating VDR: a novel approach for breast cancer therapy, PLoS One 7 (2012) e45063.
[24] N. Santos-Martinez, L. Diaz, D. Ordaz-Rosado, J. Garcia-Quiroz, D. Barrera, E. Avila, A. Halhali, H. Medina-Franco, M.J. Ibarra-Sanchez, J. Esparza-Lopez, J. Camacho, F. Larrea, R. Garcia-Becerra, Calcitriol restores antiestrogen responsiveness in estrogen receptor negative breast cancer cells: a potential new therapeutic approach, BMC Cancer 14 (2014) 230.
[25] R. Vieth, The mechanisms of vitamin D toxicity, Bone Miner. 11 (1990) 267–272.
[26] M. Koga, J.A. Eisman, R.L. Sutherland, Regulation of epidermal growth factor receptor levels by 1,25-dihydroxyvitamin D3 in human breast cancer cells, Cancer Res. 48 (1988) 2734–2739.
[27] Z. Shen, X. Zhang, J. Tang, R. Kasiappan, U. Jinwal, P. Li, S. Hann, S.V. Nicosia, J. Wu, W. Bai, The coupling of epidermal growth factor receptor down regulation by 1alpha, 25-dihydroxyvitamin D3 to the hormone-induced cell cycle arrest at the G1-S checkpoint in ovarian cancer cells, Mol. Cell Endocrinol. 338 (2011) 58–67.
[28] J. Esparza-Lopez, H. Medina-Franco, E. Escobar-Arriaga, E. Leon-Rodriguez, A. Zentella-Dehesa, M.J. Ibarra-Sanchez, Doxorubicin induces atypical NF-kappaB activation through c-Abl kinase activity in breast cancer cells, J. Cancer Res. Clin. Oncol. 139 (2013) 1625–1635.
[29] T.C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies, Pharmacol. Rev. 58 (2006) 621–681.
[30] K.K. Deeb, D.L. Trump, C.S. Johnson, Vitamin D signalling pathways in cancer: potential for anticancer therapeutics, Nat. Rev. Cancer. 7 (2007) 684–700.
[31] D.L. Trump, K.K. Deeb, C.S. Johnson, Vitamin D: considerations in the continued development as an agent for cancer prevention and therapy, Cancer J. 16 (2010) 1–9.
[32] S. Swami, A.V. Krishnan, D. Feldman, 1alpha, 25-dihydroxyvitamin D3 down-regulates estrogen receptor abundance and suppresses estrogen actions in MCF-7 human breast cancer cells, Clin. Cancer Res. 6 (2000) 3371–3379.
[33] Y. Ma, D.L. Trump, C.S. Johnson, Vitamin D in combination cancer treatment, J. Cancer 1 (2010) 101–107.
[34] M.G. Fakih, D.L. Trump, J.R. Muindi, J.D. Black, R.J. Bernardi, P.J. Creaven, J. Schwartz, M.G. Brattain, A. Hutson, R. French, C.S. Johnson, A phase I pharmacokinetic and pharmacodynamic study of intravenous calcitriol in combination with oral gefitinib in patients with advanced solid tumors, Clin. Cancer Res. 13 (2007) 1216–1223.
[35] J.R. Muindi, C.S. Johnson, D.L. Trump, R. Christy, K.L. Engler, M.G. Fakih, A phase I and pharmacokinetics study of intravenous calcitriol in combination with oral dexamethasone and gefitinib in patients with advanced solid tumors, Cancer Chemother. Pharmacol. 65 (2009) 33–40.
[36] M.H. Cohen, G.A. Williams, R. Sridhara, G. Chen, W.D. McGuinn Jr., S. Morse, A. Rahman, C. Liang, R. Lostritto, A. Baird, R. Pazdur, United States Food and Drug Administration Drug Approval summary: Gefitinib (ZD1839; Iressa) tablets, Clin. Cancer Res. 10 (2004) 1212–1218.
[37] T.M. Beer, Development of weekly high-dose calcitriol based therapy for prostate cancer, Urol. Oncol. 21 (2003) 399–405.
[38] K.R. McGaffin, S.A. Chrysogelos, Identification and characterization of a response element in the EGFR promoter that mediates transcriptional repression by 1,25-dihydroxyvitamin D3 in breast cancer cells, J. Mol. Endocrinol. 35 (2005) 117–133.
[39] J.Y. Song, C.S. Kim, J.H. Lee, S.J. Jang, S.W. Lee, J.J. Hwang, C. Lim, G. Lee, J. Seo, S.Y. Cho, J. Choi, Dual inhibition of MEK1/2 and EGFR synergistically induces caspase-3-dependent apoptosis in EGFR inhibitor-resistant lung cancer cells via BIM upregulation, Invest. New Drugs 31 (2013) 1458–1465.
[40] M.S. Cragg, J. Kuroda, H. Puthalakath, D.C. Huang, A. Strasser, Gefitinib-induced killing of NSCLC cell lines expressing mutant EGFR requires BIM and can be enhanced by BH3 mimetics, PLoS Med. 4 (2007) 1681–1689 Discussion 1690.
[41] M. Simboli-Campbell, C.J. Narvaez, K. van Weelden, M. Tenniswood, J. Welsh, Comparative effects of 1,25(OH) 2D3 and EB1089 on cell cycle kinetics and apoptosis in MCF-7 breast cancer cells, Breast Cancer Res. Treat. 42 (1997) 31–41.

21. ANEXO 2: PRODUCTIVIDAD CIENTIFICA: ARTÍCULO Y ESTANCIA DE INVESTIGACIÓN

2.- Efficacy and mechanism of action of the tyrosine kinase inhibitors gefitinib, lapatinib and neratinib in the treatment of HER2-positive breast cancer: preclinical and clinical evidence.

Segovia-Mendoza M, González-González M.E., Barrera D, Díaz L, García-Becerra R. Am J Cancer Res 2015;5(9):2531-2561

- Se anexa la publicación derivada de los estudios de doctorado en formato de revisión
- Se anexa constancia de estancia internacional de investigación realizada durante los estudios del doctorado

Am J Cancer Res 2015;5(9):2531-2561
www.ajcr.us /ISSN:2156-6976/ajcr0012755

Review Article

Efficacy and mechanism of action of the tyrosine kinase inhibitors gefitinib, lapatinib and neratinib in the treatment of HER2-positive breast cancer: preclinical and clinical evidence

Mariana Segovia-Mendoza[1,2], María E González-González[1], David Barrera[1], Lorenza Díaz[1], Rocío García-Becerra[1]

[1]Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Avenida Vasco de Quiroga No. 15, Col. Belisario Domínguez Sección XVI, Tlalpan 14080, México, D. F., México; [2]Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Circuito Interior, Cuidad Universitaria, Av. Universidad 3000, Coyoacán 04510, México D. F, México

Received July 11, 2015; Accepted July 13, 2015; Epub August 15, 2015; Published September 1, 2015

Abstract: An increasing number of tumors, including breast cancer, overexpress proteins of the epidermal growth factor receptor (EGFR) family. The interaction between family members activates signaling pathways that promote tumor progression and resistance to treatment. Human epidermal growth factor receptor type II (HER2) positive breast cancer represents a clinical challenge for current therapy. It has motivated the development of novel and more effective therapeutic EGFR family target drugs, such as tyrosine kinase inhibitors (TKIs). This review focuses on the effects of three TKIs mostly studied in HER2- positive breast cancer, lapatinib, gefitinib and neratinib. Herein, we discuss the mechanism of action, therapeutic advantages and clinical applications of these TKIs. To date, TKIs seem to be promising therapeutic agents for the treatment of HER2-overexpressing breast tumors, either as monotherapy or combined with other pharmacological agents.

Keywords: HER2-positive breast cancer, tyrosine kinase inhibitors, lapatinib, gefitinib, neratinib, epidermal growth factor receptor family

Introduction

Breast cancer is the most common malignancy in women and is a major public health issue [1, 2]. In the clinic, breast cancer is mainly classified into four molecular subtypes, luminal A/B, human epidermal growth factor receptor type II (HER2) and basal-like [3, 4]. The subtype HER2 represents 25-30% of all breast cancer cases and HER2 overexpression is strongly associated with aggressive phenotype and poor outcomes [5, 6]. The current and approved therapy for this type of cancer is trastuzumab, a humanized monoclonal antibody that binds to the extracellular domain of HER2 [7, 8]. However, *de novo* or acquired resistance to therapy occurs in some patients [9]. Consequently, new targeted therapies are in development, such as tyrosine kinase inhibitors (TKIs) [10]. This paper aims to integrate knowledge of the signaling pathways associated to the major TKIs proved on HER2-positive breast cancer, lapatinib, geftinib and neratinib. Moreover, we discuss molecular mechanisms, resistance and clinical trials for each drug, as well as their beneficial therapeutic effects and undesirable side effects.

EGFR family

The EGFR family comprises four distinct membrane tyrosine kinase receptors; EGFR/ErbB-1, HER2/ErbB-2, HER3/ErbB-3 and HER4/ErbB-4 which are activated upon ligand binding to the extracellular domain of these receptors. Afterwards, the formation of receptor homo- or hetero-dimers is induced resulting in phosphorylation of tyrosine kinases residues and cross-

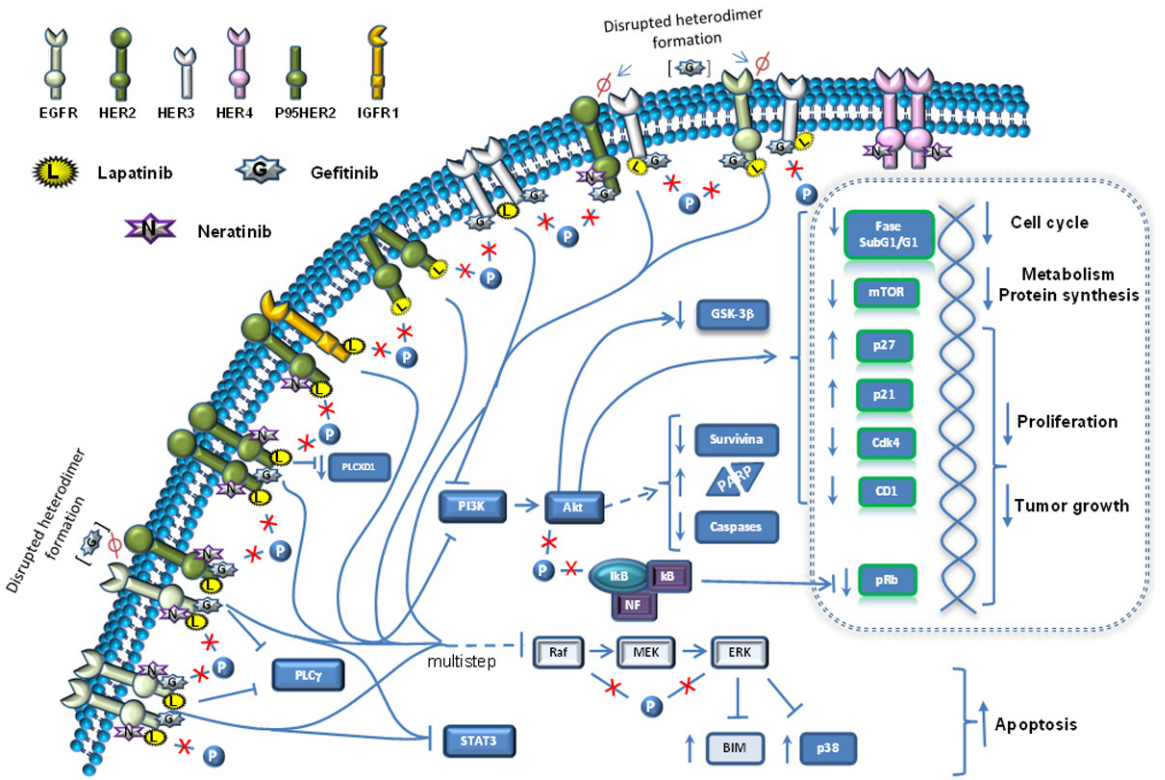


Figure 1. Schematic representation of the action of three TKIs and their interaction with receptors of the EGFR family. As TKIs are homologous to ATP, they compete for the ATP-binding domain of protein kinases preventing their phosphorylation and subsequent activation of the signal transduction pathways, leading to apoptosis, decreased cellular proliferation and eventually cell cycle arrest. Inhibition of phosphorylation of the receptors by TKIs (X); disrupted heterodimer formation by gefitinib, avoid the interaction between receptors (ϕ); upregulated (↑); down-regulated (↓).

phosphorylation, that triggers numerous signaling pathways such as phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK1/2), signal transducer and activator of transcription (STAT), phospholipase C (PLCγ), and/or the modulation of calcium channels [11], This sequence of events induces cellular responses which include proliferation, differentiation and inhibition of apoptosis, giving rise to diseases such as cancer [12].

In a wide range of epithelial cells, including breast, colon, head, neck, kidney, lung, pancreas, and prostate, the overexpression and constitutive activation of the EGFR family members, particularly EGFR and HER2, may trigger cancer initiation, metastasis, and tumor progression [13-15].

In particular, HER2 is overexpressed/amplified in 20-30% of patients with metastatic breast cancer [16]. Moreover, there is a growing evidence that heterodimer formation between receptors of EGFR members resulted in adverse response to therapy [17]. In order to block EGFRs intracellular signaling pathways in breast cancer, the development of novel therapies which include the use of TKIs is currently underway.

Tyrosine kinase inhibitors

The TKIs are oral non-peptide anilinoquinazolone compounds homologous of the adenosine triphosphate (ATP). This similarity allows them to compete for the ATP-binding domain of protein kinases preventing phosphorylation and subsequent activation of the signal transduction pathways, leading to apoptosis and decreasing cellular proliferation [18]. Moreover, TKIs target other kinase receptors due to the homology that they share with the EGFR family in the catalytic domain [19] which is highly con-

served across the kinome [20]. Whereby, the actions of TKIs in several kinases cause different effects in the therapeutic use [21, 22].

The main characteristics, mechanisms of action, causes of resistance and clinical evidences of the major TKIs proved on HER2-positive breast cancer, lapatinib, geftinib and neratinib, are described below.

Lapatinib

Lapatinib is a reversible dual TKI that selectively targets and inhibits HER2 and EGFR with proven effectiveness in clinical trials. This inhibitor has been approved by the US Food and Drug Administration (FDA) since 2007 for metastatic HER2-positive breast cancer treatment. It is commonly used in combination with the chemotherapeutic agent capecitabine on the treatment of breast cancer patients previously treated with trastuzumab, anthracyclines and taxane [23-25]. Moreover, the compound combined with letrozole, an aromatase inhibitor, has been accepted as first-line therapy for metastatic breast cancer that coexpresses hormone receptors and HER2 [26].

Mechanism of action of lapatinib: preclinical evidence

Lapatinib inhibits proliferation of several human cancer cell lines from vulva, breast, lung, and esophagus [27-29]. *In vitro* studies showed that lapatinib inhibited the activation of the three main EGFR and HER2 downstream signaling pathways, MAPK, PI3K-AKT and PLCγ, through decreased phosphorylation of target receptors and the Raf, ERK, AKT, and PLCγ1 proteins. Additionally, this TKI increased p38 expression, a stress-induced member of the MAPK pathway that is involved in apoptosis, the subG1 phase of the cell cycle (a hallmark of apoptosis), and the cyclin-dependent kinase inhibitors p21 and p27 [30-32]. Lapatinib inhibited cell proliferation and migration of breast cancer cell lines expressing different levels of EGFR and HER2; however, cells overexpressing HER2 were more sensitive to this TKI [30]. Also, lapatinib increased the pro-apoptotic protein BIM at the transcriptional level and reduced protein expression of survivin, an apoptosis inhibitor protein, which is express in 90% of all breast cancer cases and is cause of poor outcome for this pathology [33-35]. Although lapatinib is a dual TKI that targets both HER2 and EGFR, it has been demonstrated that it also inhibited phosphorylation of HER3 [36]. A resume of lapatinib mechanisms is found in **Figure 1**.

There is a high incidence of brain metastases in patients with HER2-overexpressing breast cancer even if they were treated with trastuzumab [37, 38]. Interestingly, in a preclinical mouse model, lapatinib could prevent the metastatic outgrowth of HER2-overexpressing breast cancer cells in the brain. In this *in vivo* metastasis model, lapatinib reduced the phosphorylation of HER2 but it did not affect EGFR, contrary to *in vitro* studies [30]. Moreover, EGFR small-interfering RNA (siRNA) knockdown in HER2-positive breast cancer cells did not affect the antiproliferative activity of lapatinib, whereas depletion of HER2 causes lapatinib resistance, indicating that lapatinib effects are mediated mainly through HER2 pathway [32]. The stated above suggests a direct correlation between lapatinib sensitivity and HER2 expression only.

A subgroup of HER2-overexpressing tumors also express p95HER2, an amino terminally truncated receptor, that has kinase activity but lacks the epitope recognized by trastuzumab; hence, expression of this form confers resistance to trastuzumab [39]. In addition, p95-HER2 has been considered as a biomarker of an aggressive subtype of HER2-positive breast cancer [40]. Lapatinib inhibited p95HER2, AKT, MAPK phosphorylation and the growth of cells that express the truncate receptors. Moreover, lapatinib showed antitumor activity in p95HER2 tumor xenografts [41].

Other study demonstrated that lapatinib inhibited insulin-like growth factor I (IGF-I) signaling in both trastuzumab -sensitive and -resistant HER2 overexpressing cells [42]. Cross-talk between the IGF-I receptor and HER2 in trastuzumab-resistant cells increased HER2 receptor phosphorylation [43]. Significantly, lapatinib blocked HER2 and IGF-1R crosstalk [42]. In addition, this compound also increased fragmentation of poly ADP-ribose polymerase (PARP), a protein involved in programmed cell death, and downregulated survivin expression in trastuzumab sensitive and resistant HER2 overexpressing cells [42].

Table 1. Clinical evidence with lapatinib in HER2-positive breast cancer

Therapy [Ref.]	Study type	Patient population (n)	Principal findings
Lapatinib [53]	Phase II, open-label, two-stage, two-cohorts multicenter study.	Advanced or metastatic IBC, HER2 and/or EGFR positive, refractory or recurrent after treatment with an anthracycline. Cohort A: HER2-positive (30). Cohort B: EGFR-positive and HER2 negative (15).	Lapatinib (1500 mg/day) was well tolerated in both cohorts. It showed clinical activity (CR=7%, PR=43%, OR=16.9%, PFS=14 weeks) in heavily pretreated HER2-positive but not in EGFR-positive/HER2-negative patients (PR=6.6%, PFS=4 weeks). The most common AEs in both cohorts included grade 1/2 diarrhea, musculoskeletal pain, and rash. Serious AEs included musculoskeletal pain, dyspnea, and diarrhea. PTEN status does not preclude response to lapatinib. Coexpression of phospho HER2 and phospho HER3 in tumors seems to predict for a favorable response to lapatinib.
Lapatinib [163]	Phase II, open-label, single-group, multicenter study.	Advanced or MBC with HER2 overexpression, with disease progression during TZ therapy (78).	Lapatinib demonstrated clinical activity (CBR=11.5%, TTP and PFS=15.3 weeks). Doses of 1250 and 1500 mg/day were well tolerated. AEs were rash, diarrhea, nausea, and fatigue with no grade 4 events.
Lapatinib [54]	Phase II, open-label, single-arm, two-cohorts multicenter study.	HER2-positive (cohort A=140) and negative (cohort B=89) advanced MBC prior anthracycline and/or taxane treatment.	Lapatinib dose of 1500 mg/day provided modest clinical benefit (CBR=5.7%) only in HER2-positive patients. PFS after 4 months (34 vs 18%) and OS (29.4 vs 18.6 weeks) were higher in HER2-positive than in HER2-negative patients. The most common AEs were diarrhea, nausea and rash most grade 1/2 with maximum severity of grade 3 in both cohorts. Serious AEs were diarrhea, dehydration, nausea, and vomiting. There were 4 fatal AEs.
Lapatinib [164]	Phase II, open-label, multicenter. Study was originally designed with two cohorts but cohort B was closed.	Relapsed or refractory invasive, IBC with HER2 overexpression previously treated with anthracycline and taxane plus TZ. (Cohort A=126)	Lapatinib dose of 1500 mg/day was considered potentially clinically effective (ORR=40-15% depending on criteria, PFS=14.6 weeks, OS=8.4 months) in heavily pretreated patients. Likelihood of response to lapatinib was not affected by previous treatment with TZ. The most common serious AEs were dyspnea and pleural effusion. Fatal AEs were possibly treatment related.
Lapatinib [55]	Phase II, open-label, two-cohorts multicenter studies.	Japanese patients with advanced or MBC with HER2 positive (cohort A=122) and negative (cohort B=22), with disease progression during previously therapy with anthracycline and taxane plus TZ.	Lapatinib dose of 1500 /day was well tolerated in both cohorts and effective only in HER2-positive patients. CBR (25 vs 4.5%) and OS (58.3 vs 40 weeks) were higher in cohort A than in cohort B. The most common AE was diarrhea grade 1/2. Patients with tumours harbouring an H1047R PIK3CA mutation or low expression of PTEN derived clinical benefit from lapatinib.
Lapatinib [165]	Phase II, randomized open-label, parallel-group, multicenter study.	HER2-positive advanced or MBC. Could be previously treated. (69/69).	Lapatinib doses of 1500 mg once daily or 500 mg twice daily were safe and effective without significant differences in clinical activity or the AEs profile between them (ORR=22 and 26%, CBR=29 and 33%, PFS at 4 months=60 and 67%). The most common AEs were diarrhea, rash, pruritus, and nausea, grade 1/2.

Lapatinib [56]	Phase II	HER2-positive breast cancer with brain metastases prior TZ treatment. (39).	Lapatinib doses of 750 mg twice daily provided clinical benefit measured as absence of progression in brain metastases (ORR=2.6%, SD≥16 weeks=15.4%). The most common AEs were diarrhea and fatigue grade 2/3.
Lapatinib [166]	Phase I single institution study.	HER2-overexpressing breast cancer patients without any restrictions on prior therapies including TZ or lapatinib. (34).	This study used a 3+3 dose escalation design with a starting dose cohort of 1750 mg twice daily, ending with 7000 mg twice daily. The protocol had an amendment consisted in the use of exposure enhancement strategies such as take the medication with food, inhibition of CYP3A4 with ketoconazole and dose fractionation (four times a day) using a dose of 3000 mg. The majority of AEs were grade 1/2 and diarrhea was the most common. Lapatinib dose was escalated to 7000 mg per day with no dose limiting toxicity; however, plasma lapatinib concentrations plateaued in this dose range. 6 patients achieved a response and dramatic responses were seen in 3 patients with lapatinib concentrations approaching 10000 ng/mL.
Lapatinib [167]	Phase III, randomized double blind, placebo-controlled multicenter trial.	Patients in early stages of HER2-positive invasive breast prior adjuvant chemotherapy but not TZ. (1571/1576).	Lapatinib (1500 mg) and placebo were administrated daily for 12 months. A review of HER2 status showed that only 79% of the women were HER2-positive. In this group, with a median follow-up of 47.4 months in the lapatinib subgroup and 48.3 in the placebo group disease-free survival events occurred in 13% in the lapatinib group and 17% in the placebo group. OS=6 and 7% in lapatinib and placebo groups respectively. 6% serious AEs occurred in patients taking lapatinib and 5% in patients taking placebo with higher incidences of grade 3/4 diarrhea (6 vs 1%), rash (5 vs <1%), and hepatobiliary disorders (2 vs <1%).
Lapatinib+letrozole [26]	Phase III, randomized double-blind, controlled, parallel-group, multicenter study.	Postmenopausal women with hormone receptor-positive, HER2-positive MBC. (642/644).	Combination therapy (lapatinib 1500 mg/day plus letrozole 2.5 mg/day) was superior to endocrine therapy alone (letrozole 2.5 mg/day). Respectively: PFS=8.2 vs 3.0 months, CBR=48 vs 29%. Diarrhea and rash grade 3/4 were more common in the combination arm versus monotherapy arm. 3 fatal AEs events were possibly treatment related, 1 in combination therapy, 2 in monotherapy.
Lapatinib+vinorelbine [168]	Phase I dose-escalation multicenter study.	HER2-positive, advanced or MBC, prior TZ treatment. (30).	The maximal tolerated dose recommended for combination was 1000 mg/day of lapatinib and 22.5 mg/m^2 of vinorelbine. AEs grade 1/2 were diarrhea, rash and liver enzymes elevation. AEs grade 3/4 were neutropenia, anemia, diarrhea and asthenia.
Lapatinib+capecitabine [23]	Phase III randomized two-arms, open label study.	HER2-positive locally advanced or MBC progressed after treatment with, but were not limited to, anthracycline, taxane, and TZ. (163/161).	Combination therapy (lapatinib 1250 mg/day plus capecitabine 2000 mg/m^2/day) was superior to capecitabine monotherapy (2500 mg/m^2/day). Respectively: TTP=8.4 vs 4.4 months, PFS=8.4 vs 4.1 months, ORR=22 vs 14%, CBR=44-29%. This combination was not associated with an increase in serious toxic effects. The most common AEs were diarrhea, PPE, nausea, vomiting, fatigue and rash. Grade 4 diarrhea occurred in combination therapy arm. One death related to drug toxicity in monotherapy arm.

Lapatinib+ capecitabine [169, 170]	Phase III randomized two-arms, open label study. (Updates from Geyer, 2006).	HER2-positive locally advanced or MBC progressed after treatment with, but were not limited to, anthracycline, taxane, and TZ. (198/201).	Combination therapy (lapatinib 1250 mg/day plus capecitabine 2000 mg/m^2/day) showed to be superior versus capecitabine monotherapy (2500 mg/m^2/day). Respectively: OS=75.0 vs 64.7%, TTP=27.1 vs 18.6 months, CBR=29.3 vs 17.4%. The most common AE was diarrhea in both arms.
Lapatinib+ capecitabine [171]	Phase III, randomized two-arms, open label study. (Update from Geyer 2006).	HER2-positive locally advanced or MBC progressed after treatment with, but were not limited to, anthracycline, taxane, and TZ. (198/201).	Values favored combination therapy vs monotherapy. Respectively: ORR=24 vs 14% and TTP=33.3 vs 22.9 months.
Lapatinib+ capecitabine [172]	Phase II non-randomized open-label, single-center study.	HER2-positive MBC progressed with TZ. (22).	This combination regimen (capecitabine 2000 mg/twice day for 7 days followed by a 7-day rest and lapatinib 1250 mg/day) provided clinical benefit (CBR=50%, PFS=9.4 months), was well tolerated and reduced gastrointestinal toxicity compared with standard regimen.
Lapatinib+ capecitabine [57]	Phase II open-label, multicenter study.	HER2-positive breast cancer with brain metastases. Previous treatment was allowed except with lapatinib or capecitabine and WBRT. (44).	Combination therapy (lapatinib 1250 mg/day plus capecitabine 2000 mg/m^2/day) showed to be active in terms of objective CNS response (OR=65.9%). The most common AEs were diarrhea, PPE, nausea, fatigue, rash and bilirubin increase. AEs grade 3/4 were diarrhea, PPE and fatigue. The combination therapy could change the management of selected patients with brain metastases, allowing delay to WBRT and its AEs.
Lapatinib In extension phase: lapatinib+ capecitabine [59]	Phase II open-label, two-cohorts, multicenter study.	HER2-positive breast cancer with brain metastases prior TZ and WBRT treatment. Cohort A (95): PS=0 to 1 and 1 or 2 prior TZ regimens. Cohort B (147): PS=2 and/or >2 prior TZ regimens (95/147). Extension phase: patients progressed with lapatinib. (50).	Lapatinib alone (750 mg/twice day) showed a discrete CNS clinical activity (OR=6%, PFS=2.4 months). In extension phase, combination therapy (lapatinib 1250 mg/day plus capecitabine 1000 mg/m^2/day) showed to have clinical activity (OR=20%, PFS=3.7 months). Volumetric reduction in CNS lesions was similar in both therapies. The most common AEs were PPE, diarrhea and nausea. AEs grade 3 were PPE, nausea and diarrhea.
Lapatinib+ capecitabine [173]	Single-arm open-label trial.	HER2 locally advanced or MBC that had progressed following treatment with an anthracycline, a taxane, and TZ alone or in combination. (4283).	Combination therapy (lapatinib 1250 mg/day plus capecitabine 1000 mg/m^2/twice day) showed to be safe and to offer clinical benefit to this population. Patients treated with the combination and no prior capecitabine had higher OS and PFS than patients with prior capecitabine. (41.7 and 23.9 weeks vs 36 and 18.4 weeks respectively). The most common AEs were diarrhea, vomiting, and nausea and were mainly grade 3 or higher.

Lapatinib+ capecitabine [174]	Open-label multi-center study.	Japanese patients with HER2-positive invasive and MBC previously treated with anthracyclines, taxanes and TZ. (51).	Combination therapy (lapatinib 1250 mg/day plus capecitabine 1000 mg/m^2/twice daily) was well tolerated and showed clinical activity (CBR=59%, TTP=36 weeks). The most common AEs were PPE, diarrhea and stomatitis grade 1/2.
Lapatinib+ capecitabine [175]	Single arm open-label trial.	Chinese patients with HER2-positive invasive and MBC prior therapy with a taxane and/or an anthracycline and could have received prior TZ. (52).	Combination therapy (lapatinib, 1250 mg/day plus capecitabine 2000 mg/m^2/day) showed to be well tolerated and to offer clinical benefit (CBR=57.7%, PFS=6.34 months) to heavily pretreated patients. The most common AEs were PPE, diarrhea, rash, hyperbilirubinemia, and fatigue, all grade 1/2. Grade 3/4 AEs were rash, hyperbilirubinemia, fatigue and neutropenia. PIK3CA mutation status was not associated with CBR nor PFS.
Lapatinib+ capecitabine [176]	Phase I pos hoc analysis.	HER2-positive advanced or MBC patients. (38).	Treatment schedule consisted of lapatinib 1250 mg daily, 1 hour before or after breakfast, administered as single agent for the first 10 days, then continuously, in combination with capecitabine 2000 mg/m^2, starting on day 11 (for the first cycle), and then from day 8, for 14 days out of a 21-day cycle. Cholestyramine was administered twice a day on a continuous basis, long after capecitabine and lapatinib intake. Diarrhea was the main AE: 13.2% grade 1, 10.5% grade 2, 2.6% grade 3 and no grade 4 events. ORR=34.2%, CB=55.3%, PFS=10 months. OS (1 year)=71.2%. The results are comparable with previous reports of conventional administration of the lapatinib-capecitabine regimen and led to a significant reduction in the incidence and severity of diarrhea.
Lapatinib+Pegylated liposomal doxorubicin [177]	Phase II, open-label, single-arm multicenter study.	HER2-positive with locally advanced, inoperable or MBC with disease progression after TZ therapy. (24).	Treatment consisted in 1250 mg lapatinib daily until progression plus 40 mg/m^2 of pegylated liposomal doxorubicin at every 4 weeks for a maximum of 6 cycles. This combination showed to be active and safe in HER2-positive MBC, especially suitable for patients with cardiological risk or brain metastases. ORR was 54%. PFS and OS were 5.8 and 23.3 months respectively. The one-year PFS rate was 27% and 1-year OS rate 76%. The most commonly observed AEs were diarrhea, rash and infection. No grade 4 events reported.
Lapatinib+capecitabine vs lapatinib+topotecan [58]	Phase II, open-label, two-arms randomized study.	HER2 breast cancer with brain metastases despite prior standard treatment with WBRT and/or stereotactic radiosurgery and TZ. (13/9).	No CNS-OR was observed with lapatinib (1250 mg/day) plus topotecan (3.2 mg/m^2) therapy and showed excessive toxicity (one death possible related to it). ORR=38% in the lapatinib plus capecitabine arm.
Lapatinib+capecitabine vs trastuzumab+ capecitabine [178]	Prospective non-randomized two-arms controlled study.	Chinese patients with HER2 invasive and MBC resistant to TZ and previously received taxane therapy. (60/60).	Lapatinib (1250 mg/day) plus capecitabine (2000 mg/m^2/day) therapy was superior than TZ therapy (6 mg/kg every 21 days [after the initial 8 mg/kg loading dose]) plus capecitabine (2000 mg/m^2/day) for PFS and CBR (6.0 vs 4.5 months, 48 vs 63% respectively).

Lapatinib+vinorelbine vs lapatinib+capecitabine [179]	A phase II randomized multicenter study.	HER2-positive MBC patients who had received no more than one chemotherapeutic regimen. Arm A: lapatinib plus vinorelbine. Arm B: lapatinib plus capecitabine. (70/35).	Both therapies showed similar outcomes. Combination therapies doses were: Arm A, 1250 mg/day lapatinib plus 20 mg/m^2/day vinorelbine. Arm B, 1250 mg/day lapatinib plus 2000 mg/m^2/day capecitabine. PFS for both therapies=6.2 months. OS=24.3 for arm A vs 19.4 months for arm B. 42 patients opted to cross over and second evaluation was make: PFS=3.25 months for lapatinib plus vinorelbine vs 4.0 months lapatinib plus capecitabine. The most commonly observed AEs were diarrhea, neutropenia, PPE, rash, nausea, and fatigue. There were more serious AEs in arm A than in arm B.
Lapatinib+pazopanib [180]	Phase II two-cohorts multicenter study.	HER2 relapsed or refractory IBC. Cohort 1: previous history of IBC and documented recurrence in the skin and/or other disease sites by radiologic assessments (76). Cohort 2: cutaneous disease documented with photographs (88).	Cohort 1: combination therapy (lapatinib 1500 mg/day plus pazopanib 800 mg/day) had higher clinical activity than monotherapy (lapatinib 1500 mg/day) for ORR (29 vs 45%) respectively, but was not well tolerated. Cohort 2: combination therapy (lapatinib 1000 mg/day plus pazopanib 400 mg/day) was well tolerated and was superior to monotherapy (lapatinib 1500 mg/day) for CBR (58 vs 47%) but it was not for PFS. Combination showed an increase in toxicity.
Lapatinib+pazopanib [181]	Phase II randomized two-cohorts, multicenter study.	HER2 invasive and MBC. No prior anticancer therapy (except hormonal therapy) was permitted in the randomized. Cohort 1: randomized (150). Cohort 2: nonrandomized (40).	Combination therapy in cohort 1 (lapatinib 1000 mg/day plus pazopanib 400 mg/day) was not superior to monotherapy (lapatinib 1500 mg/day) for PDR (36.2 vs 38.9%), but it was for ORR (36.2 vs 22.2%). In cohort 2: (lapatinib 1500 mg/day plus pazopanib 800 mg/day) ORR=33.3%. AEs grade 3/4 including diarrhea, hypertension and liver enzymes elevation were higher in combination in cohort 2 than in combination in cohort 1.
Lapatinib+bevacizumab [182]	Phase II open-label, multicenter study.	HER2 invasive and MBC. (52).	Lapatinib (1500 mg/day) plus bevacizumab (10 mg/kg every 2 weeks) therapy was active in terms of PFS (24.7 weeks), CBR (30.8%) and ORR (13.3%). The most common AEs were diarrhea, rash, fatigue, nausea, headache, and epistaxis, grade 1/2. AEs grade 3/4 were rash, hypertension, diarrhea, hyperbirulinemia, hydronephrosis, gastrointestinal hemorrhage and liver enzyme alteration.
Lapatinib+paclitaxel [183]	Phase III randomized two-arms, double-blind, multicenter study.	Newly diagnosed HER2 MBC. (215/215).	Lapatinib (1500 mg/day) combined with paclitaxel (80 mg/m^2 weekly) was superior to paclitaxel alone (80 mg/m^2 weekly) for OS, PFS and ORR (27.8 vs 20.5 months, 9.7 vs 6.5 months and 69 vs 50% respectively). Incidence of grades 3/4 diarrhea and neutropenia was higher in the lapatinib plus paclitaxel arm.
Lapatinib+paclitaxel [184]	Phase III randomized two-arms, double-blind, multicenter study.	HER2-negative or initially untested, locally advanced or MBC previously untreated. (291/288).	Combination therapy (paclitaxel , 175 mg/m^2 every 3 weeks plus lapatinib 1500 mg/day) was superior to monotherapy (paclitaxel, 175 mg/m^2 every 3 weeks) cómo había un subset HER2 positive si en las especificaciones dice HER2 negative or untested? in terms of ORR, CBR, TTP (63.3 vs 37.8%, 69.4 vs 40.5%, 36.4 vs 25.1 weeks, respectively). Rash and diarrhea grade 3 were higher in the combination arm.

Lapatinib+nab-paclitaxel [185]	Phase II open-label, single-arm, multicenter study.	HER2 MBC with no more than one prior chemotherapeutic regimen. (60).	The recommended doses for this combination therapy were lapatinib: 1000 mg and nab-paclitaxel 100 mg/m^2. This combination showed to offer clinical benefit (ORR=53%, PFS=39.7 weeks, TTP=41 weeks). Data were consistent with those reported for lapatinib in combination with paclitaxel.
Lapatinib+paclitaxel+ gemcitabine [186]	Phase I open-label study.	HER2 early breast cancer, no previously treated. (13).	The recommended doses for this combination were lapatinib 1000 mg/day, paclitaxel 80 mg/m^2 on days 1 and 8 and gemcitabine 1000 mg/m^2 on days 1 and 8, every 3 weeks. The most frequent AE was neutropenia grade 3/4. Other AEs of more than grade 2 were rare: liver enzyme alteration, anorexia, gastritis, abdominal pain, diarrhea and paronychia. Combination therapy was well tolerated and showed clinical benefit (PR=61.5%, CR=30.7%).
Lapatinib+TZ [187]	Phase I dose-escalation study.	HER2 advanced or MBC. (54).	The optimally tolerated regimen was lapatinib 1000 mg/day plus TZ 2 mg/kg weekly (after the initial 4 mg/kg loading dose). Combination therapy showed to offer clinical benefit (ORR=15%). The responders had received prior TZ therapy in combination with cytotoxic chemotherapy. The most frequent AEs were diarrhea, rash, fatigue and nausea. Most common grade 3 events included diarrhea, fatigue, and rash.
Lapatinib+TZ [24]	Phase III randomized two-arms open-label multicenter study.	HER2 MBC progressed in the last TZ treatment. Must have received prior anthracycline- and taxane-based regimens. (148/148).	Combination therapy (lapatinib 1000 mg/day plus TZ 2 mg/kg weekly [after the initial 4 mg/kg loading dose]) was superior to lapatinib alone (1500 mg/day) for PFS and CBR (12.0 vs 8.1 weeks and 24.7 vs 12.4% respectively). Incidence of diarrhea was higher with the combination arm.
Lapatinib+TZ [188]	Phase II single-arm, multicenter study.	HER2 invasive breast cancer. (66).	Combination therapy (TZ [4 mg/kg loading, then 2 mg/kg/week] and lapatinib 1000 mg/day) showed to be clinically active (CR=27%, pRR=22%). Only 3% of the patients experienced disease progression. The most common AEs were diarrhea, rash, fatigue and nausea, all grade 1/2. AEs grade 3/4 were liver enzymes alterations, diarrhea, rash and hypertension.
Lapatinib vs TZ [189]	Phase II randomized open-label study.	HER2 positive MBC not previously treated with chemotherapy and/or anti-HER2 agents for metastatic disease. (19).	No significant difference in terms of response at 8 weeks was observed according to treatment arm (75 and 67% MR or more with lapatinib [1500 mg daily for 8 weeks] and TZ [8 mg/kg on loading dose, followed by weekly trastuzumab at the dose of 2 mg/kg] respectively). In the overall population, persistence in protocol, PSF and OS were 3.8, 7.3 and 43 months, respectively. MBC patients bearing tumors with markers such as “HER2-enriched” subtype and/or having high HER2/p95HER2 protein expression ratio are exquisitely sensitive to anti-HER2 agents and could be candidates for studies aimed at establishing chemotherapy-free regimens.

Lapatinib+TZ+docetaxel [190]	Phase I open-label, dose-escalation, multicenter study.	HER2 MBC. (53).	Two regimens were recommended: lapatinib 1250 mg plus docetaxel 75 mg/m^2 every three weeks and lapatinib 1000 mg plus docetaxel 100 mg/m^2 every three weeks, both with weekly TZ (loading dose of 4 mg/kg followed by a fixed dose of 2 mg/kg) and including use of prophylactic granulocyte colony stimulating factor. Combination therapy showed to offer clinical benefit (ORR=64%). The most common AEs were diarrhea and nausea. Grades 3/4 were neutropenia, diarrhea, leukopenia, peripheral neuropathy, and rash.
Lapatinib+TZ+paclitaxel [191, 192]	Phase III randomized open-label, multicenter study.	HER2 invasive early breast cancer. (152/154).	Combination therapy (lapatinib 1000 mg/ day plus TZ 2 mg/kg weekly [after the initial 4 mg/kg loading dose] plus paclitaxel 80 mg/m^2/week) was superior to TZ alone (2 mg/kg weekly [after the initial 4 mg/kg loading dose) and to lapatinib alone (1500 mg/day) for CR (51.3 vs 29.5 vs 24.7% respectively). Grade 3 diarrhea and liver enzyme alteration were higher in the combination arm. 3 years EFS was 78% for lapatinib, 76% for TZ and 84% for combination. 3 years OS was 93% for lapatinib, 90% for TZ and 95% for combination. EFS and OS did not differ between treatment groups but patients who achieve CR after neoadjuvant anti-HER2 therapy have longer EFS and OS than do patients without CR.
Lapatinib+TZ+paclitaxel [193]	Phase II single-arm, multicenter study.	HER2 surgically resected invasive breast cancer. (122).	Lapatinib given concurrently with paclitaxel and TZ is feasible and did not add cardiac toxicity. The dose of lapatinib in this regimen should not exceed 750 mg daily. Diarrhea grade 3/4 was the most common serious AE. An early aggressive management of this AE is recommended.
TZ-emtansine vs capecitabine+lapatinib [194]	Phase III randomized two-arms study.	HER2-positive, unresectable locally advanced or MBC previously treated with TZ and taxane. (495/496).	TZ-emtansine (3.6 mg/kg intravenously every 3 weeks) delayed the time to symptom worsening compared with capecitabine (1000 mg/m^2 orally twice a day, on days 1 through 14 of a every 3 week cycle) plus lapatinib (1250 mg orally daily), 7.1 vs 4.6 months, respectively. In the TZ-emtansine arm, 55.3% of patients developed clinically significant improvement in symptoms vs 49.4% in the capecitabine plus lapatinib arm. Diarrhea cases were higher in the capecitabine plus lapatinib arm than in the TZ-emtansine arm.
Lapatinib+TZ+chemotherapy [195]	Phase II randomized three-arms multicenter study.	HER2 infiltrating breast cancer. (36/39/46).	Combination of chemotherapy (fluorouracil 600 mg/m^2, epirubicin 75 mg/m^2, and cyclophosphamide 600 mg/m^2 administered every 3 weeks) with lapatinib (1000 mg/day) and TZ (2 mg/kg weekly after the initial 4 mg/kg loading dose) was superior to chemotherapy plus lapatinib and chemotherapy plus TZ for CR (46.7 vs 25.0 vs 26.3% respectively). The majority of the patients required lapatinib dose reduction because high grade 3 diarrhea incidence.

Chemotherapy+TZ vs chemotherapy+lapatinib [196]	Phase II randomized two arms, open label, multicenter trial.	Stages I, II, III or inflammatory breast cancer HER2 positive. (50/52).	Chemotherapy consisted in epirubicin 90 mg/m^2 plus cyclophosphamide 600 mg/m^2 both administered on day 1 every 21 days for four cycles followed by docetaxel 100 mg/m^2 administered on day 1 every 3 weeks for four cycles. The anti-HER2 therapy was added to docetaxel as follows: patients in arm A received chemotherapy plus TZ 6 mg/kg (after a loading dose of 8 mg kg/1) on day 1 every 21 days (EC-DT). Patients in arm B received chemotherapy plus a daily dose of lapatinib 1250 mg orally (EC-DL). EC-DT exhibited higher efficacy; than EC-DL in terms of CR (52.1 vs 25.5% in breast, 47.9 vs 23.5% in breast and axila). Grade 3/4 toxicity rates were similar across arms except for diarrhea, which was more frequent in the EC-DL arm.
Chemotherapy+TZ vs chemotherapy+lapatinib [197]	Phase III randomized two arms, open label, multicenter trial.	Untreated HER2-positive operable or locally advanced breast cancer. (309/311).	Chemotherapy consisted in epirubicin 90 mg/m^2 plus cyclophosphamide 600 mg/m^2 both administered every 3 weeks for four cycles followed by docetaxel 100 mg/m^2 administered on day 1 every 3 weeks for four cycles. The anti-HER2 therapy was added to docetaxel as follows: patients in arm A received chemotherapy plus TZ 6 mg/kg (after a loading dose of 8 mg kg/1) on day 1 every 21 days (ECH-TH). Patients in arm B received chemotherapy plus a daily dose of lapatinib 1000-1250 mg orally (ECH-TL). ECH-TH exhibited higher efficacy in terms of CR (30.3 vs 22.7% respectively) than ECH-TL. Chemotherapy with trastuzumab was associated with more edema (39·1% vs 28·7%) and dyspnea (29·6 vs 21·4%), and ECL-TL with more diarrhea (75·0 vs 47·4%) and skin rash (54·9 vs 31·9%). Less AE's were reported in the ECH-TH group than in the ECL-TL group.

MBC, metastatic breast cancer; IBC, inflammatory breast cancer; CR, complete response; PR, partial response; OR, objective response; PFS, progression free survival; PTEN, tensin homologue deleted in chromosome 10; AE, adverse event; CBR, clinical benefit rate; TZ, trastuzumab; ORR, objective response rate; OS, overall survival; SD, stable disease; TTP, time to progression; CNS, central nervous system; PPE, palmar-plantar erythrodysesthesia; WBRT, whole brain radiotherapy; PS, performance status; PDR, progressive disease rate; pPR, pathological response rate; EFS, event free survival; MR, minimal response.

Table 2. Clinical evidence with neratinib in HER2 breast cancer

Therapy [Ref.]	Study type	Patient population (n)	Principal findings
Neratinib [143]	Phase II open-label, two-cohorts, multicenter study.	HER2 advanced and MBC. Cohort A: Prior TZ treatment (66). Cohort B: No prior TZ treatment (70).	Neratinib (240 mg daily) was well tolerated and had clinical activity (PFS at 16 weeks=59 and 78%, OR=24 and 56% for cohort A and cohort B respectively). The most common AEs were diarrhea, nausea, vomiting, and fatigue. Diarrhea was the most frequent grade 3/4 AE.

Neratinib+paclitaxel [142]	Phase I/II open-label, two-part study.	HER2-positive MBC and prior TZ therapy. Group A: no more than one prior chemotherapy regimen and no prior lapatinib exposure (71). Group B: no more than three prior cytotoxic chemotherapy regimens with prior lapatinib exposure permitted (31).	Combination therapy (neratinib 240 mg daily plus paclitaxel 80 mg/m^2) was well tolerated with no unexpected toxicity and had clinical activity in both groups (OR=71 and 77%, PFS=63.1 and 52.1 weeks for group A and B respectively) with a CB=82% for all evaluable patients. The most common AEs were diarrhea, neuropathy, alopecia, nausea, neutropenia, leukopenia and anemia. The most common grade 3/4 AEs were diarrhea, neutropenia, leukopenia and anemia.
Neratinib+paclitaxel +TZ [198]	Phase I open label, dose escalation, multicenter study.	HER2-positive MBC previously treated with anti-HER agent(s) and a taxane. (21).	Combination therapy consisted in neratinib (120 up to 240 mg/day) with TZ (4 mg/kg loading dose, then 2 mg/kg weekly) and paclitaxel (80 mg/m^2 days 1, 8, and 15 of a 28-day cycle). The recommended dose of neratinib for this combination therapy was 200 mg/kg. It showed to have clinical activity (OR=38%, CB=52% and median TTP=3.7 months). Common grade 3/4 AEs were diarrhea (38%), dehydration (14%), electrolyte imbalance (19%), and fatigue (19%).
Neratinib+vinorelbine [199]	Phase I/II multicenter, open-label study.	HER2-positive MBC, divided according to prior or no prior lapatinib treatment. (12/56).	Combination therapy (neratinib 240 mg plus vinorelbine 25 mg/m^2) was well tolerated with no unexpected toxicity. It showed to have clinical activity (OR=8 vs 41%, PFS=24.0 vs 47.7 weeks, CB=5 vs 39%, prior lapatinib vs no prior lapatinib respectively). The most common AEs were diarrhea, neutropenia, vomiting, nausea and fatigue. The most common grade 3/4 AEs were neutropenia (46%), diarrhea (28%), and leukopenia (17%).
Neratinib+ capecitabine [200]	Phase I/II multinational open-label study.	HER2-positive MBC or locally advanced breast cancer, previously treated with TZ and taxane. There is a sub-group previously treated with lapatinib. (61/7).	Combination therapy (neratinib 240 mg plus capecitabine 1500 mg/m^2 daily) showed to have clinical activity (OR=64 vs 57%, PFS=40.3 vs 35.9 weeks, patients with no prior lapatinib exposure vs patients previously treated with lapatinib respectively). The overall most common grade 3/4 AEs were diarrhea (26%), PPE (14%), asthenia (4%) and vomiting (4%).

MBC, metastatic breast cancer; TZ, trastuzumab; PFS, progression free survival; OR, objective response; AEs, adverse events; CB, clinical benefit; TTP, time to progression; PPE, palmar-plantar erythrodysesthesia.

In addition, lapatinib inhibited activation of nuclear factor κB (NF-κB) in HER2-overexpressing breast cancer cells [44]. The TKI inactivates NF-κB through reducing phosphorylation of its inhibitor IκB-α via blocking the PI3K/AKT cascade [44]. This fact is relevant due to co-operation between HER2 and NF-κB signaling which causes tumor resistance to radiotherapy [45].

Overexpression of EGFR and HER2 contributes to clinical radiation resistance [46] and several EGFR inhibitors sensitize tumor cells to ionizing radiation [46-48]. In this regard, lapatinib treatment enhanced the radiosensitization of EGFR- and HER2-overexpressing breast cancer cells through inhibition of MEK/ERK signaling pathway [49, 50].

In the SK-BR-3 HER2-amplified breast cancer cell line prolonged exposure to lapatinib reduced the expression and activity of the enzyme topoisomerase IIα, which renders cells resistant to the cytotoxic effects of doxorubicin, etoposide, and m-AMSA [51].

Lapatinib regulates several microRNAs (miR) that play an important role in the anti-tumor action in the HER2-postive breast carcinoma cells [52]. In this regard, lapatinib treatment upregulated miR575 and miR-1225-5 expression, contributing in this manner to downregulation of the oncogenic protein phospholipase C PLCXD1 (phosphatidylinositolspecific phospholipase-C-X-domain-containing-1), a target transcript of miR-575 and miR1225-5p [52].

Lapatinib: clinical evidence and side effects

Several clinical studies in HER2 breast cancer have evaluated safety, dosing schedules and efficacy of lapatinib as monotherapy or in combination with other therapies. The outcomes depend on population features, tumor type, stage and prior cancer treatments.

In all the studies listed in **Table 1**, lapatinib and its combinations provided clinical benefit in different grades in HER2 breast cancer patients. In contrast, HER2 negative and EGFR positive cancer patients did not experience benefit with this TKI [53-55].

In general, the addition of lapatinib to another therapy improved the efficacy compared with the drug alone and in some cases without increasing the adverse events profile or its severity.

Some studies have assessed the efficacy of lapatinib alone and in combination with capecitabine in HER2 breast cancer patients with progressive brain metastases. Only modest efficacy has been found with monotherapy while combination therapy has shown to be more effective suggesting that lapatinib plus capecitabine could be an alternative treatment for specific patients to delay whole brain radiotherapy and its adverse effects (**Table 1**) [56-59].

Lapatinib resistance and toxicity

Several cancers generate resistance to TKIs through modifications in different proteins implicated in downstream signaling of their target receptors [60]. Herein, we will revise the proteins and cellular processes known to be involved in acquiring resistance to lapatinib.

Dysregulation of the PI3K pathway is frequent in breast cancer leading to its constitutive activation [61, 62]. Mutations in the catalytic subunit of PI3K (PIK3CA) have been reported in 20-30% of breast cancer patients with HER2 amplification [63, 64]. In HER2 overexpressing cells carrying PIK3CA, the antiproliferative and proapoptotic effects of lapatinib as well as its ability to inhibit survivin and AKT phosphorylation was less pronounced than in those cells without PIK3 mutations [33, 65]. Interestingly, PIK3CA has been considered a biomarker of resistance to the EGFR family targeted therapy [66]. Another abnormal activation of the PI3K pathway is the loss of phosphatase and tensin homologue deleted in chromosome 10 (PTEN) function that has been observed in 20% to 25% of primary breast cancers [63, 67]. In this regard, it has been demonstrated that loss of PTEN expression conferred resistance to lapatinib in both PTEN knockdown cells and tumor xenografts with HER2 overexpression, through maintaining the activation of the AKT signaling pathway [65, 68]. However, another study did not find association between PTEN protein levels and resistance to lapatinib in a panel of 17 HER2-amplified cell lines [69]. This was consistent with two studies that did not show association between PTEN and lapatinib response

using cell lines and tissues of inflammatory breast cancer patients with PTEN deficiency [53, 70].

Hypoxia promotes lapatinib resistance in HER2-positive breast cancer cells by inducing high levels of hypoxia inducible factor (HIF)-1α and reducing the dual specificity phosphatase2 (DUSP2) expression that leads to activation of the ERK pathway [71].

Autophagy plays an important role in the development of drug resistance in breast cancer cells [72]. Indeed, increase of autophagosome formation was observed in lapatinib resistant breast cancer cell lines. Accordingly, treatment with autophagy inhibitors restored lapatinib sensitivity [73].

Acquired resistance to lapatinib in HER2-positive breast cancer cells can also be mediated by induction of heregulin (HRG) and subsequent formation of HER3-EGFR heterodimers that promote an HRG-HER3-EGFR-PI3K signaling axis [74].

In breast cancer, the overexpression of CD24, a glycosylphosphatidylinositol-anchored membrane protein, is associated with a poor patient prognosis [75]. Noteworthy, CD24 contributes to resistance to lapatinib in HER2-positive breast cancer cells. In fact, knockdown of CD24 downregulated HER2 expression, AKT phosphorylation and increased the sensitivity of cells to lapatinib treatment [76].

Similary, the overexpression of Neuromedin U (NmU), a neuropeptide that has been associated with poor patient outcome in HER2-overexpressing tumors, is also associated with resistance to lapatinib. NmU knockdown significantly decreased migration, motility, invasion and resistance to anoikis in HER2 overexpressing breast cancer cells [77].

In a HER2-overexpressing/estrogen receptor (ER) positive breast cancer cell line treated continuously with lapatinib, increased ER signaling was observed. This was due to activation of the transcription factor forkhead box protein O3a (FOXO3a) in response to ErbB2-PI3K-AKT signaling inhibition, resulting in autoresistance to this inhibitor [78]. Consequently, ER signaling assumed a more prominent role in cell survival during the development of acquired resistance to the TKI. Accordingly, the combination of lapatinib with antagonists of the ER such as tamoxifen or fulvestrant prevented the development of lapatinib resistance [78]. In addition, in tumor biopsies of patients with early-stage breast cancer, after 14 days of lapatinib therapy, increased FOXO3a, progesterone receptor and Bcl-2 expression was observed [78].

In human breast cancer lines and xenograft models it was demonstrated that FAM83A overexpression, an oncogenic protein, is involved in lapatinib resistance [79]. Interestingly, FAM83A downregulation decreased proliferation and rendered cells sensitive to lapatinib. The mechanism by which this protein conferred resistance to lapatinib may include the interaction of FAM38A with cRAF and PI3K, maintaining the activation of MAPK signaling. Indeed, breast cancer patients with high FAM83A expression have poor prognosis [79].

Some receptors with tyrosine kinase activity have been involved in lapatinib resistance. In this sense, the aberrant expression of AXL, recepteur d'origine nantais (RON) and fibroblast growth factor receptor 2 (FGFR2) has been reported to play an important role in lapatinib resistance through eliciting PI3K/AKT and ERK signaling activation. In fact, inhibitors that target AXL, RON and FGFR2 restored lapatinib sensitivity in HER2 breast cancer cell lines [36, 80, 81].

Protein tyrosine kinase 6 (PTK6), Src (both non-receptor tyrosine kinase proteins), the chemokine CXCL12 and its receptor (CXCR4) are involved in breast cancer progression, regulating several cellular processes involved in the malignant phenotype, such as cell proliferation, survival, invasion and migration. Current researches have demonstrated that increased levels of PTK6 and Src expression and phosphorylation respectively, are involved in lapatinib resistance through EGFR-dependent signaling. Further, Src family kinases mRNA expression was upregulated in HER2-positive tumors treated with lapatinib. In fact, treatment with Src inhibitors in combination with lapatinib reduces AKT and ERK1/2 phosphorylation restoring the sensitivity of resistant cells to lapatinib [82-85]. Moreover, lapatinib resistant cells also show high levels of expression of CXCR4. Blockade of this chemokine receptor

with a specific antibody reduced the invasive ability of lapatinib resistant cells. Notably, the combination of CXCR4 antibody and a Src inhibitor saracatinib, resulted in a greater inhibition of the invasiveness compared with either agent alone in lapatinib resistant cells. These findings suggest that blockade of Src and CXCR4 could be utilized as a therapeutic option in patients with acquired resistance to lapatinib [84].

A key feature for acquired lapatinib resistance is the fact that lapatinib treatment in HER2-positive breast cells induces the activation of several networks of kinases that contribute to induce lapatinib resistance. Efforts for dealing with this tumor response include the pharmacological inhibition of the adaptive kinome response. In this regard, inhibitors of epigenetic enzymes of BET family bromodomains suppressed the kinome reprogramming induced by lapatinib [86].

Aberrant miR-630 expression is related with lapatinib resistance in HER2-positive breast cancer cells. Overexpression of this miR reduced the metastatic phenotype and restored the drug sensitivity through decreased IGF1R expression and subsequent inhibition of the phosphorylation of HER2 and EGFR in lapatinib resistant cells [87].

In HER2-positive breast cancer cells, elevated expression of the alpha catalytic subunit of cyclic AMP-activated protein kinase A (PRKACA) confers resistance to lapatinib via increased phosphorylation of the pro-apoptotic molecule BAD [88].

The Kruppel-like transcription factors (KLFs) 4 and 5 (KLF4/5) play an import role in cell proliferation, differentiation, and transformation and have been considered as possible prognostic factors in breast cancer [89, 90]. In fact, a positive correlation between HER-2/neu and KLF5 has been described [90]. In HER2-positive breast cancer cells, lapatinib treatment upregulated KLF4/5 protein expression. In these cells, KLF4 and/or KLF5 depletion restored lapatinib sensibility and reduced basal mRNA and protein levels of the anti-apoptotic factors myeloid cell leukemia 1 (MCL1) and B-cell lymphoma-extra-large (BCL-XL) [91]. The activation of another transcription factor, the aryl hydrocarbon receptor (AhR), promotes several signaling pathways as EGFR, PI3K/AKT and ERK. In fact, the induction of apoptosis by lapatinib is inhibited by this receptor activated by its external ligand (2,3,7,8-tetrachlorodibenzo-p-dioxin), indicating its participation in lapatinib resistance [92].

In patients with residual breast cancers following administration of taxane/anthracycline-based chemotherapy used concurrently with trastuzumab, loss of HER2 gene amplification has been reported [93]. Interestingly, HER2 breast cancer cells also showed loss of HER2 expression after chronic exposure to lapatinib, conferring resistance to this inhibitor [81]. Another mechanism that limits the antitumor activity of lapatinib is the upregulation of HER3 induced by this TKI, maintaining the activation of AKT and MAPK pathways. These evidences advice the use of HER2/HER3 inhibitors combined in the treatment of HER2-positive breast cancer [94, 95].

Gefitinib

Gefitinib is a reversible EGFR TKI that has been approved by the FDA for the treatment of advanced non-small cell lung carcinoma with activating EGFR mutations [96, 97].

EGFR is overexpressed in breast cancer tissue with a positivity range of 20-70% [98, 99]. Overexpression of this receptor is associated with aggressive metastatic breast tumors. In addition, breast tumors that co-overexpress EGFR and HER2 exhibited a worse outcome than tumors that overexpressed either receptor alone [100]. Interruption of EGFR function with specific TKIs may disrupt EGFR-HER2 cross-talk, resulting in HER2 inactivation [101].

Mechanism of action of gefitinib: preclinical evidence

Gefitinib inhibits the growth of both breast cancer cell lines and human tumor xenografts expressing different levels of EGFR or HER2 [101-103]. Gefitinib effects on HER2 and EGFR coexpressing breast cancer cells are mediated by reducing basal EGFR, HER2 and HER3 phosphorylation, resulting in the blockage of downstream signaling of the AKT, MAPK and STAT3 pathways [101-105]. Also, this TKI increased p27 levels and the subG1/G1 phases of the cell cycle; reduced cyclin D1 and Cdk4. In addition,

gefitinib reduced the phosphorylation of glycogen synthase kinase 3 beta (GSK-3β, a target of the AKT kinase) [101, 106]. In EGFR-HER2 breast cancer cells, gefitinib induced cytostatic and apoptotic effects [103]. This action of gefitinib is in part mediated by increased p38 MAPK levels [102], dephosphorylation of FOXO3a with a subsequent increased of $p27^{Kip1}$, caspase 3 and BIM protein expression [105, 106]. Gefitinib has also been shown to downregulate the mTOR signaling pathway in human breast cancer cells [107].

In a similar manner as described above in the cell lines, gefitinib inhibited EGFR and MAPK phosphorylation in tumor biopsies. However, gefitinib had not effect on AKT phosphorylation or Ki67 levels. Moreover, the TKI did not increase p27 levels [108].

Gefitinib treatment disrupted the formation of the HER3/HER2 heterodimer and further association of HER3 with p85α the regulator subunit of PI3K [101]. In addition, the TKI inhibited the activation of the EGFR/HER2 and EGFR/HER3 heterodimers mediated by EGF and heregulin, respectively [102].

EGFR overexpression did not determine gefitinib sensitivity in the particular case of HER2 overexpressing breast cancer [103, 104]. In this regard, gefitinib was more potent to inhibit the proliferation of breast cancer cells with high levels of HER2 and low levels EGFR compared to those cells with high levels of EGFR without HER2 expression [101, 102, 104]. In contrast, gefitinib effects on AKT, MAPK, and p27 were not seen in EGFR-negative breast cancer cells [101]. Interestingly, inhibition of MAPK phosphorylation was observed in EGFR-negative tumor biopsies [108], suggesting that gefitinib may be inhibiting other EGFR family members [108].

In the same way as observed with lapatinib, prolonged exposure to gefitinib induced resistance to doxorubicin, etoposide, and m-AMSA through downregulation of topoisomerase IIα [51]. A graphic summary of gefitinib mechanisms of action is found in **Figure 1**.

Gefitinib: clinical evidence and side effects

There are only two phase I/II clinical studies that assess the effects of different gefitinib-combination therapies specifically in HER2-positive metastatic breast cancer patients. The first one was undertaken in 35 patients divided according to prior systemic therapy. It demonstrated that the combination of 250 mg/day of gefitinib with 2 mg/kg weekly (after the initial 4 mg/kg loading dose) of trastuzumab was safe. However, there was no greater clinical benefit compared to trastuzumab monotherapy. A possible explanation of the lack of clinical activity in this study was that coupling between HER2 and HER3 and activation of the last one would not be completely inhibited by the combination of gefitinib and trastuzumab [109]. The other study included 31 patients treated with 250 mg/day of gefitinib, 6 mg/kg every 3 weeks (after the initial 8 mg/kg loading dose) of trastuzumab and 60 mg/m^2 every 3 weeks of docetaxel. The values obtained were considered similar to those in other three drug combination regimens including trastuzumab and chemotherapy, with the advantage of less toxicity because the avoidance of a second chemotherapeutic agent. Additionally, no relationship between assessed biomarkers expression and response to therapy was found. In regard to EGFR, 95% of the biopsies showed little or no signal when evaluated by immunohistochemistry, although 47% of this subpopulation responded to the therapy [110].

Gefitinib alone or in combination with other drugs has been assessed in several clinical studies with HER2-negative breast cancer patients. The results are inconsistent but in some cases there is a correlation between the outcome and prior therapies administrated. We discuss these studies below.

In a clinical trial conducted in women with stage IIIb/IV advanced breast cancer resistant to chemotherapy, gefitinib monotherapy showed only a stabilization of disease for at least 3 months, neither partial nor complete response were achieved. Interestingly, a good correlation between the degree of inhibition of EGFR phosphorylation in skin and in breast tumors after gefitinib treatment was reported. Therefore, the inefficacy of this TKI could be explained by the lack of EGFR tumor dependence [108]. In other study, gefitinib was not effective in patients with recurrent or refractory breast cancer previously treated with taxane and anthracycline [111].

Moreover, studies in patients with ER and/or progesterone receptor positive breast adenocarcinoma [112, 113], primary untreated breast cancer [114] and metastatic breast cancer [115], it was found that the addition of gefitinib to standard treatments (chemotherapy or hormonal therapy) did not further increase the clinical benefit. However, in some cases it caused higher toxicity and serious adverse events such as dehydration, diarrhea, fatigue, stomatitis, hypokalemia and neutropenia.

In contrast, Polychronis et al., reported that gefitinib alone and in combination with anastrozol was effective for reducing the tumor size in ER-EGFR positive breast cancer naive patients. The most frequently adverse effects presented with both therapies were rash and gastrointestinal disorders such as diarrhea, dry mouth, nausea and loose stools [116]. Ciardiaello et al., also reported good response in patients with metastatic breast cancer treated with gefitinib plus docetaxel, who had not previously received any kind of therapy for metastatic disease. The most common adverse effects in this study were neutropenia, diarrhea and nausea [117]. Interestingly, Osborne et al, found that the gefitinib plus tamoxifen combination was effective only in patients with newly diagnosed metastases or those who had recurred one year or more after stopping adjuvant therapy with tamoxifen but not in patients with recurrent disease during or after adjuvant treatment with aromatase inhibitors or those progressing after first-line aromatase inhibitor treatment for metastatic disease [118].

Additionally, Kalykaki et al., demonstrated that gefitinib was effective to reduce the number of EGFR positive and negative circulating tumor cells in patients with metastatic breast cancer [119].

The most common adverse effects of gefitinib as monotherapy in breast cancer patients are diarrhea, rush, dry skin, pruritus, dry mouth, nausea, vomiting and fatigue [108, 111, 116].

Gefinib resistance and toxicity

The constitutive activation of signaling pathways downstream of EGFR contributes to gefitinib resistance [104, 120, 121]. Normanno et al., demonstrated that hiperactivation of the MAPK pathway is involved in both the intrinsic and acquired gefitinib resistance in breast cancer cells. Interestingly, blockade of the MAPK activity resulted in growth inhibition and induction of apoptosis. In addition, constitutive activation of p42-MAPK was related with gefitinib resistance [120, 121].

Other study related with intrinsic gefitinib resistance demonstrated that this TKI increased the expression of genes codifying for HER specific ligands and induced active import and nuclear accumulation of the HER ligand neuregulin, suggesting its possible transcriptional role [122]. Moreover, HER3 and consequently PI3K/AKT signaling evade inhibition by gefitinib *in vitro* and *in vivo* [123].

Regarding autophagy, gefitinib induced this cellular process in various gefitinib-sensitive and -insensitive breast cancer cell lines. Treatment with gefitinib in combination with agents that inhibit late-stage autophagy such as hydroxychloroquine or bafilomycin A1 increased efficacy of gefitinib *in vitro* and *in vivo* [107].

Alternatively, other studies suggest that the upregulation of the PI3K pathway caused by loss of PTEN activity is involved in gefitinib resistance in breast cancer [104, 124, 125]. In fact, in an EGFR overexpressing breast cancer cell line which lacks PTEN function, gefitinib abolished the phosphorylation of both EGFR and MAPK but not AKT [124]. Moreover, gefitinib treatment in cells that PTEN function was restored resulted in the inhibition of AKT, GSK-3β and the translation repressor protein 4EBP1 phosphorylation [124], restoration of gefitinib sensitivity, arrest in the G1 phase [125], and relocalization of FOXO3a into the nucleus. This transcriptional factor is implicated in cell cycle arrest and apoptosis [126].

Other mechanisms implicated in gefitinib resistance in breast cancer involved the interplay between EGFR and different receptors. In this regard, hepatocyte growth factor receptor (MET), a tyrosine kinase receptor implicated in breast cancer progression, confers gefitinib resistance through increased EGFR phosphorylation induced by the activation of the kinase c-Src. In fact, an inhibitor of MET kinase activity decreased both EGFR phosphorylation and cell proliferation in the presence of gefitinib in HER2-positive and EGFR overexpressing breast cancer cells [127].

As with lapatinib, FAM83A dysregulation has been show to promote gefitinib insensitivity in breast cancer cells [79].

The crosstalk between G-protein coupled receptors (GPCR) and EGFR [128]; K-RAS activation (a downstream effector of EGFR signaling) [129] and EGFR mutations [130] have all been involved with gefitinib resistance in lung cancer. In this regard, GPCRs-EGFR interaction has also been reported in breast cancer [131], Moreover, K-RAS gene mutations were found in several adenocarcinomas [132-134]. Interestingly, EGFR mutations have not been reported neither in primary breast carcinomas nor in several breast cancer cell lines [135]. These issues have not yet been addressed in gefitinib resistant breast cancer cells and will have to be further analyzed.

Neratinib

Neratinib is another oral, but irreversible TKI, known as a pan-inhibitor because interacts with the catalytic domain of several EGFR family members (EGFR, HER2 and HER4) and blocks their downstream signaling pathways [136]. Neratinib covalently binds a specific and shared cysteine residue in the ATP-binding pocket of the receptors in the EGFR family [137]. In particular, neratinib binds to cysteine residues Cys-773 and Cys-805 in HER1 and HER2, respectively [138].

Neratinib derives from structural modifications of EKB-569, another potent and irreversible EGFR inhibitor [136, 139]. Neratinib has significant activity in naïve and previously exposed to trastuzumab patients [140], making it an alternative treatment for HER2-positive breast cancer. Currently, this TKI is in clinical trials and has been used to treat solid tumors and metastatic HER2 breast cancer [141-144].

Mechanism of action of neratinib: preclinical evidence

There are some reports that describe the mechanism of action of neratinib in breast cancer. A pioneering work from Rabindran et al., showed that neratinib inhibited proliferation and EGFR, HER2, HER4, AKT and MEK phosphorylation in HER2 overexpressing breast cancer cell lines [136, 145]. The regulation of downstream signal transduction by neratinib leads to arrest at the G1-S phase transition resulting in increased p27 levels and decreased phosphorylated retinoblastoma protein (pRb) and cyclin D1 expression. Interestingly, neratinib showed less antiproliferative activity in cell lines that express neither HER2 nor EGFR [136]. Moreover, HER2-positive breast cancer cell lines are more likely to respond to neratinib than EGFR-positive cells or HER2 non-amplified cell lines [145]. Another antineoplastic mechanism for neratinib in cancer cell lines is that it can reverse membrane-bound ATP transporters-mediated multidrug resistance [146]. The inhibition of multidrug resistance via ATP transporters by neratinib may be an alternative mechanism that could improve the response to chemotherapy agents used in HER2-positive breast cancer.

Similarly, neratinib enhanced the therapeutic response and counteracted trastuzumab resistance by decreased trastuzumab-induced HER4 nuclear translocation in HER2-positive breast cancer [145, 147]. A resume of neratinib mechanisms is found in **Figure 1**.

Neratinib: clinical evidence and side effects

Neratinib has proved to be a promising therapy for HER2 metastatic breast cancer in five recent phase I/II clinical studies listed in **Table 2**. Nevertheless, in a phase II trial, Martin et al. compared two treatments: neratinib versus lapatinib plus capecitabine with inconclusive results, since neither inferiority nor superiority of the monotherapy versus combination therapy could be demonstrated. However, they confirmed relevant single agent clinical activity and acceptable overall tolerability of neratinib [148]. These studies have warranted further studies of this TKI and its combination with other therapies. In this regard, several clinical trials phase I/II (ClinicalTrials.gov identifiers: NCT01008150, NCT01111825, NCT01670877, NCT00398567, NCT00741260, NCT00915018, NCT01494662, NCT00146172) and two phase III (NCT00878709 and NCT01808573) in HER2-positive breast cancer population are in progress.

Neratinib resistance and toxicity

Seyhan et al., using a pool-based lentiviral genome-wide functional RNAi screen identified the following genes involved in neratinib resistance: oncogenesis (RAB33A, RAB6A and

BCL2L14), transcription factors (FOXP4, TFEC, ZNF), cellular ion transporters (CLIC3, TRAPPC2P1, P2RX2), protein ubiquitination (UBL5), cell cycle (CCNF9) and genes known to interact with breast cancer-associated genes (CCNF, FOXP4, TFEC, several ZNF factors, GNA13, IGFBP1, PMEPA1, SOX5, RAB33A, RAB6A, FXR1, DDO, TFEC, OLFM2) [149]. This analysis helps to the identification of new biomarkers related to neratinib treatment that could eventually make more effective the use of combination targeted HER2 therapy.

In a similar manner as other TKIs, neratinib also may generate resistance. In this regard, NmU overexpression and low miR-630 levels are associated with innate or acquired-resistance to neratinib in HER2-positive breast cancer cells [77, 87].

In HER2-posive breast cancer cell lines, despite the initial inhibition of phosphorylation of all members of the EGFR family by neratinib, its continued exposure resulted in reactivation of HER3 and AKT signaling. This process is thought to be involved in the developing of resistance to neratinib. Interestingly, the combination of trastuzumab and neratinib blocked the reactivation of HER3 and AKT compared to neratinib alone [145].

Conclusion

Overexpression of EGFR family members and activation of their signaling pathways have been associated with the development of breast cancer and poor prognostic [150]. Interaction between these receptors is related with resistance to current therapy becoming a challenge for HER2-positive breast cancer treatment [100, 151]. Targeted therapy against cell signaling pathways of EGFR family members has been introduced in the clinic in recent years since it generates fewer side effects than conventional therapy [152]. In this regard, TKIs have been developed such as lapatinb, gefitinib and neratinb. These inhibitors have a similar mechanism of action; specifically, they compete for the ATP binding pocket of the EGFR family. These agents can inhibit the same signaling pathways such as PI3K/AKT, MAPK, PLCγ, and STAT [104, 153-155]. However, the differences in their responses, and their selectivity in HER2-positive breast cancer, could depend on their dissociation constant (kd) to EGFR family members, and their molecular interactions with these proteins. The biological consequences of the binding of TKIs with EGFR family members are poorly understood, and several researchers are currently examining the different TKIs interactions within the human kinome [156]. In addition, it has been described that TKIs can interact with different dimerization complexes with active or inactive forms of EGFRs [157-159]. Also, TKIs can modify the amount of EGFR family ligands [122].

However, if the three compounds have similar mechanisms of action, what is the difference between them that could explain the different responses in the clinic? A current work of Sánchez-Martín et al, in 2012 gives us an explanation about the chemical mechanism that led to increased efficacy of lapatinib and neratinib action in breast cancer versus gefitinib action. The reversible and irreversible inhibitors, lapatinib and neratinib; respectively, have in their structure an aromatic group that binds into the ATP pocket of the EGFR family while maintain it in a closed conformation, reducing the flexibility of the kinase receptor, which ultimately limits the dimerization with other receptors. Unlike lapatinib and neratinib, gefitinib lacks an aromatic group in its structure, which maintains EGFR family ATP pocket in an open conformation, favoring the dimerization with other kinase receptors [160]. Moreover, a recent work of O'Neill et al, in 2013 studied a panel of genes implicated in cell cycle progression, tumorigenesis and invasiveness, which were upregulated after treatment with neratinib in HER2 overexpressing breast cancer cells. This work demonstrated that the genic regulation induced by neratinib was similar to that seen with trastuzumab and the combination of lapatinib and capecitabine in HER2-positive breast cancer cells. In contrast, gefitinib treatment resulted in a completely different expression pattern [161, 162]. This is important because the expression of genes could be predictors of the response in some patients [161]. In summary, a different gene expression profile induced by each one of the TKIs could explain the difference in the treatment response.

The TKIs have shown sustainable features to block the signaling of the EGFR family members, inhibit cell proliferation and induce apop-

tosis in HER2-positive breast cancer subtype. However, the search for novel therapeutic strategies comprising the use of these or other TKIs, in combination with different antineoplastic agents could further improve the prognostic in cancer patients. In order to enhance the clinical response to the TKIs therapy, we consider that tumor molecular analyses, which include the characterization of the EGFR family members, and the search for mutated or overexpressed proteins related with TKI resistance, must be performed.

Acknowledgements

This work was supported by Instituto Científico Pfizer, México, and by the Consejo Nacional de Ciencia y Tecnología (CONACyT), México grant number 154215 to DBH. MSM is a Ph.D, student from the Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, México, and recipient of a fellowship from CONACyT (324486) and PAEP, UNAM, México.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Rocío García-Becerra, Departamento de Biología de la Reproducción, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Avenida Vasco de Quiroga No. 15, Col. Belisario Domínguez Sección XVI, Tlalpan 14080, México, D.F., México. Tel: (525) 54.87.09.00. Ext. 2417; E-mail: rocio.garciab@quetzal.innsz.mx

References

[1] Beiki O, Hall P, Ekbom A and Moradi T. Breast cancer incidence and case fatality among 4.7 million women in relation to social and ethnic background: a population-based cohort study. Breast Cancer Res 2012; 14: R5.

[2] Globocan. Cancer Incidence and Mortality Worldwide. IARC CancerBase (http://globocan.iarc.fr) 2008; No. 10:

[3] Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO and Botstein D. Molecular portraits of human breast tumours. Nature 2000; 406: 747-752.

[4] Sotiriou C and Pusztai L. Gene-expression signatures in breast cancer. N Engl J Med 2009; 360: 790-800.

[5] Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989; 244: 707-712.

[6] Dawood S, Broglio K, Buzdar AU, Hortobagyi GN and Giordano SH. Prognosis of women with metastatic breast cancer by HER2 status and trastuzumab treatment: an institutional-based review. J Clin Oncol 2010; 28: 92-98.

[7] Brower V. Cardiotoxicity Debated for Anthracyclines and Trastuzumab in Breast Cancer. J Natl Cancer Inst 2013; 105: 835-6.

[8] Dieras V, Vincent-Salomon A, Degeorges A, Beuzeboc P, Mignot L and de Cremoux P. [Trastuzumab (Herceptin) and breast cancer: mechanisms of resistance]. Bull Cancer 2007; 94: 259-266.

[9] Nahta R and Esteva FJ. HER2 therapy: molecular mechanisms of trastuzumab resistance. Breast Cancer Res 2006; 8: 215.

[10] Agrawal A, Gutteridge E, Gee JM, Nicholson RI and Robertson JF. Overview of tyrosine kinase inhibitors in clinical breast cancer. Endocr Relat Cancer 2005; 12 Suppl 1: S135-144.

[11] Wang YN and Hung MC. Nuclear functions and subcellular trafficking mechanisms of the epidermal growth factor receptor family. Cell Biosci 2012; 2: 13.

[12] Yarden Y. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. Eur J Cancer 2001; 37 Suppl 4: S3-8.

[13] Asgeirsson KS, Agrawal A, Allen C, Hitch A, Ellis IO, Chapman C, Cheung KL and Robertson JF. Serum epidermal growth factor receptor and HER2 expression in primary and metastatic breast cancer patients. Breast Cancer Res 2007; 9: R75.

[14] Wells A. EGF receptor. Int J Biochem Cell Biol 1999; 31: 637-643.

[15] Gullick WJ. Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. Br Med Bull 1991; 47: 87-98.

[16] Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987; 235: 177-182.

[17] Zaczek A, Brandt B and Bielawski KP. The diverse signaling network of EGFR, HER2, HER3 and HER4 tyrosine kinase receptors and the consequences for therapeutic approaches. Histol Histopathol 2005; 20: 1005-1015.

[18] Traxler P. Tyrosine kinases as targets in cancer therapy - successes and failures. Expert Opin Ther Targets 2003; 7: 215-234.

[19] Fabian MA, Biggs WH 3rd, Treiber DK, Atteridge CE, Azimioara MD, Benedetti MG, Carter TA,

Ciceri P, Edeen PT, Floyd M, Ford JM, Galvin M, Gerlach JL, Grotzfeld RM, Herrgard S, Insko DE, Insko MA, Lai AG, Lelias JM, Mehta SA, Milanov ZV, Velasco AM, Wodicka LM, Patel HK, Zarrinkar PP and Lockhart DJ. A small molecule-kinase interaction map for clinical kinase inhibitors. Nat Biotechnol 2005; 23: 329-336.

[20] Hanks SK, Quinn AM and Hunter T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 1988; 241: 42-52.

[21] Adnane L, Trail PA, Taylor I and Wilhelm SM. Sorafenib (BAY 43-9006, Nexavar), a dual-action inhibitor that targets RAF/MEK/ERK pathway in tumor cells and tyrosine kinases VEGFR/PDGFR in tumor vasculature. Methods Enzymol 2006; 407: 597-612.

[22] Martinelli G, Soverini S, Rosti G and Baccarani M. Dual tyrosine kinase inhibitors in chronic myeloid leukemia. Leukemia 2005; 19: 1872-1879.

[23] Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, Jagiello-Gruszfeld A, Crown J, Chan A, Kaufman B, Skarlos D, Campone M, Davidson N, Berger M, Oliva C, Rubin SD, Stein S and Cameron D. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med 2006; 355: 2733-2743.

[24] Blackwell KL, Burstein HJ, Storniolo AM, Rugo H, Sledge G, Koehler M, Ellis C, Casey M, Vukelja S, Bischoff J, Baselga J and O'Shaughnessy J. Randomized study of Lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. J Clin Oncol 2010; 28: 1124-1130.

[25] Tevaarwerk AJ and Kolesar JM. Lapatinib: a small-molecule inhibitor of epidermal growth factor receptor and human epidermal growth factor receptor-2 tyrosine kinases used in the treatment of breast cancer. Clin Ther 2009; 31 Pt 2: 2332-2348.

[26] Johnston S, Pippen J, Jr., Pivot X, Lichinitser M, Sadeghi S, Dieras V, Gomez HL, Romieu G, Manikhas A, Kennedy MJ, Press MF, Maltzman J, Florance A, O'Rourke L, Oliva C, Stein S and Pegram M. Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor-positive metastatic breast cancer. J Clin Oncol 2009; 27: 5538-5546.

[27] Rusnak DW, Lackey K, Affleck K, Wood ER, Alligood KJ, Rhodes N, Keith BR, Murray DM, Knight WB, Mullin RJ and Gilmer TM. The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines in vitro and in vivo. Mol Cancer Ther 2001; 1: 85-94.

[28] Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, Owens G, Alligood KJ and Spector NL. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. Oncogene 2002; 21: 6255-6263.

[29] Wainberg ZA, Anghel A, Desai AJ, Ayala R, Luo T, Safran B, Fejzo MS, Hecht JR, Slamon DJ and Finn RS. Lapatinib, a dual EGFR and HER2 kinase inhibitor, selectively inhibits HER2-amplified human gastric cancer cells and is synergistic with trastuzumab in vitro and in vivo. Clin Cancer Res 2010; 16: 1509-1519.

[30] Gril B, Palmieri D, Bronder JL, Herring JM, Vega-Valle E, Feigenbaum L, Liewehr DJ, Steinberg SM, Merino MJ, Rubin SD and Steeg PS. Effect of lapatinib on the outgrowth of metastatic breast cancer cells to the brain. J Natl Cancer Inst 2008; 100: 1092-1103.

[31] Konecny GE, Pegram MD, Venkatesan N, Finn R, Yang G, Rahmeh M, Untch M, Rusnak DW, Spehar G, Mullin RJ, Keith BR, Gilmer TM, Berger M, Podratz KC and Slamon DJ. Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. Cancer Res 2006; 66: 1630-1639.

[32] Zhang D, Pal A, Bornmann WG, Yamasaki F, Esteva FJ, Hortobagyi GN, Bartholomeusz C and Ueno NT. Activity of lapatinib is independent of EGFR expression level in HER2-overexpressing breast cancer cells. Mol Cancer Ther 2008; 7: 1846-1850.

[33] Tanizaki J, Okamoto I, Fumita S, Okamoto W, Nishio K and Nakagawa K. Roles of BIM induction and survivin downregulation in lapatinib-induced apoptosis in breast cancer cells with HER2 amplification. Oncogene 2011; 30: 4097-4106.

[34] Ryan BM, Konecny GE, Kahlert S, Wang HJ, Untch M, Meng G, Pegram MD, Podratz KC, Crown J, Slamon DJ and Duffy MJ. Survivin expression in breast cancer predicts clinical outcome and is associated with HER2, VEGF, urokinase plasminogen activator and PAI-1. Ann Oncol 2006; 17: 597-604.

[35] Jha K, Shukla M and Pandey M. Survivin expression and targeting in breast cancer. Surg Oncol 2012; 21: 125-131.

[36] Liu L, Greger J, Shi H, Liu Y, Greshock J, Annan R, Halsey W, Sathe GM, Martin AM and Gilmer TM. Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL. Cancer Res 2009; 69: 6871-6878.

[37] Bendell JC, Domchek SM, Burstein HJ, Harris L, Younger J, Kuter I, Bunnell C, Rue M, Gelman

R and Winer E. Central nervous system metastases in women who receive trastuzumab-based therapy for metastatic breast carcinoma. Cancer 2003; 97: 2972-2977.

[38] Ono M, Ando M, Yunokawa M, Nakano E, Yonemori K, Matsumoto K, Kouno T, Shimizu C, Tamura K, Katsumata N and Fujiwara Y. Brain metastases in patients who receive trastuzumab-containing chemotherapy for HER2-overexpressing metastatic breast cancer. Int J Clin Oncol 2009; 14: 48-52.

[39] Arribas J, Baselga J, Pedersen K and Parra-Palau JL. p95HER2 and breast cancer. Cancer Res 2011; 71: 1515-1519.

[40] Saez R, Molina MA, Ramsey EE, Rojo F, Keenan EJ, Albanell J, Lluch A, Garcia-Conde J, Baselga J and Clinton GM. p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. Clin Cancer Res 2006; 12: 424-431.

[41] Scaltriti M, Rojo F, Ocana A, Anido J, Guzman M, Cortes J, Di Cosimo S, Matias-Guiu X, Ramon y Cajal S, Arribas J and Baselga J. Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. J Natl Cancer Inst 2007; 99: 628-638.

[42] Nahta R, Yuan LX, Du Y and Esteva FJ. Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling. Mol Cancer Ther 2007; 6: 667-674.

[43] Wakeling AE, Barker AJ, Davies DH, Brown DS, Green LR, Cartlidge SA and Woodburn JR. Specific inhibition of epidermal growth factor receptor tyrosine kinase by 4-anilinoquinazolines. Breast Cancer Res Treat 1996; 38: 67-73.

[44] Ma C, Zuo W, Wang X, Wei L, Guo Q and Song X. Lapatinib inhibits the activation of NF-kappaB through reducing phosphorylation of IkappaB-alpha in breast cancer cells. Oncol Rep 2013; 29: 812-818.

[45] Ahmed KM, Cao N and Li JJ. HER-2 and NF-kappaB as the targets for therapy-resistant breast cancer. Anticancer Res 2006; 26: 4235-4243.

[46] Sartor CI. Epidermal growth factor family receptors and inhibitors: radiation response modulators. Semin Radiat Oncol 2003; 13: 22-30.

[47] Tanaka T, Munshi A, Brooks C, Liu J, Hobbs ML and Meyn RE. Gefitinib radiosensitizes non-small cell lung cancer cells by suppressing cellular DNA repair capacity. Clin Cancer Res 2008; 14: 1266-1273.

[48] Ochs JS. Rationale and clinical basis for combining gefitinib (IRESSA, ZD1839) with radiation therapy for solid tumors. Int J Radiat Oncol Biol Phys 2004; 58: 941-949.

[49] Zhou H, Kim YS, Peletier A, McCall W, Earp HS and Sartor CI. Effects of the EGFR/HER2 kinase inhibitor GW572016 on EGFR- and HER2-overexpressing breast cancer cell line proliferation, radiosensitization, and resistance. Int J Radiat Oncol Biol Phys 2004; 58: 344-352.

[50] Sambade MJ, Camp JT, Kimple RJ, Sartor CI and Shields JM. Mechanism of lapatinib-mediated radiosensitization of breast cancer cells is primarily by inhibition of the Raf>MEK>ERK mitogen-activated protein kinase cascade and radiosensitization of lapatinib-resistant cells restored by direct inhibition of MEK. Radiother Oncol 2009; 93: 639-644.

[51] Bhosle J, Kiakos K, Porter AC, Wu J, Makris A, Hartley JA and Hochhauser D. Treatment with gefitinib or lapatinib induces drug resistance through downregulation of topoisomerase IIalpha expression. Mol Cancer Ther 2013; 12: 2897-2908.

[52] Fisher JN, Terao M, Fratelli M, Kurosaki M, Paroni G, Zanetti A, Gianni M, Bolis M, Lupi M, Tsykin A, Goodall GJ and Garattini E. MicroRNA networks regulated by all-trans retinoic acid and Lapatinib control the growth, survival and motility of breast cancer cells. Oncotarget 2015; 6: 13176-200.

[53] Johnston S, Trudeau M, Kaufman B, Boussen H, Blackwell K, LoRusso P, Lombardi DP, Ben Ahmed S, Citrin DL, DeSilvio ML, Harris J, Westlund RE, Salazar V, Zaks TZ and Spector NL. Phase II study of predictive biomarker profiles for response targeting human epidermal growth factor receptor 2 (HER-2) in advanced inflammatory breast cancer with lapatinib monotherapy. J Clin Oncol 2008; 26: 1066-1072.

[54] Burstein HJ, Storniolo AM, Franco S, Forster J, Stein S, Rubin S, Salazar VM and Blackwell KL. A phase II study of lapatinib monotherapy in chemotherapy-refractory HER2-positive and HER2-negative advanced or metastatic breast cancer. Ann Oncol 2008; 19: 1068-1074.

[55] Toi M, Iwata H, Fujiwara Y, Ito Y, Nakamura S, Tokuda Y, Taguchi T, Rai Y, Aogi K, Arai T, Watanabe J, Wakamatsu T, Katsura K, Ellis CE, Gagnon RC, Allen KE, Sasaki Y and Takashima S. Lapatinib monotherapy in patients with relapsed, advanced, or metastatic breast cancer: efficacy, safety, and biomarker results from Japanese patients phase II studies. Br J Cancer 2009; 101: 1676-1682.

[56] Lin NU, Carey LA, Liu MC, Younger J, Come SE, Ewend M, Harris GJ, Bullitt E, Van den Abbeele AD, Henson JW, Li X, Gelman R, Burstein HJ, Kasparian E, Kirsch DG, Crawford A, Hochberg F and Winer EP. Phase II trial of lapatinib for brain metastases in patients with human epi-

dermal growth factor receptor 2-positive breast cancer. J Clin Oncol 2008; 26: 1993-1999.

[57] Bachelot T, Romieu G, Campone M, Dieras V, Cropet C, Dalenc F, Jimenez M, Le Rhun E, Pierga JY, Goncalves A, Leheurteur M, Domont J, Gutierrez M, Cure H, Ferrero JM and Labbe-Devilliers C. Lapatinib plus capecitabine in patients with previously untreated brain metastases from HER2-positive metastatic breast cancer (LANDSCAPE): a single-group phase 2 study. Lancet Oncol 2013; 14: 64-71.

[58] Lin NU, Eierman W, Greil R, Campone M, Kaufman B, Steplewski K, Lane SR, Zembryki D, Rubin SD and Winer EP. Randomized phase II study of lapatinib plus capecitabine or lapatinib plus topotecan for patients with HER2-positive breast cancer brain metastases. J Neurooncol 2011; 105: 613-620.

[59] Lin NU, Dieras V, Paul D, Lossignol D, Christodoulou C, Stemmler HJ, Roche H, Liu MC, Greil R, Ciruelos E, Loibl S, Gori S, Wardley A, Yardley D, Brufsky A, Blum JL, Rubin SD, Dharan B, Steplewski K, Zembryki D, Oliva C, Roychowdhury D, Paoletti P and Winer EP. Multicenter phase II study of lapatinib in patients with brain metastases from HER2-positive breast cancer. Clin Cancer Res 2009; 15: 1452-1459.

[60] Sierra JR, Cepero V and Giordano S. Molecular mechanisms of acquired resistance to tyrosine kinase targeted therapy. Mol Cancer 2010; 9: 75.

[61] Brugge J, Hung MC and Mills GB. A new mutational AKTivation in the PI3K pathway. Cancer Cell 2007; 12: 104-107.

[62] Coughlin CM, Johnston DS, Strahs A, Burczynski ME, Bacus S, Hill J, Feingold JM, Zacharchuk C and Berkenblit A. Approaches and limitations of phosphatidylinositol-3-kinase pathway activation status as a predictive biomarker in the clinical development of targeted therapy. Breast Cancer Res Treat 2010; 124: 1-11.

[63] Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, Yu JS, Malmstrom PO, Mansukhani M, Enoksson J, Hibshoosh H, Borg A and Parsons R. PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res 2005; 65: 2554-2559.

[64] Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, Carey M, Hu Z, Guan Y, Sahin A, Symmans WF, Pusztai L, Nolden LK, Horlings H, Berns K, Hung MC, van de Vijver MJ, Valero V, Gray JW, Bernards R, Mills GB and Hennessy BT. An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. Cancer Res 2008; 68: 6084-6091.

[65] Eichhorn PJ, Gili M, Scaltriti M, Serra V, Guzman M, Nijkamp W, Beijersbergen RL, Valero V, Seoane J, Bernards R and Baselga J. Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235. Cancer Res 2008; 68: 9221-9230.

[66] Barton S, Starling N and Swanton C. Predictive molecular markers of response to epidermal growth factor receptor(EGFR) family-targeted therapies. Curr Cancer Drug Targets 2010; 10: 799-812.

[67] Isakoff SJ, Engelman JA, Irie HY, Luo J, Brachmann SM, Pearline RV, Cantley LC and Brugge JS. Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. Cancer Res 2005; 65: 10992-11000.

[68] Wang L, Zhang Q, Zhang J, Sun S, Guo H, Jia Z, Wang B, Shao Z, Wang Z and Hu X. PI3K pathway activation results in low efficacy of both trastuzumab and lapatinib. BMC Cancer 2011; 11: 248.

[69] O'Brien NA, Browne BC, Chow L, Wang Y, Ginther C, Arboleda J, Duffy MJ, Crown J, O'Donovan N and Slamon DJ. Activated phosphoinositide 3-kinase/AKT signaling confers resistance to trastuzumab but not lapatinib. Mol Cancer Ther 2010; 9: 1489-1502.

[70] Xia W, Husain I, Liu L, Bacus S, Saini S, Spohn J, Pry K, Westlund R, Stein SH and Spector NL. Lapatinib antitumor activity is not dependent upon phosphatase and tensin homologue deleted on chromosome 10 in ErbB2-overexpressing breast cancers. Cancer Res 2007; 67: 1170-1175.

[71] Karakashev SV and Reginato MJ. Hypoxia/HIF1alpha induces lapatinib resistance in ERBB2-positive breast cancer cells via regulation of DUSP2. Oncotarget 2015; 6: 1967-1980.

[72] Vazquez-Martin A, Oliveras-Ferraros C and Menendez JA. Autophagy facilitates the development of breast cancer resistance to the anti-HER2 monoclonal antibody trastuzumab. PLoS One 2009; 4: e6251.

[73] Chen S, Li X, Feng J, Chang Y, Wang Z and Wen A. Autophagy facilitates the Lapatinib resistance of HER2 positive breast cancer cells. Med Hypotheses 2011; 77: 206-208.

[74] Xia W, Petricoin EF 3rd, Zhao S, Liu L, Osada T, Cheng Q, Wulfkuhle JD, Gwin WR, Yang X, Gallagher RI, Bacus S, Lyerly HK and Spector NL. An heregulin-EGFR-HER3 autocrine signaling axis can mediate acquired lapatinib resistance in HER2+ breast cancer models. Breast Cancer Res 2013; 15: R85.

[75] Kristiansen G, Winzer KJ, Mayordomo E, Bellach J, Schluns K, Denkert C, Dahl E, Pilarsky C, Altevogt P, Guski H and Dietel M. CD24 expression is a new prognostic marker in breast cancer. Clin Cancer Res 2003; 9: 4906-4913.

[76] Hosonaga M, Arima Y, Sugihara E, Kohno N and Saya H. Expression of CD24 is associated with HER2 expression and supports HER2-Akt signaling in HER2-positive breast cancer cells. Cancer Sci 2014; 105: 779-787.

[77] Rani S, Corcoran C, Shiels L, Germano S, Breslin S, Madden S, McDermott MS, Browne BC, O'Donovan N, Crown J, Gogarty M, Byrne AT and O'Driscoll L. Neuromedin U: a candidate biomarker and therapeutic target to predict and overcome resistance to HER-tyrosine kinase inhibitors. Cancer Res 2014; 74: 3821-3833.

[78] Xia W, Bacus S, Hegde P, Husain I, Strum J, Liu L, Paulazzo G, Lyass L, Trusk P, Hill J, Harris J and Spector NL. A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. Proc Natl Acad Sci U S A 2006; 103: 7795-7800.

[79] Lee SY, Meier R, Furuta S, Lenburg ME, Kenny PA, Xu R and Bissell MJ. FAM83A confers EGFR-TKI resistance in breast cancer cells and in mice. J Clin Invest 2012; 122: 3211-3220.

[80] Wang Q, Quan H, Zhao J, Xie C, Wang L and Lou L. RON confers lapatinib resistance in HER2-positive breast cancer cells. Cancer Lett 2013; 340: 43-50.

[81] Azuma K, Tsurutani J, Sakai K, Kaneda H, Fujisaka Y, Takeda M, Watatani M, Arao T, Satoh T, Okamoto I, Kurata T, Nishio K and Nakagawa K. Switching addictions between HER2 and FGFR2 in HER2-positive breast tumor cells: FGFR2 as a potential target for salvage after lapatinib failure. Biochem Biophys Res Commun 2011; 407: 219-224.

[82] Park SH, Ito K, Olcott W, Katsyv I, Halstead-Nussloch G and Irie HY. PTK6 inhibition promotes apoptosis of Lapatinib-resistant Her2 breast cancer cells by inducing Bim. Breast Cancer Res 2015; 17: 86.

[83] Formisano L, Nappi L, Rosa R, Marciano R, D'Amato C, D'Amato V, Damiano V, Raimondo L, Iommelli F, Scorziello A, Troncone G, Veneziani B, Parsons SJ, De Placido S and Bianco R. Epidermal growth factor-receptor activation modulates Src-dependent resistance to lapatinib in breast cancer models. Breast Cancer Res 2014; 16: R45.

[84] De Luca A, D'Alessio A, Gallo M, Maiello MR, Bode AM and Normanno N. Src and CXCR4 are involved in the invasiveness of breast cancer cells with acquired resistance to lapatinib. Cell Cycle 2014; 13: 148-156.

[85] Rexer BN, Ham AJ, Rinehart C, Hill S, Granja-Ingram Nde M, Gonzalez-Angulo AM, Mills GB, Dave B, Chang JC, Liebler DC and Arteaga CL. Phosphoproteomic mass spectrometry profiling links Src family kinases to escape from HER2 tyrosine kinase inhibition. Oncogene 2011; 30: 4163-4174.

[86] Stuhlmiller TJ, Miller SM, Zawistowski JS, Nakamura K, Beltran AS, Duncan JS, Angus SP, Collins KA, Granger DA, Reuther RA, Graves LM, Gomez SM, Kuan PF, Parker JS, Chen X, Sciaky N, Carey LA, Earp HS, Jin J and Johnson GL. Inhibition of Lapatinib-Induced Kinome Reprogramming in ERBB2-Positive Breast Cancer by Targeting BET Family Bromodomains. Cell Rep 2015; 11: 390-404.

[87] Corcoran C, Rani S, Breslin S, Gogarty M, Ghobrial IM, Crown J and O'Driscoll L. miR-630 targets IGF1R to regulate response to HER-targeting drugs and overall cancer cell progression in HER2 over-expressing breast cancer. Mol Cancer 2014; 13: 71.

[88] Moody SE, Schinzel AC, Singh S, Izzo F, Strickland MR, Luo L, Thomas SR, Boehm JS, Kim SY, Wang ZC and Hahn WC. PRKACA mediates resistance to HER2-targeted therapy in breast cancer cells and restores anti-apoptotic signaling. Oncogene 2015; 34: 2061-71.

[89] Chen CJ, Lin SE, Lin YM, Lin SH, Chen DR and Chen CL. Association of expression of kruppel-like factor 4 and kruppel-like factor 5 with the clinical manifestations of breast cancer. Pathol Oncol Res 2012; 18: 161-168.

[90] Tong D, Czerwenka K, Heinze G, Ryffel M, Schuster E, Witt A, Leodolter S and Zeillinger R. Expression of KLF5 is a prognostic factor for disease-free survival and overall survival in patients with breast cancer. Clin Cancer Res 2006; 12: 2442-2448.

[91] Farrugia MK, Sharma SB, Lin CC, McLaughlin SL, Vanderbilt DB, Ammer AG, Salkeni MA, Stoilov P, Agazie YM, Creighton CJ and Ruppert JM. Regulation of anti-apoptotic signaling by Kruppel-like factors 4 and 5 mediates lapatinib resistance in breast cancer. Cell Death Dis 2015; 6: e1699.

[92] Bekki K, Vogel H, Li W, Ito T, Sweeney C, Haarmann-Stemmann T, Matsumura F and Vogel CF. The aryl hydrocarbon receptor (AhR) mediates resistance to apoptosis induced in breast cancer cells. Pestic Biochem Physiol 2015; 120: 5-13.

[93] Mittendorf EA, Wu Y, Scaltriti M, Meric-Bernstam F, Hunt KK, Dawood S, Esteva FJ, Buzdar AU, Chen H, Eksambi S, Hortobagyi GN, Baselga J and Gonzalez-Angulo AM. Loss of

HER2 amplification following trastuzumab-based neoadjuvant systemic therapy and survival outcomes. Clin Cancer Res 2009; 15: 7381-7388.

[94] Garrett JT, Olivares MG, Rinehart C, Granja-Ingram ND, Sanchez V, Chakrabarty A, Dave B, Cook RS, Pao W, McKinely E, Manning HC, Chang J and Arteaga CL. Transcriptional and posttranslational up-regulation of HER3 (ErbB3) compensates for inhibition of the HER2 tyrosine kinase. Proc Natl Acad Sci U S A 2011; 108: 5021-5026.

[95] Amin DN, Sergina N, Ahuja D, McMahon M, Blair JA, Wang D, Hann B, Koch KM, Shokat KM and Moasser MM. Resiliency and vulnerability in the HER2-HER3 tumorigenic driver. Sci Transl Med 2010; 2: 16ra17.

[96] Berardi R, Santoni M, Morgese F, Ballatore Z, Savini A, Onofri A, Mazzanti P, Pistelli M, Pierantoni C, De Lisa M, Caramanti M, Pagliaretta S, Pellei C and Cascinu S. Novel small molecule EGFR inhibitors as candidate drugs in non-small cell lung cancer. Onco Targets Ther 2013; 6: 563-576.

[97] Esteva FJ. Monoclonal antibodies, small molecules, and vaccines in the treatment of breast cancer. Oncologist 2004; 9 Suppl 3: 4-9.

[98] Sainsbury JR, Farndon JR, Harris AL and Sherbet GV. Epidermal growth factor receptors on human breast cancers. Br J Surg 1985; 72: 186-188.

[99] Ferrero JM, Ramaioli A, Largillier R, Formento JL, Francoual M, Ettore F, Namer M and Milano G. Epidermal growth factor receptor expression in 780 breast cancer patients: a reappraisal of the prognostic value based on an eight-year median follow-up. Ann Oncol 2001; 12: 841-846.

[100] Harris AL, Nicholson S, Sainsbury JR, Farndon J and Wright C. Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. J Steroid Biochem 1989; 34: 123-131.

[101] Moulder SL, Yakes FM, Muthuswamy SK, Bianco R, Simpson JF and Arteaga CL. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo. Cancer Res 2001; 61: 8887-8895.

[102] Anido J, Matar P, Albanell J, Guzman M, Rojo F, Arribas J, Averbuch S and Baselga J. ZD1839, a specific epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, induces the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers and prevents heregulin signaling in HER2-overexpressing breast cancer cells. Clin Cancer Res 2003; 9: 1274-1283.

[103] Campiglio M, Locatelli A, Olgiati C, Normanno N, Somenzi G, Vigano L, Fumagalli M, Menard S and Gianni L. Inhibition of proliferation and induction of apoptosis in breast cancer cells by the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor ZD1839 ('Iressa') is independent of EGFR expression level. J Cell Physiol 2004; 198: 259-268.

[104] Moasser MM, Basso A, Averbuch SD and Rosen N. The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. Cancer Res 2001; 61: 7184-7188.

[105] Krol J, Francis RE, Albergaria A, Sunters A, Polychronis A, Coombes RC and Lam EW. The transcription factor FOX03a is a crucial cellular target of gefitinib (Iressa) in breast cancer cells. Mol Cancer Ther 2007; 6: 3169-3179.

[106] Segovia-Mendoza M, Diaz L, Gonzalez-Gonzalez ME, Martinez-Reza I, Garcia-Quiroz J, Prado-Garcia H, Ibarra-Sanchez MJ, Esparza-Lopez J, Larrea F and Garcia-Becerra R. Calcitriol and its analogues enhance the antiproliferative activity of gefitinib in breast cancer cells. J Steroid Biochem Mol Biol 2015; 148: 122-131.

[107] Dragowska WH, Weppler SA, Wang JC, Wong LY, Kapanen AI, Rawji JS, Warburton C, Qadir MA, Donohue E, Roberge M, Gorski SM, Gelmon KA and Bally MB. Induction of autophagy is an early response to gefitinib and a potential therapeutic target in breast cancer. PLoS One 2013; 8: e76503.

[108] Baselga J, Albanell J, Ruiz A, Lluch A, Gascon P, Guillem V, Gonzalez S, Sauleda S, Marimon I, Tabernero JM, Koehler MT and Rojo F. Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. J Clin Oncol 2005; 23: 5323-5333.

[109] Arteaga CL, O'Neill A, Moulder SL, Pins M, Sparano JA, Sledge GW and Davidson NE. A phase I-II study of combined blockade of the ErbB receptor network with trastuzumab and gefitinib in patients with HER2 (ErbB2)-overexpressing metastatic breast cancer. Clin Cancer Res 2008; 14: 6277-6283.

[110] Somlo G, Martel CL, Lau SK, Frankel P, Ruel C, Gu L, Hurria A, Chung C, Luu T, Morgan R Jr, Leong L, Koczywas M, McNamara M, Russell CA and Kane SE. A phase I/II prospective, single arm trial of gefitinib, trastuzumab, and docetaxel in patients with stage IV HER-2 positive metastatic breast cancer. Breast Cancer Res Treat 2012; 131: 899-906.

[111] von Minckwitz G, Jonat W, Fasching P, du Bois A, Kleeberg U, Luck HJ, Kettner E, Hilfrich J, Eiermann W, Torode J and Schneeweiss A. A multicentre phase II study on gefitinib in taxane- and anthracycline-pretreated metastatic

breast cancer. Breast Cancer Res Treat 2005; 89: 165-172.
[112] Smith IE, Walsh G, Skene A, Llombart A, Mayordomo JI, Detre S, Salter J, Clark E, Magill P and Dowsett M. A phase II placebo-controlled trial of neoadjuvant anastrozole alone or with gefitinib in early breast cancer. J Clin Oncol 2007; 25: 3816-3822.
[113] Massarweh S, Tham YL, Huang J, Sexton K, Weiss H, Tsimelzon A, Beyer A, Rimawi M, Cai WY, Hilsenbeck S, Fuqua S and Elledge R. A phase II neoadjuvant trial of anastrozole, fulvestrant, and gefitinib in patients with newly diagnosed estrogen receptor positive breast cancer. Breast Cancer Res Treat 2011; 129: 819-827.
[114] Guarneri V, Frassoldati A, Ficarra G, Puglisi F, Andreetta C, Michelotti A, Cresti N, Boni C, Bisagni G, Berardi R, Battelli N, Santoro A, Banna G, Bottini A, Di Blasio B, Maiorana A, Piacentini F, Giovannelli S, Jovic G and Conte P. Phase II, randomized trial of preoperative epirubicin-paclitaxel +/- gefitinib with biomarker evaluation in operable breast cancer. Breast Cancer Res Treat 2008; 110: 127-134.
[115] Engebraaten O, Edvardsen H, Lokkevik E, Naume B, Kristensen V, Ottestad L and Natarajan V. Gefitinib in Combination with Weekly Docetaxel in Patients with Metastatic Breast Cancer Caused Unexpected Toxicity: Results from a Randomized Phase II Clinical Trial. ISRN Oncol 2012; 2012: 176789.
[116] Polychronis A, Sinnett HD, Hadjiminas D, Singhal H, Mansi JL, Shivapatham D, Shousha S, Jiang J, Peston D, Barrett N, Vigushin D, Morrison K, Beresford E, Ali S, Slade MJ and Coombes RC. Preoperative gefitinib versus gefitinib and anastrozole in postmenopausal patients with oestrogen-receptor positive and epidermal-growth-factor-receptor-positive primary breast cancer: a double-blind placebo-controlled phase II randomised trial. Lancet Oncol 2005; 6: 383-391.
[117] Ciardiello F, Troiani T, Caputo F, De Laurentiis M, Tortora G, Palmieri G, De Vita F, Diadema MR, Orditura M, Colantuoni G, Gridelli C, Catalano G, De Placido S and Bianco AR. Phase II study of gefitinib in combination with docetaxel as first-line therapy in metastatic breast cancer. Br J Cancer 2006; 94: 1604-1609.
[118] Osborne CK, Neven P, Dirix LY, Mackey JR, Robert J, Underhill C, Schiff R, Gutierrez C, Migliaccio I, Anagnostou VK, Rimm DL, Magill P and Sellers M. Gefitinib or placebo in combination with tamoxifen in patients with hormone receptor-positive metastatic breast cancer: a randomized phase II study. Clin Cancer Res 2011; 17: 1147-1159.
[119] Kalykaki A, Agelaki S, Kallergi G, Xyrafas A, Mavroudis D and Georgoulias V. Elimination of EGFR-expressing circulating tumor cells in patients with metastatic breast cancer treated with gefitinib. Cancer Chemother Pharmacol 2014; 73: 685-693.
[120] Normanno N, De Luca A, Maiello MR, Campiglio M, Napolitano M, Mancino M, Carotenuto A, Viglietto G and Menard S. The MEK/MAPK pathway is involved in the resistance of breast cancer cells to the EGFR tyrosine kinase inhibitor gefitinib. J Cell Physiol 2006; 207: 420-427.
[121] Normanno N, Campiglio M, Maiello MR, De Luca A, Mancino M, Gallo M, D'Alessio A and Menard S. Breast cancer cells with acquired resistance to the EGFR tyrosine kinase inhibitor gefitinib show persistent activation of MAPK signaling. Breast Cancer Res Treat 2008; 112: 25-33.
[122] Ferrer-Soler L, Vazquez-Martin A, Brunet J, Menendez JA, De Llorens R and Colomer R. An update of the mechanisms of resistance to EGFR-tyrosine kinase inhibitors in breast cancer: Gefitinib (Iressa) -induced changes in the expression and nucleo-cytoplasmic trafficking of HER-ligands (Review). Int J Mol Med 2007; 20: 3-10.
[123] Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM and Moasser MM. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature 2007; 445: 437-441.
[124] She QB, Solit D, Basso A and Moasser MM. Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3'-kinase/Akt pathway signaling. Clin Cancer Res 2003; 9: 4340-4346.
[125] Bianco R, Shin I, Ritter CA, Yakes FM, Basso A, Rosen N, Tsurutani J, Dennis PA, Mills GB and Arteaga CL. Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. Oncogene 2003; 22: 2812-2822.
[126] Dijkers PF, Medema RH, Pals C, Banerji L, Thomas NS, Lam EW, Burgering BM, Raaijmakers JA, Lammers JW, Koenderman L and Coffer PJ. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol Cell Biol 2000; 20: 9138-9148.
[127] Mueller KL, Hunter LA, Ethier SP and Boerner JL. Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells. Cancer Res 2008; 68: 3314-3322.
[128] Kuzumaki N, Suzuki A, Narita M, Hosoya T, Nagasawa A, Imai S, Yamamizu K, Morita H,

Suzuki T, Okada Y, Okano HJ, Yamashita JK and Okano H. Multiple analyses of G-protein coupled receptor (GPCR) expression in the development of gefitinib-resistance in transforming non-small-cell lung cancer. PLoS One 2012; 7: e44368.

[129] Pao W, Wang TY, Riely GJ, Miller VA, Pan Q, Ladanyi M, Zakowski MF, Heelan RT, Kris MG and Varmus HE. KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. PLoS Med 2005; 2: e17.

[130] Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M and Varmus H. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004; 101: 13306-13311.

[131] Hart S, Fischer OM, Prenzel N, Zwick-Wallasch E, Schneider M, Hennighausen L and Ullrich A. GPCR-induced migration of breast carcinoma cells depends on both EGFR signal transactivation and EGFR-independent pathways. Biol Chem 2005; 386: 845-855.

[132] Han C, Zou H, Ma J, Zhou Y and Zhao J. [Comparison of EGFR and KRAS status between primary non-small cell lung cancer and corresponding metastases: a systematic review and meta-analysis]. Zhongguo Fei Ai Za Zhi 2010; 13: 882-891.

[133] Han CB, Li F, Ma JT and Zou HW. Concordant KRAS mutations in primary and metastatic colorectal cancer tissue specimens: a meta-analysis and systematic review. Cancer Invest 2012; 30: 741-747.

[134] Santarpia L, Qi Y, Stemke-Hale K, Wang B, Young EJ, Booser DJ, Holmes FA, O'Shaughnessy J, Hellerstedt B, Pippen J, Vidaurre T, Gomez H, Valero V, Hortobagyi GN, Symmans WF, Bottai G, Di Leo A, Gonzalez-Angulo AM and Pusztai L. Mutation profiling identifies numerous rare drug targets and distinct mutation patterns in different clinical subtypes of breast cancers. Breast Cancer Res Treat 2012; 134: 333-343.

[135] Bhargava R, Gerald WL, Li AR, Pan Q, Lal P, Ladanyi M and Chen B. EGFR gene amplification in breast cancer: correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations. Mod Pathol 2005; 18: 1027-1033.

[136] Rabindran SK, Discafani CM, Rosfjord EC, Baxter M, Floyd MB, Golas J, Hallett WA, Johnson BD, Nilakantan R, Overbeek E, Reich MF, Shen R, Shi X, Tsou HR, Wang YF and Wissner A. Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase. Cancer Res 2004; 64: 3958-3965.

[137] Wissner A, Overbeek E, Reich MF, Floyd MB, Johnson BD, Mamuya N, Rosfjord EC, Discafani C, Davis R, Shi X, Rabindran SK, Gruber BC, Ye F, Hallett WA, Nilakantan R, Shen R, Wang YF, Greenberger LM and Tsou HR. Synthesis and structure-activity relationships of 6,7-disubstituted 4-anilinoquinoline-3-carbonitriles. The design of an orally active, irreversible inhibitor of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor-2 (HER-2). J Med Chem 2003; 46: 49-63.

[138] Wissner A and Mansour TS. The development of HKI-272 and related compounds for the treatment of cancer. Arch Pharm (Weinheim) 2008; 341: 465-477.

[139] Tsou HR, Overbeek-Klumpers EG, Hallett WA, Reich MF, Floyd MB, Johnson BD, Michalak RS, Nilakantan R, Discafani C, Golas J, Rabindran SK, Shen R, Shi X, Wang YF, Upeslacis J and Wissner A. Optimization of 6,7-disubstituted-4-(arylamino)quinoline-3-carbonitriles as orally active, irreversible inhibitors of human epidermal growth factor receptor-2 kinase activity. J Med Chem 2005; 48: 1107-1131.

[140] R. Swaby KB, Jiang Z, Sun Y, Dieras V, Zaman K, Zacharchuk C, Powell C, Abbas R, Thakuria M. Neratinib in combination with trastuzumab for the treatment of advanced breast cancer: A phase I/II study. J Clin Oncol 2009; 27:15s.

[141] Ito Y, Suenaga M, Hatake K, Takahashi S, Yokoyama M, Onozawa Y, Yamazaki K, Hironaka S, Hashigami K, Hasegawa H, Takenaka N and Boku N. Safety, efficacy and pharmacokinetics of neratinib (HKI-272) in Japanese patients with advanced solid tumors: a Phase 1 dose-escalation study. Jpn J Clin Oncol 2012; 42: 278-286.

[142] Chow LW, Xu B, Gupta S, Freyman A, Zhao Y, Abbas R, Vo Van ML and Bondarenko I. Combination neratinib (HKI-272) and paclitaxel therapy in patients with HER2-positive metastatic breast cancer. Br J Cancer 2013; 108: 1985-1993.

[143] Burstein HJ, Sun Y, Dirix LY, Jiang Z, Paridaens R, Tan AR, Awada A, Ranade A, Jiao S, Schwartz G, Abbas R, Powell C, Turnbull K, Vermette J, Zacharchuk C and Badwe R. Neratinib, an irreversible ErbB receptor tyrosine kinase inhibitor, in patients with advanced ErbB2-positive breast cancer. J Clin Oncol 2010; 28: 1301-1307.

[144] Wong KK, Fracasso PM, Bukowski RM, Lynch TJ, Munster PN, Shapiro GI, Janne PA, Eder JP, Naughton MJ, Ellis MJ, Jones SF, Mekhail T, Zacharchuk C, Vermette J, Abbas R, Quinn S, Powell C and Burris HA. A phase I study with

neratinib (HKI-272), an irreversible pan ErbB receptor tyrosine kinase inhibitor, in patients with solid tumors. Clin Cancer Res 2009; 15: 2552-2558.

[145] Canonici A, Gijsen M, Mullooly M, Bennett R, Bouguern N, Pedersen K, O'Brien NA, Roxanis I, Li JL, Bridge E, Finn R, Siamon D, McGowan P, Duffy MJ, O'Donovan N, Crown J and Kong A. Neratinib overcomes trastuzumab resistance in HER2 amplified breast cancer. Oncotarget 2013; 4: 1592-1605.

[146] Zhao XQ, Xie JD, Chen XG, Sim HM, Zhang X, Liang YJ, Singh S, Talele TT, Sun Y, Ambudkar SV, Chen ZS and Fu LW. Neratinib reverses ATP-binding cassette B1-mediated chemotherapeutic drug resistance in vitro, in vivo, and ex vivo. Mol Pharmacol 2012; 82: 47-58.

[147] Nafi S, Generali D, Kramer-Marek G, Gijsen M, Strina C, Cappelletti M, Andreis D, Haider S, Li JL, Bridges E, Capala J, Ioannis R, Harris AL and Kong A. Nuclear HER4 mediates acquired resistance to trastuzumab and is associated with poor outcome in HER2 positive breast cancer. Oncotarget 2014; [Epub ahead of print].

[148] Martin M, Bonneterre J, Geyer CE Jr, Ito Y, Ro J, Lang I, Kim SB, Germa C, Vermette J, Wang K and Awada A. A phase two randomised trial of neratinib monotherapy versus lapatinib plus capecitabine combination therapy in patients with HER2+ advanced breast cancer. Eur J Cancer 2013; 49: 3763-3772.

[149] Seyhan AA, Varadarajan U, Choe S, Liu W and Ryan TE. A genome-wide RNAi screen identifies novel targets of neratinib resistance leading to identification of potential drug resistant genetic markers. Mol Biosyst 2012; 8: 1553-1570.

[150] Navolanic PM, Steelman LS and McCubrey JA. EGFR family signaling and its association with breast cancer development and resistance to chemotherapy (Review). Int J Oncol 2003; 22: 237-252.

[151] Dua R, Zhang J, Nhonthachit P, Penuel E, Petropoulos C and Parry G. EGFR over-expression and activation in high HER2, ER negative breast cancer cell line induces trastuzumab resistance. Breast Cancer Res Treat 2010; 122: 685-697.

[152] Arora A and Scholar EM. Role of tyrosine kinase inhibitors in cancer therapy. J Pharmacol Exp Ther 2005; 315: 971-979.

[153] Bunn PA Jr and Franklin W. Epidermal growth factor receptor expression, signal pathway, and inhibitors in non-small cell lung cancer. Semin Oncol 2002; 29: 38-44.

[154] Bachawal SV, Wali VB and Sylvester PW. Enhanced antiproliferative and apoptotic response to combined treatment of gamma-tocotrienol with erlotinib or gefitinib in mammary tumor cells. BMC Cancer 2010; 10: 84.

[155] Bachawal SV, Wali VB and Sylvester PW. Combined gamma-tocotrienol and erlotinib/gefitinib treatment suppresses Stat and Akt signaling in murine mammary tumor cells. Anticancer Res 2010; 30: 429-437.

[156] Karaman MW, Herrgard S, Treiber DK, Gallant P, Atteridge CE, Campbell BT, Chan KW, Ciceri P, Davis MI, Edeen PT, Faraoni R, Floyd M, Hunt JP, Lockhart DJ, Milanov ZV, Morrison MJ, Pallares G, Patel HK, Pritchard S, Wodicka LM and Zarrinkar PP. A quantitative analysis of kinase inhibitor selectivity. Nat Biotechnol 2008; 26: 127-132.

[157] Stamos J, Sliwkowski MX and Eigenbrot C. Structure of the epidermal growth factor receptor kinase domain alone and in complex with a 4-anilinoquinazoline inhibitor. J Biol Chem 2002; 277: 46265-46272.

[158] Wood ER, Truesdale AT, McDonald OB, Yuan D, Hassell A, Dickerson SH, Ellis B, Pennisi C, Horne E, Lackey K, Alligood KJ, Rusnak DW, Gilmer TM and Shewchuk L. A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. Cancer Res 2004; 64: 6652-6659.

[159] Bublil EM, Pines G, Patel G, Fruhwirth G, Ng T and Yarden Y. Kinase-mediated quasi-dimers of EGFR. FASEB J 2010; 24: 4744-4755.

[160] Sanchez-Martin M and Pandiella A. Differential action of small molecule HER kinase inhibitors on receptor heterodimerization: therapeutic implications. Int J Cancer 2012; 131: 244-252.

[161] O'Neill F, Madden SF, Clynes M, Crown J, Doolan P, Aherne ST and O'Connor R. A gene expression profile indicative of early stage HER2 targeted therapy response. Mol Cancer 2013; 12: 69.

[162] O'Neill F, Madden SF, Aherne ST, Clynes M, Crown J, Doolan P and O'Connor R. Gene expression changes as markers of early lapatinib response in a panel of breast cancer cell lines. Mol Cancer 2012; 11: 41.

[163] Blackwell KL, Pegram MD, Tan-Chiu E, Schwartzberg LS, Arbushites MC, Maltzman JD, Forster JK, Rubin SD, Stein SH and Burstein HJ. Single-agent lapatinib for HER2-overexpressing advanced or metastatic breast cancer that progressed on first- or second-line trastuzumab-containing regimens. Ann Oncol 2009; 20: 1026-1031.

[164] Kaufman B, Trudeau M, Awada A, Blackwell K, Bachelot T, Salazar V, DeSilvio M, Westlund R, Zaks T, Spector N and Johnston S. Lapatinib monotherapy in patients with HER2-ov-

erexpressing relapsed or refractory inflammatory breast cancer: final results and survival of the expanded HER2+ cohort in EGF103009, a phase II study. Lancet Oncol 2009; 10: 581-588.

[165] Gomez HL, Doval DC, Chavez MA, Ang PC, Aziz Z, Nag S, Ng C, Franco SX, Chow LW, Arbushites MC, Casey MA, Berger MS, Stein SH and Sledge GW. Efficacy and safety of lapatinib as first-line therapy for ErbB2-amplified locally advanced or metastatic breast cancer. J Clin Oncol 2008; 26: 2999-3005.

[166] Chien AJ, Munster PN, Melisko ME, Rugo HS, Park JW, Goga A, Auerback G, Khanafshar E, Ordovas K, Koch KM and Moasser MM. Phase I dose-escalation study of 5-day intermittent oral lapatinib therapy in patients with human epidermal growth factor receptor 2-overexpressing breast cancer. J Clin Oncol 2014; 32: 1472-1479.

[167] Goss PE, Smith IE, O'Shaughnessy J, Ejlertsen B, Kaufmann M, Boyle F, Buzdar AU, Fumoleau P, Gradishar W, Martin M, Moy B, Piccart-Gebhart M, Pritchard KI, Lindquist D, Chavarri-Guerra Y, Aktan G, Rappold E, Williams LS, Finkelstein DM; TEACH investigators. Adjuvant lapatinib for women with early-stage HER2-positive breast cancer: a randomised, controlled, phase 3 trial. Lancet Oncol 2013; 14: 88-96.

[168] Brain E, Isambert N, Dalenc F, Dieras V, Bonneterre J, Rezai K, Jimenez M, Mefti-Lacheraf F, Cottura E, Tresca P, Vanlemmens L, Mahier-Ait Oukhatar C, Lokiec F and Fumoleau P. Phase I study of lapatinib plus vinorelbine in patients with locally advanced or metastatic breast cancer overexpressing HER2. Br J Cancer 2012; 106: 673-677.

[169] Cameron D, Casey M, Oliva C, Newstat B, Imwalle B and Geyer CE. Lapatinib plus capecitabine in women with HER-2-positive advanced breast cancer: final survival analysis of a phase III randomized trial. Oncologist 2010; 15: 924-934.

[170] Cameron D, Casey M, Press M, Lindquist D, Pienkowski T, Romieu CG, Chan S, Jagiello-Gruszfeld A, Kaufman B, Crown J, Chan A, Campone M, Viens P, Davidson N, Gorbounova V, Raats JI, Skarlos D, Newstat B, Roychowdhury D, Paoletti P, Oliva C, Rubin S, Stein S and Geyer CE. A phase III randomized comparison of lapatinib plus capecitabine versus capecitabine alone in women with advanced breast cancer that has progressed on trastuzumab: updated efficacy and biomarker analyses. Breast Cancer Res Treat 2008; 112: 533-543.

[171] Kaufman B, Stein S, Casey MA and Newstat BO. Lapatinib in combination with capecitabine in the management of ErbB2-positive (HER2-positive) advanced breast cancer. Biologics 2008; 2: 61-65.

[172] Gajria D, Gonzalez J, Feigin K, Patil S, Chen C, Theodoulou M, Drullinsky P, D'Andrea G, Lake D, Norton L, Hudis CA and Traina TA. Phase II trial of a novel capecitabine dosing schedule in combination with lapatinib for the treatment of patients with HER2-positive metastatic breast cancer. Breast Cancer Res Treat 2012; 131: 111-116.

[173] Capri G, Chang J, Chen SC, Conte P, Cwiertka K, Jerusalem G, Jiang Z, Johnston S, Kaufman B, Link J, Ro J, Schutte J, Oliva C, Parikh R, Preston A, Rosenlund J, Selzer M, Zembryki D and De Placido S. An open-label expanded access study of lapatinib and capecitabine in patients with HER2-overexpressing locally advanced or metastatic breast cancer. Ann Oncol 2010; 21: 474-480.

[174] Iwata H, Fujii H, Masuda N, Mukai H, Nishimura Y, Katsura K, Ellis CE, Gagnon RC and Nakamura S. Efficacy, safety, pharmacokinetics and biomarker findings in patients with HER2-positive advanced or metastatic breast cancer treated with lapatinib in combination with capecitabine: results from 51 Japanese patients treated in a clinical study. Breast Cancer 2015; 22: 192-200.

[175] Xu BH, Jiang ZF, Chua D, Shao ZM, Luo RC, Wang XJ, Liu DG, Yeo W, Yu SY, Newstat B, Preston A, Martin AM, Chi HD and Wang L. Lapatinib plus capecitabine in treating HER2-positive advanced breast cancer: efficacy, safety, and biomarker results from Chinese patients. Chin J Cancer 2011; 30: 327-335.

[176] Gamucci T, Moscetti L, Mentuccia L, Pizzuti L, Mauri M, Zampa G, Pavese I, Sperduti I, Vaccaro A and Vici P. Optimal tolerability and high efficacy of a modified schedule of lapatinib-capecitabine in advanced breast cancer patients. J Cancer Res Clin Oncol 2014; 140: 221-226.

[177] Pircher M, Mlineritsch B, Fridrik MA, Dittrich C, Lang A, Petru E, Weltermann A, Thaler J, Hufnagl C, Gampenrieder SP, Rinnerthaler G, Ressler S, Ulmer H and Greil R. Lapatinib-plus-pegylated liposomal doxorubicin in advanced HER2-positive breast cancer following trastuzumab: a phase II trial. Anticancer Res 2015; 35: 517-521.

[178] Bian L, Wang T, Zhang S and Jiang Z. Trastuzumab plus capecitabine vs. lapatinib plus capecitabine in patients with trastuzumab resistance and taxane-pretreated metastatic breast cancer. Tumour Biol 2013; 34: 3153-3158.

[179] Janni W, Sarosiek T, Karaszewska B, Pikiel J, Staroslawska E, Potemski P, Salat C, Brain E,

Caglevic C, Briggs K, Desilvio M, Marini L and Papadimitriou C. A phase II, randomized, multicenter study evaluating the combination of lapatinib and vinorelbine in women with ErbB2 overexpressing metastatic breast cancer. Breast Cancer Res Treat 2014; 143: 493-505.

[180] Cristofanilli M, Johnston SR, Manikhas A, Gomez HL, Gladkov O, Shao Z, Safina S, Blackwell KL, Alvarez RH, Rubin SD, Ranganathan S, Redhu S and Trudeau ME. A randomized phase II study of lapatinib + pazopanib versus lapatinib in patients with HER2+ inflammatory breast cancer. Breast Cancer Res Treat 2013; 137: 471-482.

[181] Johnston SR, Gomez H, Stemmer SM, Richie M, Durante M, Pandite L, Goodman V and Slamon D. A randomized and open-label trial evaluating the addition of pazopanib to lapatinib as first-line therapy in patients with HER2-positive advanced breast cancer. Breast Cancer Res Treat 2013; 137: 755-766.

[182] Rugo HS, Chien AJ, Franco SX, Stopeck AT, Glencer A, Lahiri S, Arbushites MC, Scott J, Park JW, Hudis C, Nulsen B and Dickler MN. A phase II study of lapatinib and bevacizumab as treatment for HER2-overexpressing metastatic breast cancer. Breast Cancer Res Treat 2012; 134: 13-20.

[183] Guan Z, Xu B, DeSilvio ML, Shen Z, Arpornwirat W, Tong Z, Lorvidhaya V, Jiang Z, Yang J, Makhson A, Leung WL, Russo MW, Newstat B, Wang L, Chen G, Oliva C and Gomez H. Randomized trial of lapatinib versus placebo added to paclitaxel in the treatment of human epidermal growth factor receptor 2-overexpressing metastatic breast cancer. J Clin Oncol 2013; 31: 1947-1953.

[184] Di Leo A, Gomez HL, Aziz Z, Zvirbule Z, Bines J, Arbushites MC, Guerrera SF, Koehler M, Oliva C, Stein SH, Williams LS, Dering J, Finn RS and Press MF. Phase III, double-blind, randomized study comparing lapatinib plus paclitaxel with placebo plus paclitaxel as first-line treatment for metastatic breast cancer. J Clin Oncol 2008; 26: 5544-5552.

[185] Yardley DA, Hart L, Bosserman L, Salleh MN, Waterhouse DM, Hagan MK, Richards P, DeSilvio ML, Mahoney JM and Nagarwala Y. Phase II study evaluating lapatinib in combination with nab-paclitaxel in HER2-overexpressing metastatic breast cancer patients who have received no more than one prior chemotherapeutic regimen. Breast Cancer Res Treat 2013; 137: 457-464.

[186] Park IH, Lee KS, Kang HS, Kim SW, Lee S, Jung SY, Kwon Y, Shin KH, Ko K, Nam BH and Ro J. A phase Ib study of preoperative lapatinib, paclitaxel, and gemcitabine combination therapy in women with HER2 positive early breast cancer. Invest New Drugs 2012; 30: 1972-1977.

[187] Storniolo AM, Pegram MD, Overmoyer B, Silverman P, Peacock NW, Jones SF, Loftiss J, Arya N, Koch KM, Paul E, Pandite L, Fleming RA, Lebowitz PF, Ho PT and Burris HA 3rd. Phase I dose escalation and pharmacokinetic study of lapatinib in combination with trastuzumab in patients with advanced ErbB2-positive breast cancer. J Clin Oncol 2008; 26: 3317-3323.

[188] Rimawi MF, Mayer IA, Forero A, Nanda R, Goetz MP, Rodriguez AA, Pavlick AC, Wang T, Hilsenbeck SG, Gutierrez C, Schiff R, Osborne CK and Chang JC. Multicenter phase II study of neoadjuvant lapatinib and trastuzumab with hormonal therapy and without chemotherapy in patients with human epidermal growth factor receptor 2-overexpressing breast cancer: TBCRC 006. J Clin Oncol 2013; 31: 1726-1731.

[189] Montemurro F, Prat A, Rossi V, Valabrega G, Sperinde J, Peraldo-Neia C, Donadio M, Galvan P, Sapino A, Aglietta M, Baselga J and Scaltriti M. Potential biomarkers of long-term benefit from single-agent trastuzumab or lapatinib in HER2-positive metastatic breast cancer. Mol Oncol 2014; 8: 20-26.

[190] Crown J, Kennedy MJ, Tresca P, Marty M, Espie M, Burris HA, Desilvio M, Lau MR, Kothari D, Koch KM and Dieras V. Optimally tolerated dose of lapatinib in combination with docetaxel plus trastuzumab in first-line treatment of HER2-positive metastatic breast cancer. Ann Oncol 2013; 24: 2005-2011.

[191] Baselga J, Bradbury I, Eidtmann H, Di Cosimo S, de Azambuja E, Aura C, Gomez H, Dinh P, Fauria K, Van Dooren V, Aktan G, Goldhirsch A, Chang TW, Horvath Z, Coccia-Portugal M, Domont J, Tseng LM, Kunz G, Sohn JH, Semiglazov V, Lerzo G, Palacova M, Probachai V, Pusztai L, Untch M, Gelber RD, Piccart-Gebhart M and Neo AST. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. Lancet 2012; 379: 633-640.

[192] de Azambuja E, Holmes AP, Piccart-Gebhart M, Holmes E, Di Cosimo S, Swaby RF, Untch M, Jackisch C, Lang I, Smith I, Boyle F, Xu B, Barrios CH, Perez EA, Azim HA Jr, Kim SB, Kuemmel S, Huang CS, Vuylsteke P, Hsieh RK, Gorbunova V, Eniu A, Dreosti L, Tavartkiladze N, Gelber RD, Eidtmann H and Baselga J. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with

pathological complete response. Lancet Oncol 2014; 15: 1137-1146.
[193] Moreno-Aspitia A, Dueck AC, Ghanem-Canete I, Patel T, Dakhil S, Johnson D, Franco S, Kahanic S, Colon-Otero G, Tenner KS, Rodeheffer R, McCullough AE, Jenkins RB, Palmieri FM, Northfelt D and Perez EA. RC0639: phase II study of paclitaxel, trastuzumab, and lapatinib as adjuvant therapy for early stage HER2-positive breast cancer. Breast Cancer Res Treat 2013; 138: 427-435.
[194] Welslau M, Dieras V, Sohn JH, Hurvitz SA, Lalla D, Fang L, Althaus B, Guardino E and Miles D. Patient-reported outcomes from EMILIA, a randomized phase 3 study of trastuzumab emtansine (T-DM1) versus capecitabine and lapatinib in human epidermal growth factor receptor 2-positive locally advanced or metastatic breast cancer. Cancer 2014; 120: 642-651.
[195] Guarneri V, Frassoldati A, Bottini A, Cagossi K, Bisagni G, Sarti S, Ravaioli A, Cavanna L, Giardina G, Musolino A, Untch M, Orlando L, Artioli F, Boni C, Generali DG, Serra P, Bagnalasta M, Marini L, Piacentini F, D'Amico R and Conte P. Preoperative chemotherapy plus trastuzumab, lapatinib, or both in human epidermal growth factor receptor 2-positive operable breast cancer: results of the randomized phase II CHER-LOB study. J Clin Oncol 2012; 30: 1989-1995.
[196] Alba E, Albanell J, de la Haba J, Barnadas A, Calvo L, Sanchez-Rovira P, Ramos M, Rojo F, Burgues O, Carrasco E, Caballero R, Porras I, Tibau A, Camara MC and Lluch A. Trastuzumab or lapatinib with standard chemotherapy for HER2-positive breast cancer: results from the GEICAM/2006-14 trial. Br J Cancer 2014; 110: 1139-1147.
[197] Untch M, Loibl S, Bischoff J, Eidtmann H, Kaufmann M, Blohmer JU, Hilfrich J, Strumberg D, Fasching PA, Kreienberg R, Tesch H, Hanusch C, Gerber B, Rezai M, Jackisch C, Huober J, Kuhn T, Nekljudova V, von Minckwitz G; German Breast Group (GBG); Arbeitsgemeinschaft Gynäkologische Onkologie-Breast (AGO-B) Study Group.Lapatinib versus trastuzumab in combination with neoadjuvant anthracycline-taxane-based chemotherapy (GeparQuinto, GBG 44): a randomised phase 3 trial. Lancet Oncol 2012; 13: 135-144.
[198] Jankowitz RC, Abraham J, Tan AR, Limentani SA, Tierno MB, Adamson LM, Buyse M, Wolmark N and Jacobs SA. Safety and efficacy of neratinib in combination with weekly paclitaxel and trastuzumab in women with metastatic HER2positive breast cancer: an NSABP Foundation Research Program phase I study. Cancer Chemother Pharmacol 2013; 72: 1205-1212.
[199] Awada A, Dirix L, Manso Sanchez L, Xu B, Luu T, Dieras V, Hershman DL, Agrapart V, Ananthakrishnan R and Staroslawska E. Safety and efficacy of neratinib (HKI-272) plus vinorelbine in the treatment of patients with ErbB2-positive metastatic breast cancer pretreated with anti-HER2 therapy. Ann Oncol 2013; 24: 109-116.
[200] Saura C, Garcia-Saenz JA, Xu B, Harb W, Moroose R, Pluard T, Cortes J, Kiger C, Germa C, Wang K, Martin M, Baselga J and Kim SB. Safety and efficacy of neratinib in combination with capecitabine in patients with metastatic human epidermal growth factor receptor 2-positive breast cancer. J Clin Oncol 2014; 32: 3626-3633.

DREXEL UNIVERSITY
College of
Medicine
Biochemistry and Molecular Biology

May 31, 2016

To whom it may concern,

This letter is to confirm that Master Mariana Segovia Mendoza, a graduate student completing her doctorate degree at the National Autonomous University of Mexico, was in my laboratory at Drexel University College of Medicine, Philadelphia, P.A. U.S.A., from March 2016 until May 2016. Master Segovia performed biomedical research activities related to breast cancer in my lab including cell culture, immunofluorescence and western blot analysis. The results of her research during her stay in my lab will be included in a publication for a scientific article within the next 6 months.

Feel free to contact me if you have further question.

Sincerely,

Mauricio J. Reginato, Ph.D.

Professor of Biochemisty and Molecular Biology

245 N. 15th Street, MS #497, Philadelphia, PA 19102-1192 **TEL** 215.762.3554 **FAX** 215.762.4452

Mauricio.Reginato@drexelmed.edu