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TESIS:

**“Evaluación del efecto antimetastásico de los ciclodipeptidos de
Pseudomonas aeruginosa PAO1 en un modelo murino de cáncer de
mama”**

**Que para obtener el grado de Doctora en
Ciencias en Biología Experimental**

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RESUMEN

En la actualidad existen cientos de tipos de cáncer y la complejidad de estas enfermedades, aunada al hecho de que un diagnóstico en etapas avanzadas o de metástasis suele significar un mal pronóstico y poco éxito en el tratamiento, hace necesario seguir investigando nuevas opciones terapéuticas. El cáncer de mama es el principal causante de muertes por neoplasias en mujeres en México y en el mundo y representa un importante problema de salud pública. Los ciclodipéptidos producidos por la bacteria *Pseudomonas aeruginosa* PAO1 (PAO1-CDPs) han demostrado tener un efecto antineoplásico, induciendo apoptosis e inhibiendo diversas vías de señalización implicadas en el cáncer en diversas líneas celulares cancerosas, así como en tumores murinos de melanoma no metastásicos. El principal objetivo de este proyecto fue evaluar el efecto de los PAO1-CDPs en un modelo de cáncer de mama con células humanas y en un estado avanzado del cáncer con lesiones metastásicas. Utilizando ratones inmunosuprimidos, generamos tumores metastásicos con células MDA-MB-231 de cáncer de mama triple negativo, altamente metastásicas, y se sometieron a un esquema de tratamiento con los CDPs, solos o en combinación con metotrexato (MTX), en etapa temprana del tumor y en etapa avanzada (metástasis). Los resultados mostraron una disminución significativa de los tumores primarios en los grupos tratados con los ciclodipéptidos, tanto solos como en combinación con el MTX, en contraste con los que no recibieron tratamiento alguno y con los tratados solo con MTX. Además, se observó un menor número y tamaño de focos metastásicos en pulmón, hígado y huesos analizados por histología. También se determinaron los niveles séricos de AST, ALT y LDH como marcadores de daño tisular e, interesantemente, se encontraron drásticamente elevados en los grupos de tumor sin tratamiento y tratados con MTX, a diferencia de los grupos tratados con CDPs, que mostraron niveles de las tres enzimas similares a los del control. Al analizar algunas proteínas involucradas en vías de señalización clave en el proceso canceroso y metastásico, observamos una disminución de su actividad tras la administración de los PAO1-CDPs. Estos resultados sugieren un efecto antimetástico de los PAO1-CDPs, aun en cánceres de fenotipo agresivo como el cáncer de mama triple negativo, para lo cual planeamos diseñar más ensayos que nos permitan obtener más información del mecanismo molecular de estos compuestos, al inhibir la proliferación e invasión de células cancerosas.

Palabras clave: cáncer de mama, metástasis, ciclodipéptidos, invasión, antineoplásico.

ABSTRACT

There are currently hundreds of types of cancer, and the complexity of these diseases, coupled with the fact that a diagnosis in advanced or metastatic stages often means a poor prognosis and limited treatment success, makes it necessary to continue investigating new therapeutic options. Breast cancer is the leading cause of cancer-related death in women in Mexico and around the world, representing a significant public health problem. Cyclodipeptides produced by the bacterium *Pseudomonas aeruginosa* PAO1 (PAO1-CDPs) have been shown to have an antineoplastic effect by inducing apoptosis and inhibiting various signaling pathways implicated in cancer in various cancer cell lines, as well as in non-metastatic murine melanoma tumors. The main objective of this project was to test the effect of PAO1-CDPs in a breast cancer model with human cells and in an advanced stage of cancer with metastatic lesions. Using immunosuppressed mice, we generated metastatic tumors with the highly metastatic triple-negative breast cancer cell line MDA-MB-231. We subjected them to a treatment regimen with CDPs alone or in combination with Methotrexate (MTX) at the early stage of the tumor and the advanced stage (metastasis). The results showed a significant decrease in primary tumors in groups treated with cyclodipeptides alone or in combination with MTX, compared with those who received no treatment or MTX alone. In addition, a lower number and size of metastatic foci were observed in the lung, liver, and bones analyzed by histology. Serum levels of AST, ALT, and LDH were also determined as markers of tissue damage, and, notably, they were elevated in the untreated and MTX-treated groups. In contrast, the CDP-treated groups showed levels of the three enzymes comparable to those in the control. When analyzing proteins involved in key signaling pathways in cancer and metastasis, we observed decreased activity upon treatment with PAO1-CDPs. These results suggest an anti-metastatic effect of PAO1-CDPs even in aggressive cancers such as triple-negative breast cancer. To this end, we plan to develop additional assays to gain further insight into the molecular mechanisms underlying these compounds' inhibition of cancer cell proliferation and invasion.

Keywords: breast cancer, metastasis, cyclodipeptides, invasion, antineoplastic.

INTRODUCCIÓN

Cáncer es un término genérico que se refiere a decenas de enfermedades que afectan a muchos tejidos y tipos celulares, caracterizadas por una proliferación descontrolada (Hoskin y Ramamoorthy, 2008). Es un padecimiento multifactorial, en el que las causas de su desarrollo van desde factores ambientales y agentes infecciosos, como virus y bacterias, hasta hábitos de vida e incluso la historia familiar de la incidencia de ciertos tipos de cáncer. Hasta hace algunos años se pensaba que el origen del cáncer debía exclusivamente al factor genético que implica mutación de genes importantes para mantener el equilibrio entre división y muerte celular, sin embargo ahora se sabe que también puede originarse por factores epigenéticos que consisten en cambios reversibles y heredables en la regulación génica que ocurren sin un cambio en la secuencia del ADN, dichos cambios pueden ser ARNs de interferencia (iARNs), metilación del ADN y modificación de histonas (Meng *et al*, 2012).

Es importante destacar que no todos los tumores dan origen a cáncer. Las neoplasias pueden clasificarse en benignas y malignas, diferenciándose principalmente por su comportamiento biológico. En las neoplasias benignas, las células suelen dividirse lentamente y son similares a las normales; los tejidos mantienen su arquitectura y el tumor está restringido a la zona donde se inició su proliferación, con un límite definido respecto de los tejidos que lo rodean. Esta característica es tal vez la más importante, puesto que los tumores benignos no son capaces de diseminarse a otros sitios del organismo. Por su parte, las neoplasias malignas o cancerosas están constituidas por células que tienden a perder su diferenciación y a dividirse rápidamente o de manera errática. Tienen la capacidad de infiltrar e invadir tejidos sanos cercanos y, eventualmente, dispersarse en el organismo a través del sistema circulatorio o linfático, dando origen a focos secundarios de implantación o metástasis (Segatori, tesis doctoral, 2014).

EPIDEMIOLOGÍA

La frecuencia de los distintos tipos de cáncer varía según el sexo y la edad. Aproximadamente el 30 % de las defunciones por cáncer son prevenibles; además, se reportó que el consumo del tabaco es el principal factor de riesgo de cáncer. Otros factores involucrados en el desarrollo de esta enfermedad son los contaminantes ambientales, la exposición a la luz UV y otras radiaciones.

Las estadísticas recientes, según la Organización Mundial de la Salud (OMS), revelan que el cáncer es la principal causa de muerte a nivel mundial y la tercera causa de muerte en México (por enfermedades no infecciosas), solo después de las enfermedades del corazón y la diabetes mellitus. En los últimos años se han registrado más de 8.2 millones de muertes en el mundo y se pronostica que las muertes por cáncer sigan aumentando, estimándose en 12 millones para el año 2030.

El cáncer en la actualidad comprende más de 100 tipos y cada uno de ellos presentan diferentes características patológicas y comportamiento, esto sumado a que la inestabilidad genética de las células tumorales genera un fenotipo muy cambiante incluso dentro de un mismo tumor, hace difícil el comprendido de los eventos moleculares claves para la supervivencia de las células malignas y la identificación de los blancos moleculares para la generación de fármacos anti cáncer que sean eficaces para los cánceres de difícil tratamiento. Los tipos de cáncer de mayor prevalencia, sin distinguir sexo y/o edad, son el cáncer de próstata, de mama, cervicouterino, leucemias, hígado, tiroides, cavidad oral y pulmón, riñón y vejiga, y el cáncer de piel (melanoma) (www.cancer.gov, National Cancer Institute, NCI).

CARACTERÍSTICAS DISTINTIVAS DEL CÁNCER

El cáncer es una enfermedad compleja cuyas bases moleculares se establecen en el ciclo celular, el cual, al verse desregulado por mutaciones en proteínas importantes que constituyen los diferentes puntos de control.

Se pueden destacar, de manera general, dos grupos de genes que participan en la conversión de la célula en un fenotipo canceroso: los genes supresores de tumores, que se encargan de detectar daños o anormalidades en el ADN y detener el ciclo celular hasta que estos sean reparados, y los genes reparadores de daño, que trabajan en conjunto con ellos. El segundo grupo lo constituyen los protooncogenes que participan en vías de crecimiento, proliferación y supervivencia celular, y que en las células malignas se encuentran sobreexpresados. Un desequilibrio entre estos grupos de genes da como resultado diversas características que les permiten a las células cancerosas dividirse desmedidamente y resistir la muerte, entre otras. Las mutaciones en ambos tipos de genes, junto con modificaciones en su expresión (regulación epigenética), llevan a la transformación de una célula normal en una maligna (Cooper G y Hausman R, 2010; Weinberg, 1989).

En 2011, Hanagan y Weinberg realizaron una revisión que agrupa las características consideradas como marcadores principales del cáncer, es decir, procesos que se ven afectados en las células tumorales, independientemente del tipo de cáncer, y que ayudan a la célula a proliferar, resistir a la muerte e invadir nuevos tejidos. Algunas de las características distintivas del cáncer se mencionan a continuación (Figura 1):

Mantienen la señalización proliferativa. Las células cancerosas pueden tener mutaciones en genes que activan vías de señalización de división y crecimiento celulares, lo que provoca que estas vías estén constantemente encendidas o que las señales que detienen los procesos proliferativos (evasión de supresores de crecimiento) no sean correctamente reconocidas, lo que permite la **inmortalidad replicativa**.

Desregulación del metabolismo energético. El crecimiento excesivo del tumor impide que las células más profundas accedan al oxígeno, lo que debería provocar su muerte paulatina. Esto puede ocurrir, pero solo en un pequeño porcentaje de las células. La mayoría de las células tumorales realizan un cambio en su metabolismo, siendo capaces de obtener energía a través de glucólisis anaeróbica, conocido como efecto Warburg, que pese a tener cierta desventaja energéticamente hablando, las provee de otras características que le ayudan a sobrevivir, por ejemplo, cambios en el pH que crean un microambiente tumoral óptimo para la **activación de genes prometastásicos** (Ward *et al*, 2012; Gatenby y Gillies, 2004).

Inducción de la angiogénesis. Para acceder a nutrientes y a la oxigenación, las células activan mecanismos que favorecen la formación de nuevos vasos sanguíneos a partir de vasos preexistentes, como la liberación de interleucinas y factores de crecimiento.

Evaden el sistema inmune. De manera natural, el sistema inmune elimina continuamente células dañadas o malignas, evitando que estas sigan dividiéndose y generen una población de células con una importante **acumulación de mutaciones y alta inestabilidad genética**. Cuando una célula maligna presenta mutaciones en moléculas de superficie celular, puede pasar desapercibida para las células del sistema inmune y así evitar su neutralización. Sin embargo, se sabe que el sistema inmune

puede desempeñar un papel dual en el desarrollo de tumores, pues la persistencia de la respuesta inflamatoria de manera crónica favorece la activación de vías de supervivencia celular y el microambiente proinflamatorio aporta interleucinas y factores de crecimiento que favorecen el desarrollo del tumor.

Resistencia a la muerte celular. Para mantener la homeostasis celular, se cuenta con diferentes mecanismos regulatorios, entre ellos las vías de muerte, como la apoptosis, un proceso que ocurre cuando las células requieren recambio o presentan daño al ADN, para evitar una progenie celular defectuosa. Una sobreactivación de vías de supervivencia o un mal funcionamiento de proteínas que inhiben estas vías o activan la apoptosis evita que las células mueran, lo que dificulta el control de la proliferación.

Invasión y metástasis. Tal vez la característica que más distingue una neoplasia maligna de una benigna es que, en un estado avanzado del cáncer, las células sufren cambios fenotípicos que les permiten transvasarse a la sangre y albergarse en nuevos tejidos. La transición epitelio-mesénquima (TEM), si bien es fundamental durante el desarrollo embrionario, en el cáncer participa en la pérdida de las uniones adherentes (como las N-cadherinas, las cateninas y los anillos de actina) que las mantienen unidas a otras células del tumor, lo que le permite adaptarse a nuevos nichos distantes y dificulta un tratamiento exitoso.

El estudio detallado de estas características permite identificar alteraciones en las vías de señalización y en las moléculas que participan en ellas, así como biomarcadores del fenotipo canceroso útiles para diseñar nuevas terapias específicas, los cuales constituyen posibles blancos terapéuticos.

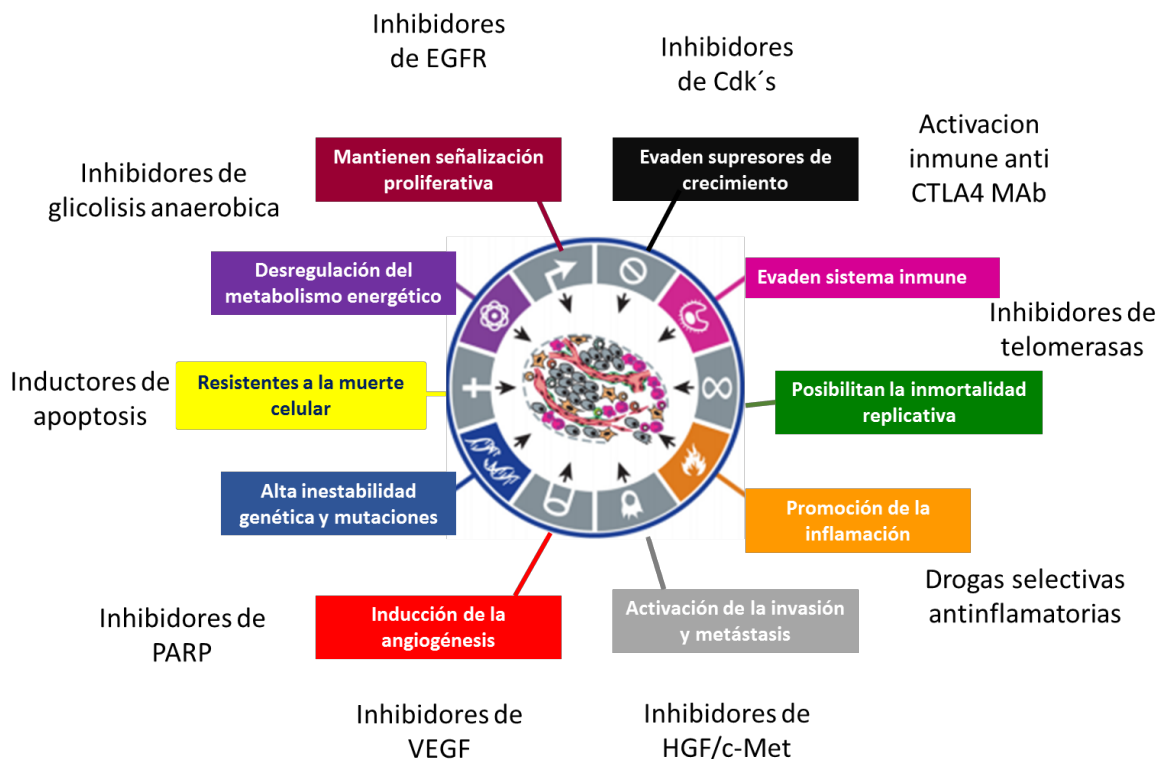


Figura 1. Características distintivas de las células cancerosas. Modificado de Hanahan, 2022.

MICROAMBIENTE TUMORAL

Dentro de la complejidad del cáncer y de los múltiples factores involucrados en su progresión, se encuentra el microambiente tumoral, cuyo papel fundamental se sabe que abarca desde el establecimiento de una metástasis hasta la respuesta de los tumores a los fármacos anticancerígenos. Para ello, es importante definir primero qué es el microambiente tumoral.

El microambiente tumoral consiste en un espacio de interacción entre las células neoplásicas y las células mesenquimales no malignas y algunos componentes del sistema inmune, entre ellas; fibroblastos, adipocitos, células endoteliales, células dendríticas, macrófagos, neutrófilos, linfocitos, etc., además de ciertos componentes de la matriz extracelular que se ven modificados por dichas interacciones, tales como quimiocinas, citocinas, factores de crecimiento, metaloproteasas, enzimas inflamatorias, entre otros (Tabla 1). Este microambiente se encarga de regular las funciones esenciales de supervivencia y crecimiento del tumor, participando en todas las etapas de la carcinogénesis al estimular y favorecer el crecimiento descontrolado de las células malignas (Arneth, 2019).

Existe una gran controversia sobre cómo el sistema inmune, que inicialmente desempeña un papel protector frente a las células cancerosas, puede, en etapas avanzadas del tumor, favorecer su progresión e invasión. Hay muchas investigaciones que reportan que, en las primeras etapas del tumor, las células de este son poco estimulantes y blancos débiles del sistema inmune y con el tiempo se vuelven resistentes a la respuesta inmune innata y luego comienzan a alterar la respuesta adaptativa, evitando así la maduración y funcionamiento de los linfocitos T, que terminan formando parte importante del microambiente tumoral (Dong *et al*, 2002 y Wang *et al*, 2004)

También se ha reportado, por ejemplo, que los fibroblastos que, en condiciones fisiológicas, protegen el tejido frente a las neoplasias, evitando su escape al mantener la continuidad tisular, en presencia de un tumor avanzado e influenciados por el propio microambiente, sufren cambios que les permiten excretar moléculas que favorecen la proliferación, la invasión y la metástasis.

Otro de los componentes importantes y mayoritario en casi todos los casos son los macrófagos o macrófagos asociados al tumor (TAMs, por sus siglas en inglés: Tumor-associated macrophages), los cuales favorecen la invasión celular y la expansión tumoral mediante la producción de moléculas que promueven el remodelamiento del tejido, tales como EGF, MMP9, MT1-MMP y MMP2, y de moléculas proinflamatorias como TNF- α , CXCL10 e IL1 β (Birbrai *et al*, 2014).

Tabla 1. Células en el microambiente tumoral. Modificado de Arneth, 2019.

CELULAS PARTICIPANTES	PRINCIPALES MARCADORES O TIPO	FUNCIONES
Linfocitos T	CD8 ⁺ y CD4 ⁺	Algunas son protumorigénicas, mientras que otras restringen el tumor.
Natural killer y NKT	Receptores NKG2, receptores	Usan receptores inhibitorios,

	Ly49, NK1, CD94, CD161, CD56 Y NKG2A	de adhesión, de activación y de citocinas para identificar blancos celulares y células sanas de repuesto
Macrófagos	M1 y M2	Crean un estroma que favorece la invasión y expansión de células neoplásicas
Macrófagos M1	Antitumorígenicas	
Macrófagos M2	Inmunosupresor y protumorigénico	Pueden promover la progresión del tumor
Fibroblastos asociados a cáncer (FACs)	Actina de músculo liso α , proteína activadora de fibroblastos, vimentina, desmina y PDGFR α y β	Contribuyen a la proliferación celular por mantenimiento continuo de señales de crecimiento y propagación en sitios primarios y metastásicos
Células madre cancerosas	Células madre tumorales y DPSCs	Favorecen la tumorigénesis a través de capacidades únicas de localización en sitios primarios y metastásicos
Quimiocinas	CXCL14 y CXCL12	Son usualmente sobreexpresados en miofibroblastos y células mioepiteliales, estas moléculas pueden unirse a receptores celulares epiteliales para incrementar la migración, invasión y proliferación
Integrinas	α M β 2, α X β 2 α L β 2 α β 2, α 4 β 7 y α E β 7	Estas se unen a la matriz extracelular en el microambiente tumoral
Selectinas	Factor de crecimiento epidérmico, ST3Gal6 y P-selectina	Son vitales moléculas de adhesión vascular que afectan el desarrollo de las células
Cadherinas	Protocaderina, N-caderina desmocolina, desmogleína	Estas moléculas median la formación de uniones homofílicas de manera calcio-dependiente
Tregs	CD4, FOXP3 y CD25	Promueven la generación y función de células de memoria CD8 ⁺
Super familia de inmunoglobulinas	Receptores antigénicos de superficie celular, correceptoras y moléculas coestimuladoras	Median la formación de uniones homo y heterofílicas
Células derivadas de médula ósea	Diversas funciones promotoras de crecimiento tumoral	Funciones promotoras de crecimiento tumoral que

		incluyen expresión de factores de crecimiento, promoción de la formación de venas en el tumor y creación de nichos de células madre
Células supresoras derivadas de mieloides	Se expanden en situaciones patológicas como el cáncer como resultado de una hematopoyesis alterada	Poseen fuerte actividad inmunosupresora especialmente en células mieloides

TRANSICIÓN EPITELIO MESÉNQUIMA

La transición epitelio-mesénquima (TEM) es el proceso mediante el cual una célula epitelial adquiere temporalmente el fenotipo de una célula mesenquimal como respuesta a un estímulo interno o externo (Hay ED,1995). Este proceso puede ocurrir de manera fisiológica, como la morfogénesis y la reparación de heridas, y de manera patológica, como la fibrosis de órganos o el cáncer (Lee *et al.*, 2006).

Para comprender cómo ocurre este proceso, clave para la progresión tumoral, es importante conocer las características diferenciales entre los fenotipos epiteliales y mesenquimales. Las células epiteliales poseen una interacción cohesiva a través de diversas uniones intercelulares que les permite mantener una forma definida, con polaridad apical-basal en su membrana, formar varias capas de células y limitar su movilidad. Además, poseen marcadores específicos como citoqueratina, integrinas epiteliales, E-cadherina y β -catenina. Por otro lado, las células con fenotipo mesenquimal carecen de estas proteínas de unión intercelular, por lo que, a su vez, pierden la polaridad apical-basal y presentan cambios en el citoesqueleto de actina, que forma filopodios y las vuelve móviles, en dicho fenotipo, los marcadores principales son vimentina, N-cadherina e integrinas mesenquimales, además de una gran cantidad de metaloproteasas de la matriz extracelular (Dongre y Weinberg, 2019).

En general, la transición epitelio-mesénquima consta de tres fases: fase no migratoria, premigratoria y migratoria. Cada una consta de diversos pasos y características que son consecuencia de la expresión de diferentes factores de transcripción y de vías de señalización específicas.

- Fase no migratoria: las células de fenotipo epitelial se encuentran en la parte más superficial de los tejidos, tienen una forma definida y una polaridad apical-basal que depende de diversos tipos de uniones intercelulares, entre las que podemos encontrar uniones adherentes, uniones gap, uniones estrechas y desmosomas. Estas uniones son de naturaleza proteica y, además de formar interacciones célula-célula y célula-membrana basal, pueden interactuar con activadores de vías de señalización, como β -catenina. En esta fase, se observa una alta expresión de E-cadherina, uno de los principales marcadores epiteliales, y se ha reportado que una baja expresión de esta está relacionada con el inicio de la TEM y, por ende, con la metástasis (Dietmar, 2015).

- Fase premigratoria: aquí las células se encuentran en una transición justo antes de iniciar la TEM; para ello pierden la polaridad apical-basal típica debido a la represión y activación simultáneas de proteínas de adhesión, como E-cadherina, claudinas y ocludinas, y de proteínas mesenquimales, como vimentina, N-cadherina y metaloproteasas, respectivamente. También se produce la liberación de β -catenina, encargada de mediar la unión entre el citoesqueleto y las proteínas transmembranales, que puede migrar al núcleo para asociarse con diversos factores de transcripción e iniciar la TEM. Algunas de las vías de señalización que pueden activarse en esta fase son TGF- β , IL-6, EGF, PDGF, Hedgehog, WNT y Notch, factores que pueden liberarse por linfocitos, macrófagos, fibroblastos o por las propias células tumorales. Otras de las rutas involucradas durante el desarrollo de este evento son la vía Erk-MAP, la cinasa inducida por Ras-Raf, las GTPasas de tipo Rho y la vía de IP3-cinasa/AKT (Troncoso, 2017).
- Fase migratoria: en este punto las células han perdido su morfología y uniones intercelulares por completo, ahora poseen filamentos de citoqueratina en su citoesqueleto que les permite la formación de filopodios, se ha reprimido la expresión de E-cadherina y hay una alta expresión de N-cadherina y vimentina, las responsables de que haya una débil afinidad intercelular y en conjunto esto permitirá la movilidad de las células cancerosas hacia los vasos sanguíneos o linfáticos circundantes. Se ha reportado que Rho es un factor importante en la generación de la fuerza contráctil y en el movimiento del cuerpo y de la cola de la célula, ya que actúa sobre la actina, componente del citoesqueleto, para formar las fibras de estrés. Asimismo, existe evidencia de que el complejo CDC42-RIF induce la formación de los filopodios (Van Aelst, 2002). Tal vez uno de los muchos factores de transcripción que pueden mediar en el inicio de la TEM es Snail1, cuyas funciones más destacadas son reprimir la expresión de E-cadherina y promover la expresión de metaloproteasas de matriz, como MT1-MMP, MT2-MMP y MMP9, para facilitar la degradación de la matriz extracelular (Tsai y Yang, 2013; Radisky *et al.*, 2005).

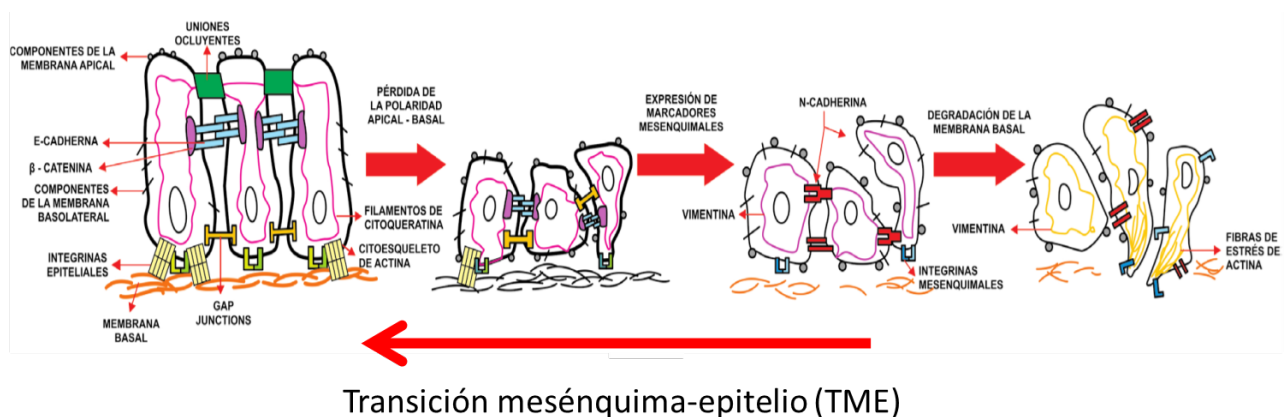


Figura 2. Transición epitelio-mesénquima. Se esquematizan las modificaciones fenotípicas que experimentan las células tumorales para adquirir características mesenquimales e invadir tejidos adyacentes o distantes. Modificado de Benedetti y Reyes, 2015.

EL PROCESO METASTÁSICO

Es la principal causa de muerte en personas con cáncer, ya que al menos en México más del 60 % de los casos de cáncer son diagnosticados en etapas avanzadas de la enfermedad, es decir, que suele haber tumores secundarios en otros órganos y tejidos, lo cual se considera un mal pronóstico y suele ser señal de baja probabilidad de éxito con los tratamientos aprobados actualmente, o bien, una alta probabilidad de resistencia al tratamiento pues las células cancerosas durante el proceso de metástasis suelen adquirir modificaciones importantes que las vuelven cada vez más difíciles de tratar.

Durante el proceso metastásico, ocurre una serie de procesos que van condicionando a las células para mejorar sus mecanismos de supervivencia, adaptación y resistencia a la muerte. Tal vez uno de los mecanismos más descritos en el desarrollo de metástasis es la TEM; sin embargo, existen muchos otros procesos importantes para que la propagación del tumor ocurra con éxito.

De forma muy general, un tumor primario que ha logrado prosperar, proliferar y crecer evadiendo la respuesta del sistema inmune y adaptándose a las condiciones del tejido donde se encuentra, ahora con ayuda de su microambiente tumoral es capaz de iniciar el desprendimiento de unos pocos grupos de células que se intravasan a los sistemas circulatorios del cuerpo (sanguíneo y linfático) y alcanzan así todos los sitios del cuerpo donde, solo en aquellos con las condiciones adecuadas formarán un nuevo nicho metastásico y una vez bien instaladas las células tumorales comenzarán a proliferar de nuevo como tumores secundarios o metástasis (figura 3).

Como sabemos, el único problema con la diseminación del cáncer no es que haya más de un tumor, sino que, a medida que se disemina, las células tumorales van adquiriendo características que les proporcionan mejores estrategias para evadir cualquier intento de neutralizarlas; se vuelven resistentes a los tratamientos y adquieren un fenotipo más agresivo. En las últimas décadas se ha hecho un gran esfuerzo por conocer cada vez mejor cómo estas células malignas van cambiando y qué factores promueven la metástasis y los cambios que las hacen tan resistentes.

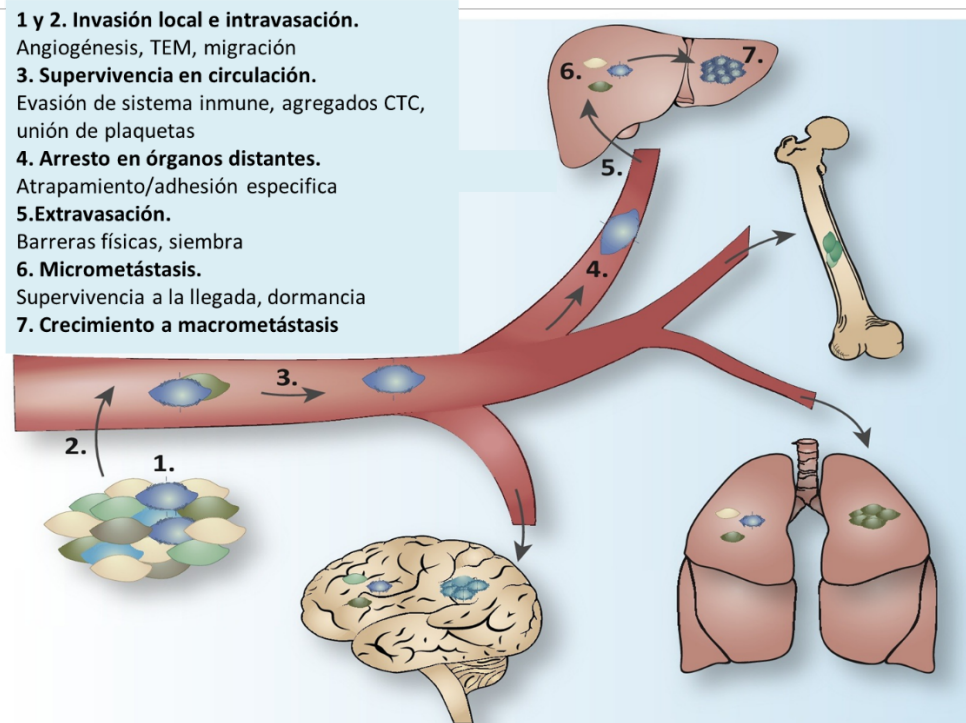


Figura 3. La cascada metastásica. Se esquematizan los pasos del proceso de metástasis, desde el tumor primario hasta los órganos distantes del cuerpo. Modificado de Obenauf y Massagué, 2015.

FACTORES PROMETASTÁSICOS

Dada la complejidad de las células cancerosas, su relación con su entorno y con otros tipos celulares, así como la exposición a tan diversos estímulos, podemos enumerar los principales factores que promueven el desarrollo de metástasis.

Microambiente tumoral

Como ya mencionamos anteriormente, el entorno del tumor y las complejas interacciones con las células mesenquimales, la matriz extracelular, el sistema inmune y algunas enzimas inflamatorias le proporcionan todo lo necesario para prosperar e iniciar procesos de invasión. Hay estudios que demuestran que cambios fenotípicos en fibroblastos y, principalmente, en macrófagos provocan la síntesis de quimiocinas y factores de crecimiento, lo que favorece la exacerbación de las vías de señalización de proliferación y el inicio de la TEM (Feig *et al.*, 2013).

Angiogénesis

Claramente, como todas las células, las células cancerosas requieren un aporte constante de nutrientes y oxígeno debido a su proliferación sostenida, pero, además, para acceder a otros sitios distantes, los sistemas circulatorios son el medio necesario para desplazarse y establecer nuevos nichos metastásicos. Se ha reportado que la angiogénesis es uno de los marcadores distintivos del cáncer, pero ahora se sabe que también constituye un factor importante que determinará la posibilidad de desarrollar metástasis, siendo muy probable que en tumores altamente vascularizados ya se hayan originado tumores secundarios. Algunos de los marcadores más estudiados para

determinar el grado de angiogénesis son VEGF, CD34 y Gro α . Se sabe que la interacción de las células tumorales con células mesenquimales de su microambiente promueve la liberación de estos factores angiogénicos, lo que podría implicar una alta probabilidad de que se inicie la metástasis.

Estromagénesis

La estromagénesis es un proceso dinámico en el que, bajo el control de la célula epitelial, la matriz extracelular se remodela de manera orquestada, creando un ambiente más propicio para el crecimiento tumoral (Beacham y Cukierman, 2005). El nicho tumoral alberga aproximadamente el 80% de los fibroblastos, que han cambiado su fenotipo a fibroblastos activados o asociados al cáncer (CAFs), es decir, adquieren características estimuladas por procesos inflamatorios, similares a las observadas durante la reparación de heridas, lo que crea condiciones favorables para el tumor. También se ha reportado que los tumores con mayor contenido de estroma son más agresivos (Rykala *et al.*, 2011).

Sistema inmune

Durante mucho tiempo hubo un gran conflicto sobre si el sistema inmune, cuyo papel fisiológico es proteger al organismo frente a células malignas, podía ser un factor importante que favoreciera su diseminación. Ahora no es tan difícil entender cómo ocurre este fenómeno, pues sabemos que el sistema inmune responde a una serie de estímulos y, dependiendo de la naturaleza e intensidad de cada uno, así será su respuesta. Varios estudios demuestran que, en una primera respuesta del sistema inmune, se excretan citocinas proinflamatorias que, finalmente, desencadenan condiciones que el tumor aprovecha para prosperar. Algunos de los marcadores más relevantes en metástasis son los macrófagos asociados al tumor y los fibroblastos asociados al cáncer, así como los linfocitos T y la presencia de las interleucinas 6 y 8 (Pavakis *et al.*, 2009).

CÁNCER DE MAMA

El cáncer de mama es una enfermedad clínicamente heterogénea y aproximadamente el 10-15 % de las pacientes con cáncer de mama presentan un fenotipo agresivo y desarrollan metástasis distantes dentro de los 3 años posteriores a la detección del tumor primario. Es el tipo de cáncer más frecuente en mujeres y ocupa uno de los primeros lugares entre las causas de defunción en pacientes oncológicos a nivel mundial, además del principal tipo de tumor maligno causante de muerte en mujeres. Según datos del Instituto Nacional del Cáncer (2021), en el mundo cada año ocurren 458 mil muertes por cáncer de mama debido a que es altamente heterogéneo en los planos molecular y clínico.

Dentro de los factores de riesgo para desarrollar cáncer de mama están la edad, donde la mayoría de los casos se presentan en mujeres mayores de 50 años; la raza, las mujeres de raza blanca son más propensas a este tipo de cáncer; hábitos y estilo de vida, por ejemplo; el consumo de alcohol y tabaco, la obesidad y el tipo de alimentación; antecedentes familiares de cáncer de mama o factores genéticos.

La mayoría de los casos de cáncer de mama son esporádicos, es decir, se desarrollan debido del daño genético de una persona que ocurre por casualidad después del nacimiento. No hay riesgo de transmitir este gen a los hijos de la persona, ya que los factores ambientales son la causa subyacente del cáncer de mama esporádico.

Los casos de cáncer de mama hereditario son menos frecuentes y representan entre el 5 % y el 10 % de los casos de cáncer de mama. Los casos de cáncer de mama hereditario se presentan cuando se heredan mutaciones dentro de una familia, del padre o de la madre al hijo. Muchas de estas mutaciones se localizan en genes de supresión de tumores, como BRCA1, BRCA2 y PALB2. Existen otras mutaciones en genes o enfermedades hereditarias que pueden aumentar el riesgo de que una persona padezca cáncer de mama, pero son mucho menos frecuentes que los genes BRCA1 o BRCA2 y no aumentan tanto el riesgo de cáncer de mama, por ejemplo: *MLH1*, *MSH2*, *MSH6* y *PMS2*, *PTEN*, *TP53*, *STK11*, *ATM*, *CDH1*, *PALB2*, *CHEK2*.

Según la ASCO (American Society of Clinical Oncology), existen otros factores considerados como predisponentes al cáncer de mama que, aunque no son 100 % determinantes, son importantes al formar la historia clínica del paciente, como: antecedentes personales de cáncer de ovario, menstruación temprana o menopausia tardía, edad del primer embarazo, terapia de reemplazo hormonal después de la menopausia, uso de anticonceptivos orales, densidad mamaria y exposición a la radiación a temprana edad.

CLASIFICACIÓN

El cáncer de mama es una enfermedad bastante heterogénea que puede clasificarse según diferentes aspectos, como la histopatología, el grado, el tamaño tumoral, el estado ganglionar e invasión linfovascular, las características moleculares, entre otros, lo cual se refleja en las variaciones observadas en la respuesta a los tratamientos actualmente conocidos. Los avances recientes en la biología molecular y en la secuenciación del genoma nos han permitido conocer la heterogeneidad de esta enfermedad tan compleja, diferenciando subgrupos con características biológicas, clínicas y pronósticas totalmente distintas (Tabla 2).

Una de las clasificaciones más utilizadas actualmente es la que se basa en la expresión de receptores hormonales de progesterona (RP) y de estrógeno (RE), la sobreexpresión de HER2 (receptor 2 del factor de crecimiento epidérmico humano) y el marcador de proliferación Ki-67.

Luminal

Es el más frecuente, con una frecuencia de alrededor del 75-80 % entre los tumores de mama. Son tumores que expresan receptores de estrógeno (RE). Tienen una alta expresión de genes asociados a las células epiteliales lumbinales del ducto mamario, como las citoqueratinas 8 y 18. Dentro de este grupo diferenciamos dos subtipos con pronóstico diferente:

- Luminal A: RE (+), RP $\geq$ 20, HER2(-), Ki67 $\leq$ 20 %. Constituye el 50-60 % de los lumbinales. Es el subtipo de mejor pronóstico, con menos incidencia de recaídas y mayor índice de supervivencia. Presenta una alta tasa de respuesta a la hormonoterapia y un beneficio más limitado con la quimioterapia. Por lo tanto, en este grupo se recomienda, según las Guías de ESMO6 ([Sociedad Europea de Oncología Médica](#)) y NCCN ([National Comprehensive Cancer Network](#)), el uso de plataformas genéticas para determinar qué pacientes se beneficiarían de tratamiento quimioterápico adyuvante en función del riesgo de progresión. La recaída es más frecuente a nivel óseo, con una tasa menor en el sistema

nervioso visceral y en el sistema nervioso central (SNC). Asimismo, presentan mayor supervivencia en caso de recaída.

-Luminal B: RE (+), RP (+/-), HER2(+/-), Ki67 > 20 %. Representa entre el 10 % y el 20 % de los tumores luminales. Presenta una baja expresión moderada de receptores estrogénicos y una mayor expresión de genes de proliferación y de ciclo celular. Representa el grupo de tumores luminales de peor pronóstico

HER2 positivo: RE (-), RP (-), HER (+)

Tienen peor pronóstico si los comparamos con los luminales y son candidatos a tratamiento dirigido anti-HER2, que incluye el trastuzumab, el trastuzumab combinado con emtasina (T-DM1), pertuzumab y los inhibidores de la tirosina quinasa como lapatinib y neratinib, entre otros. Tienen una alta tasa de respuesta a los esquemas de quimioterapia. La localización ósea es la más habitual en la enfermedad diseminada y también es más frecuente en este subtipo que en el grupo anterior. Además, es el grupo que más frecuentemente afecta al SNC durante las recaídas.

Triple negativo (CMTN): RE (-), RP (-), HER2(-)

Constituye el 10-15 %. Son tumores que expresan < 1 % de RE y RP por inmunohistoquímica (IHQ) y HER2-. Su comportamiento es agresivo con altas tasas de recurrencia a distancia. La población afectada suele ser más joven. En la enfermedad localizada, la quimioterapia es el único tratamiento disponible.

Tabla 2. Clasificación del cáncer de mama. Modificado de Tsang y Tse, 2020.

Tipo intrínseco	Fenotipo inmunohistoquímico	Hallazgos moleculares	Pronóstico
LUMINAL A	RE+, RP ≥20%, HER2-, Ki67 bajo	Mutaciones en PI3KCA, MAPK3K1 y GATA3	Bueno-intermedio
LUMINAL B	RE+, RP <20% / o HER2+ / o Ki67 alto	Similar a luminal A, pero con alta prevalencia de inactivación en vías TP53 y RB, así como transcripción relacionada a Myc y FOXM1	Bueno-intermedio
HER2	RE-, RP-, HER2+	Amplificación HER2 y señalización EGFR/HER2	Pobre-intermedio
TIPO BASAL (TRIPLE NEGATIVO)	RE-, RP-, HER2-	Mutaciones en TP53; pérdidas en RB1 y BRCA1; amplificación de MYC; sobreactivación de vía PI3K/AKT	Muy pobre

CÁNCER DE MAMA TRIPLE NEGATIVO

El cáncer de mama triple negativo es muy agresivo; presenta baja tasa de supervivencia y tasas muy altas de recurrencia, principalmente antes de los 3 años, cuando predominan las metástasis viscerales en hígado, pulmón y sistema nervioso central, con una elevada mortalidad, sobre todo cuando hay enfermedad residual.

Son más prevalentes en mujeres más jóvenes, de origen africano e hispano, y se observa un enriquecimiento de estos tumores en pacientes con mutación en BRCA1. De hecho, entre los cánceres que se desarrollan con mutación en BRCA1, el 80-85 % corresponde al tipo triple negativo.

En 2011 Lehmann *et al.*, examinaron una base de datos de expresión génica y clasificaron los CMTN en seis subtipos moleculares de la enfermedad triple negativo: tipo basal 1, tipo basal 2, inmunomodulador, tipo mesenquimal, MSL (mesenquimal stem-like) y luminal AR, los cuales para el 2016 fueron rectificados a solo 4 subgrupos (BL1, BL2, M y LAR), pues han comprobado que los subtipos IM y MSL simplemente representan tumores con importante infiltración linfocitaria y células mesenquimales asociadas a tumor, respectivamente.

– Subtipo Basal-like (BL): presenta la expresión de genes propios de las células mioepiteliales basales del acino mamario. Por IHQ, se observa la expresión de las citoqueratinas 5 y 6, asociada a mutaciones en BRCA1 y EGFR, con alta tasa de proliferación y pronóstico desfavorable.

– Subtipo inmunoenriquecido (IM). Caracterizado por la activación de genes involucrados en la inmunidad celular, como las vías de señalización de las células T, B, de natural killer y de las células dendríticas.

- Subtipos mesenquimales (M) y MSL: presentan una importante expresión de genes relacionados con la diferenciación celular, la movilidad celular y los receptores que interactúan con la matriz extracelular. El subtipo MSL está enriquecido con genes asociados a la transición epitelial-mesenquimal (cáncer metaplásico).

- Subtipo receptor de andrógeno luminal (LAR): Presenta alteraciones en las vías de regulación hormonal, como la de estrógenos/andrógenos, con niveles elevados del receptor de andrógenos. Se están ensayando terapias de bloqueo androgénico, con bicalutamida o enzutatamida.

OPCIONES TERAPEUTICAS

A pesar de los esfuerzos de investigación de varios grupos de trabajo en el diagnóstico y tratamiento, cuestiones como la prevención, la recurrencia, la progresión del tumor y la resistencia a la quimioterapia aún quedan por dilucidar por completo. Además de un especial esfuerzo por encontrar nuevas opciones terapéuticas para el tratamiento del cáncer de mama en etapas avanzadas.

En los primeros intentos por “curar” el cáncer, hace ya bastantes años, se utilizaban la cirugía, la quimioterapia y la radioterapia como opciones de primera línea; sin embargo, poco a poco se fueron conociendo varios y graves efectos adversos de estas terapias que comprometían la calidad de vida de los pacientes y que muchas veces llegaban a resultar más graves que la propia enfermedad. A pesar de que actualmente se siguen usando (Tabla 3), ya que han mejorado gracias a la innovación científica, también han surgido nuevas alternativas, como la inmunoterapia y la terapia dirigida con fármacos de origen natural, que prometen buenos resultados y menores efectos adversos.

Entre las opciones usadas con frecuencia en pacientes con cáncer de mama triple negativo se encuentran los taxanos, las antraciclinas, el metotrexato, el carboplatino, los inhibidores de PD-1 o PD-L1 y los inhibidores de PARP (Bardia A *et al.*, 2021). Sin embargo, se han observado pobres resultados en el tratamiento de las metástasis con quimioterapia sola, por lo que también se ha optado por tratamientos combinados con inmunoterapia o con terapia dirigida (INC, 2021).

Tabla 3. Ejemplos de quimioterapias usadas en el cáncer y sus mecanismos de acción. Modificada de Szadvari *et al*, 2016.

Bleomicina	- Produce la escisión oxidativa del ADN y, posiblemente, del ARN. Media la degradación del ADN, de los ARNm y de la cromatina activa. Provoca la detención en G2, la inhibición de la síntesis de ADN y ARN e induce la apoptosis.	Bimonte <i>et al.</i> , 2015; van der Kuyl <i>et al.</i> , 2002.
Capecitabina	- Es un profármaco que, tanto en tejidos tumorales como en tejidos normales, se convierte en el fármaco activo fluorouracilo (5-FU). Se observa una mayor concentración de la enzima de conversión en células cancerosas y en el hígado.	Walko y Lindley, 2005.

Ciclofosfamida	- Su efecto citotóxico se debe a la reticulación de las cadenas de ADN y a la subsiguiente inhibición de la división celular. Además, posee efectos anticancerígenos inmunomoduladores.	Le y Jaffee 2012; Mukhtar y Woodhouse 2010.
Doxorubicina	- Se intercala en el ADN (inhibe las polimerasas de ADN y ARN) e inhibe la reparación del ADN mediada por la topoisomerasa II durante el proceso de eliminación del superenrollamiento del ADN. Generación de radicales libres que daña el ADN, las membranas celulares y las proteínas. Hay otros posibles mecanismos involucrados.	Thorn <i>et al.</i> , 2011; Pommier, 2013; Tacar <i>et al.</i> , 2013.
Fluorouracil	- Es un análogo de pirimidina que interfiere con la síntesis y la reparación del ADN al inhibir la timidilato sintasa (que ejerce la llamada muerte sin timina). Afecta la función normal de los ácidos nucleicos debido a su incorporación incorrecta en el ADN y el ARN. Se han propuesto otros posibles mecanismos (p. ej., interferencia en la regulación del ciclo celular, inhibición de la angiogénesis, etc.).	Zhang <i>et al.</i> , 2008; Wyatt and Wilson, 2009.
Gemcitabina	- Es un análogo de la desoxicitidina que induce apoptosis al inhibir la síntesis de bases del ADN y su incorporación al ADN, lo que impide la síntesis de ADN.	Mini <i>et al.</i> , 2006.
Hidroxiurea	- Inhibición de la síntesis de ADN mediante la inhibición de la ribonucleótido reductasa (enzima responsable de la síntesis de desoxirribonucleótidos).	Torrents 2014; Yarbrow 1992.
Irinotecan	- Inhibición de la topoisomerasa 1 y, por consiguiente, roturas del ADN. Inducción de la detención/retraso de G2 y de la muerte celular.	Xu y Villalona-Calero 2002.
Metotrexato (MTX)	- Pertenece a la categoría de fármacos llamados antifolatos. Inhibe la conversión de dihidrofolato a tetrahidrofolato, necesaria para la síntesis de nucleótidos, en particular de la timidina. Por lo tanto, se inhibe la síntesis de ADN y ARN.	Wong y Choi, 2015; Schweitzer <i>et al.</i> , 1990; Bleyer, 1978.
Oxaliplatino	- Su mecanismo de acción es similar al de otras sustancias a base de platino, como el cisplatino. Causa	

	daño predominante al ADN mediante reticulación intercatenaria/intracatenaria. Otros efectos incluyen la detención de la síntesis de ADN y ARNm e inducción de mecanismos inmunológicos citotóxicos.	
Paclitaxel (Pac)	-Interfiere con la dinámica de los microtúbulos debido a su efecto estabilizador en el ensamblaje de estos. Provoca una segregación cromosómica defectuosa y bloquea la finalización del ciclo celular.	Alcindor and Beauger, 2011.
Vinblastina (Vinb)	-Ejerce un efecto inhibitor sobre la formación de fragmentos de microtúbulos en los centrosomas. Otro fármaco dirigido a la tubulina, como Pac, interfiere con la dinámica de los microtúbulos al inhibir su ensamblaje. Ejerce un efecto positivo sobre la formación de fragmentos de microtúbulos en los centrosomas.	Ganguly <i>et al.</i> , 2010.
		Ganguly <i>et al.</i> , 2010.

Con el avance de las investigaciones y la innovación en modelos preclínicos y clínicos, se han desarrollado tanto estrategias de tratamiento combinadas como pruebas con nuevos fármacos que prometen mejores resultados y menores efectos adversos que la radioterapia y la quimioterapia.

Actualmente se ha prestado especial interés a las moléculas de origen biológico, obtenidas a partir de plantas o microorganismos; incluso, existen amplias revisiones sobre los péptidos microbianos con propiedades anticancerígenas prometedoras. La importancia de estudiar este tipo de moléculas radica en que, a pesar de existir un importante número de tratamientos contra el cáncer, la gran heterogeneidad de esta enfermedad hace que siga habiendo muchos pacientes que no responden a los tratamientos, sobre todo aquellos diagnosticados en etapas avanzadas o con fenotipos muy agresivos.

Los péptidos cíclicos, por ejemplo, son un grupo diverso de pequeñas moléculas que han sido aprobados para varios usos terapéuticos, entre ellos, actividades como las antibióticas, antifúngicas y anticáncer (Costa y Fernández, 2023). En lo que concierne al cáncer, los péptidos cíclicos han mostrado efectos favorables al inhibir vías de señalización implicadas en la progresión de varios tipos de cáncer.

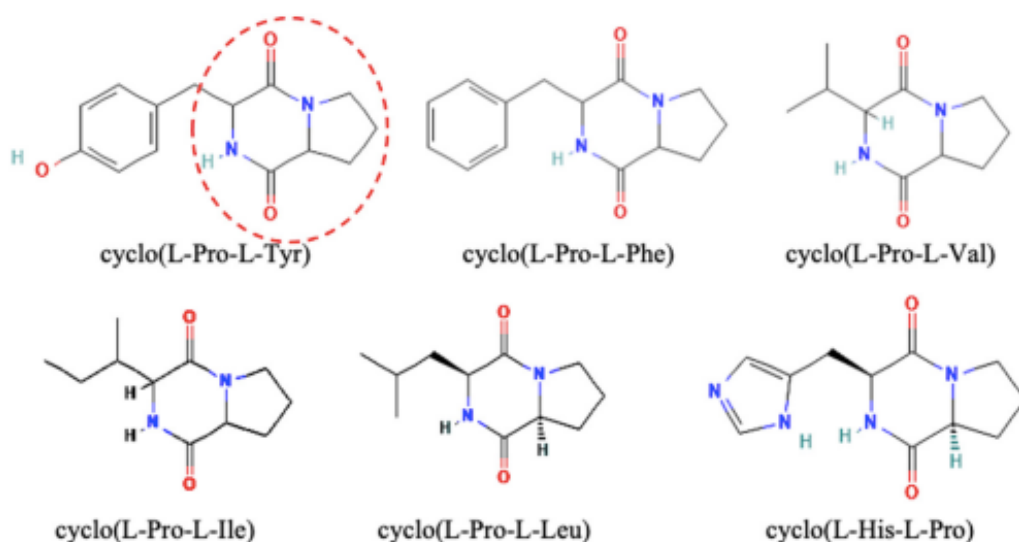


Figura 4. Estructura molecular de ciclodipeptidos bacterianos. Tomado de Martínez-Alcántar *et al.*, 2025.

CICLODIPEPTIDOS DE *Pseudomonas aeruginosa* PAO1

Los ciclodipeptidos o dicetopiperazinas son moléculas conformadas por dos aminoácidos unidos en forma cíclica por dos enlaces peptídicos, que pueden ser producidos por una amplia variedad de organismos o ser producidos por síntesis química, sin embargo, son aquellos producidos por los que se ha reportado una amplia variedad de funciones como por ejemplo, inductores de crecimiento en plantas, reguladores de *Quorum sensing* e incluso su potencial uso como antimicrobianos o en el tratamiento de diferentes enfermedades como diabetes, obesidad, enfermedades neurológicas y cáncer (Martínez-Alcantar *et al.*, 2024). Tal vez uno de los aspectos más importantes de estas pequeñas moléculas es su capacidad para interactuar con un gran número de blancos moleculares.

Actualmente, los CDPs son considerados agentes terapéuticos prometedores para la inhibición de las cinasas proteicas, al imitar la interacción de estas con sus ligandos naturales, ya que la desregulación de dichas cinasas puede ocasionar un amplio número de patologías. Por ejemplo, la vía HGF/MET es crucial para procesos de proliferación, invasión, angiogénesis, evasión inmune y resistencia a fármacos en células tumorales, lo que favorece la progresión del cáncer; sin embargo, aún no existen inhibidores selectivos de la interacción HGF/MET (Martínez-Alcantar *et al.*, 2024). Interesantemente, nuestro grupo de trabajo ha reportado que los PAO1-CDPs podrían estar modulando esa interacción en células de cáncer cervicouterino y de mama (Hernández-Padilla *et al.*, 2024).

Pseudomonas aeruginosa PAO1 sintetiza una mezcla de ciclodipeptidos compuesta por Ciclo(L-Pro-L-Tyr), Ciclo(L-Pro-L-Val), Ciclo(L-Pro-L-Leu) y Ciclo(L-Pro-L-Phe), y actualmente se ha investigado ampliamente tanto su participación como reguladores del sistema de *Quorum sensing*, como su potencial uso como anticancerígenos y en el tratamiento de la obesidad y de la enfermedad del hígado graso no alcohólico (Figueroa-Guzmán: artículo en revisión y patente No. 426355).

ANTECEDENTES

Los ciclodipeptidos, debido a sus características químicas y a la variedad de actividades biológicas, han cobrado interés en la investigación y, aun más, en sus posibles aplicaciones biotecnológicas. En nuestro grupo de trabajo se inició el estudio de los CDPs de *P. aeruginosa* PAO1 y, en 2011, se encontró que estos compuestos poseían actividad promotora del crecimiento en plantas, utilizando como modelo *A. thaliana*. Dicho efecto se atribuyó a su parecido con las auxinas, más específicamente con el ácido indolacético (IAA), y se propuso la posible interacción de los CDPs con los receptores de auxinas (Ortíz-Castro *et al.*, 2011). Asimismo, se investigó la relación de los CDPs con el sistema de quorum sensing de *P. aeruginosa* y cómo son sintetizados por posibles péptidos sintetasa no ribosomales (PSNR) (Omar González *et al.*, 2016).

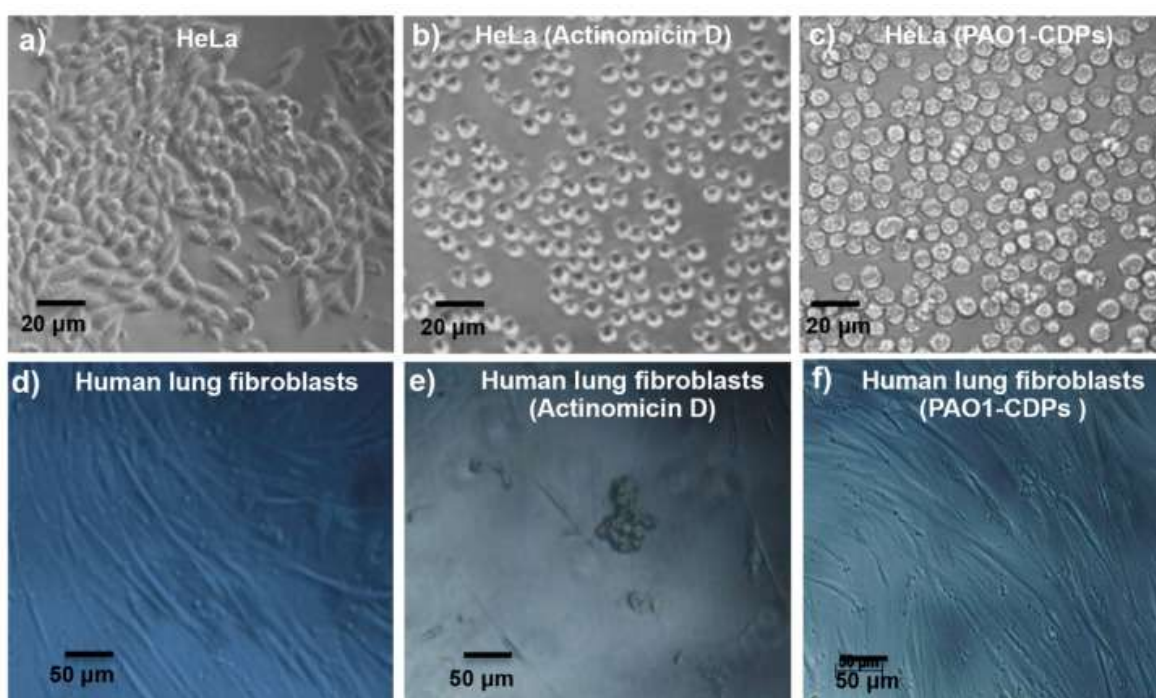
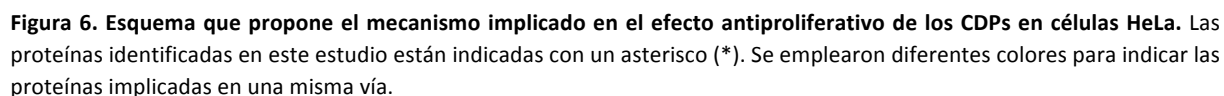


Figura 5. Efecto citotóxico de los PAO1-CDPs en células cancerosas y sanas. Se muestran fotografías representativas de células HeLa, de una línea de cáncer cervicouterino y de fibroblastos de pulmón, correspondientes a células sanas inmortalizadas y tratadas con PAO1-CDPs.

En 2015, Vázquez-Rivera y colaboradores, reportaron que la mezcla de los CDPs producidos por *P. aeruginosa* PAO1 promueven apoptosis en las líneas celulares cancerosas HeLa y CaCo-2 de manera dosis dependiente, con una concentración inhibitoria 50 % (IC₅₀) de 0.53 y 0.66 mg/mL respectivamente (figura 4) y como seguimiento de esta investigación, se encontró que el mecanismo por el cual los CDPs de *P. aeruginosa* PAO1 inducen apoptosis en células HeLa, es la vía intrínseca y que esto ocurre por la inhibición de la fosforilación de la proteína Akt, involucrada en la inhibición de la vía apoptótica (Hernández-Padilla *et al.*, 2017). Además, en 2020 se reveló el efecto multiblanco de los CDPs en diversas proteínas cinasas, incluidas mTORC1 y mTORC2. Además, se reportó que los ciclodipeptidos producidos por *P. aeruginosa* PAO1 no tenían efecto citotóxico en fibroblastos de

Posteriormente, se realizó un estudio en el que se evaluaron estos ciclodipéptidos en ratones C57BL/6 con melanoma no metastásico, obteniéndose una reducción del tamaño de los tumores de hasta el 80 % y una disminución del marcador tumoral lactato deshidrogenasa (LDH) en el suero. De manera interesante, también mejoraron los parámetros hematológicos y los niveles de enzimas de daño tisular, como alanina aminotransferasa (ALT) y aspartato aminotransferasa (AST), en los ratones tratados con 0.01 mg/g de peso corporal de los CDPs. Además de observar que los ciclodipéptidos tenían un mejor efecto al administrarse con β -ciclodextrinas (figura 6), se realizó un microarreglo para la detección de 21 proteínas involucradas en el fenotipo canceroso, lo que permitió una considerable disminución en la expresión de antígeno nuclear de células en proliferación (PCNA), inhibidor de cinasa dependiente de ciclina 1B (P27kip1), N-cadherina, factor inducible por hipoxia (HIF1 α) y Survivina, proteínas implicadas en vías de señalización de proliferación, de metabolismo energético y de metástasis (Durán-Maldonado *et al.*, 2020).



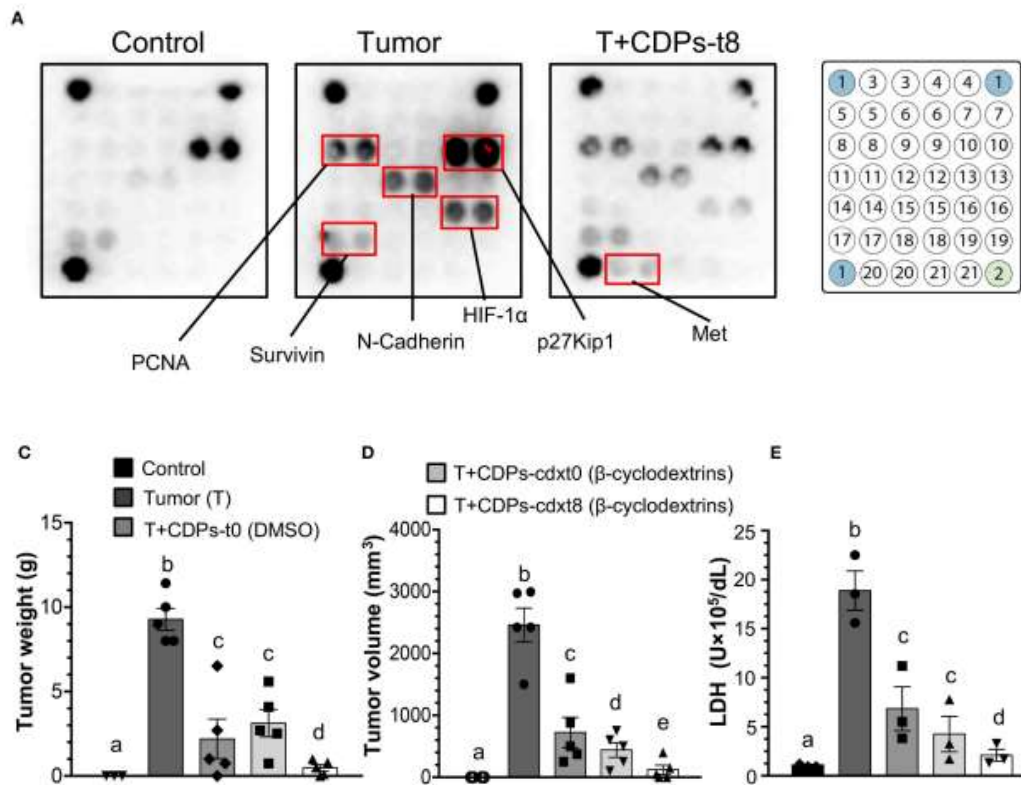


Figura 7. Los PAO1-CDPs tienen un efecto antiproliferativo en el modelo murino de melanoma. A) ensayo de detección de proteínas relacionadas con el fenotipo canceroso mediante microarreglo en lisados de tumor de melanoma murino; C) Gráfica del peso de los melanomas en ratones con y sin tratamiento con CDPs; D) Gráfica del volumen tumoral en ratones tratados con CDPs usando β-ciclodextrinas como vehículo; E) cuantificación de lactato deshidrogenasa (LDH) en suero de ratones con melanoma y con tratamiento con CDPs.

En 2022, se realizó un estudio transcriptómico en células HeLa tratadas con los PAO1-CDPs. Este análisis permitió identificar 151 genes diferencialmente expresados al alza o a la baja durante la exposición a estos péptidos; al ser sometidos a una base de datos de ontología, estos genes revelaron estar involucrados en la traducción de señales en el cáncer y en vías metabólicas como PI3K-Akt, mTOR, FoxO, Wnt, MAPK, P53, TGF-β, Notch, apoptosis, EMT y CSC. Adicionalmente, como se muestra en la figura 7, el análisis reveló que los ciclodipéptidos modifican la expresión de genes de la vía del mevalonato y de la biosíntesis del colesterol, lo que, en consecuencia, disminuye los niveles de colesterol total como parte del efecto antineoplásico en células HeLa (Lázaro-Mixteco *et al.*, 2022).

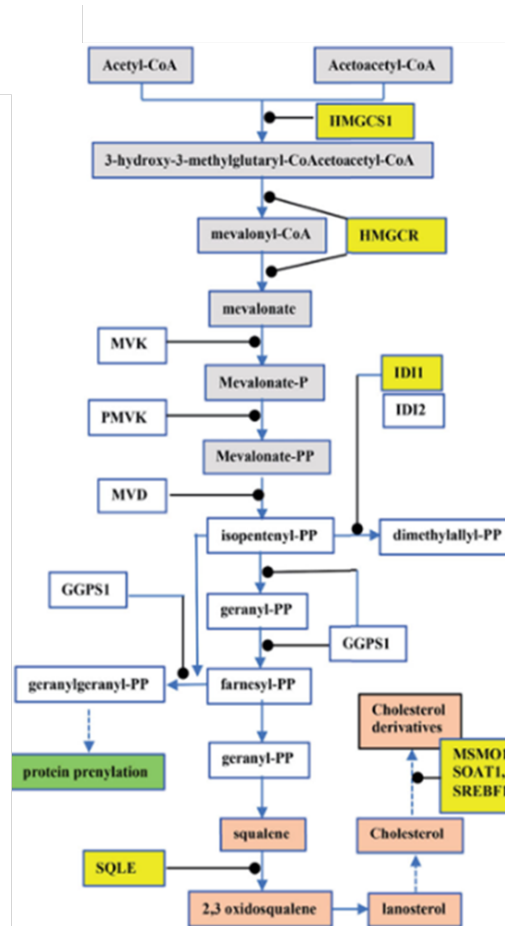
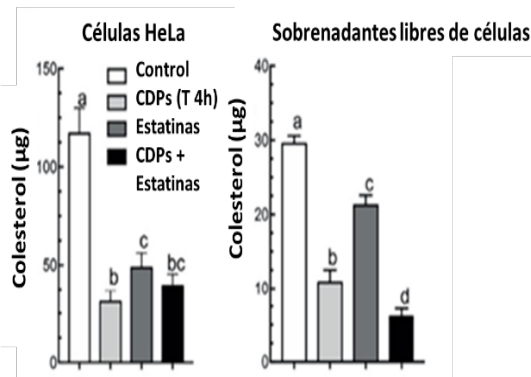


Figura 8. Los PAO1-CDPs bloquean la vía de síntesis del colesterol y del mevalonato en células HeLa. A) Células HeLa tratadas con 0.01 mg/mL y/o estatinas, así como los sobrenadantes libres de células, mostraron una disminución significativa del colesterol total; B) Se muestran en amarillo los genes cuya expresión se modificó por la exposición a los CDPs. Modificado de Lázaro-Mixteco *et al.*, 2022).

Otro trabajo publicado en 2024 por Hernández-Padilla *et al.*, sugieren que la actividad antiproliferativa y citotóxica de los CDP en células HeLa involucra al receptor HGF/MET aguas arriba de la vía PI3K/Akt/mTOR; ensayos con las líneas celulares humanas de cáncer de mama MCF-7 y MDA-MB-231 respaldaron este hallazgo. Así, también, el mecanismo de transducción de señales puede estar relacionado con la inhibición de la fosforilación del receptor EGF/MET en el sitio de unión del sustrato, mediante un mecanismo de inhibición similar al de los fármacos antineoplásicos Gefitinib y Foretinib (figura 8).

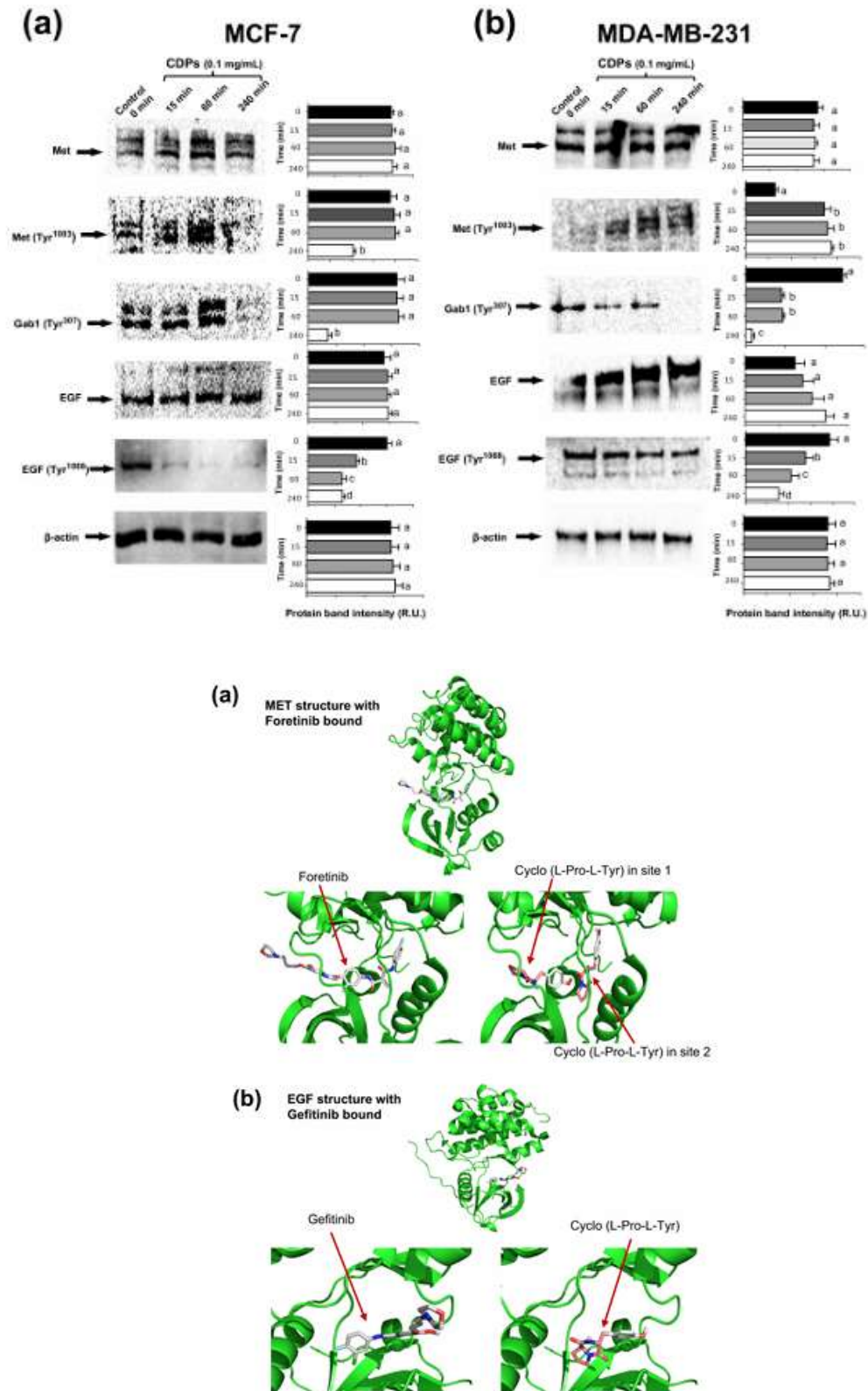


Figura 9. Efecto de los PAO1-CDPS sobre la fosforilación del receptor MET/EGF en células de cáncer de mama. En el panel superior se muestran los western blots de los lisados de las células MCF-7 y MDA-MB-231.

En el panel inferior se muestran imágenes de docking molecular entre las estructuras de MET y EGF y los CDPs Foretinib y Gefitinib. Tomado de Hernández-Padilla *et al.*, 2024.

Así mismo, diversos antecedentes recopilados en una revisión sobre péptidos cíclicos como moduladores de proteínas cinasas en diferentes enfermedades humanas nos muestran que éstas moléculas desde hace varias décadas han mostrado características químicas clave que los hacen potenciales fármacos contra enfermedades crónicas degenerativas como diabetes y obesidad, neurodegenerativas como el Alzheimer y Parkinson así como diversos tipos de cáncer, en donde incluso se enlistó un gran número de compuestos aprobados por la FDA para su uso como tratamientos en humanos y otros tantos que se encuentran actualmente en investigación (Martínez Alcántar *et al.*, 2025).

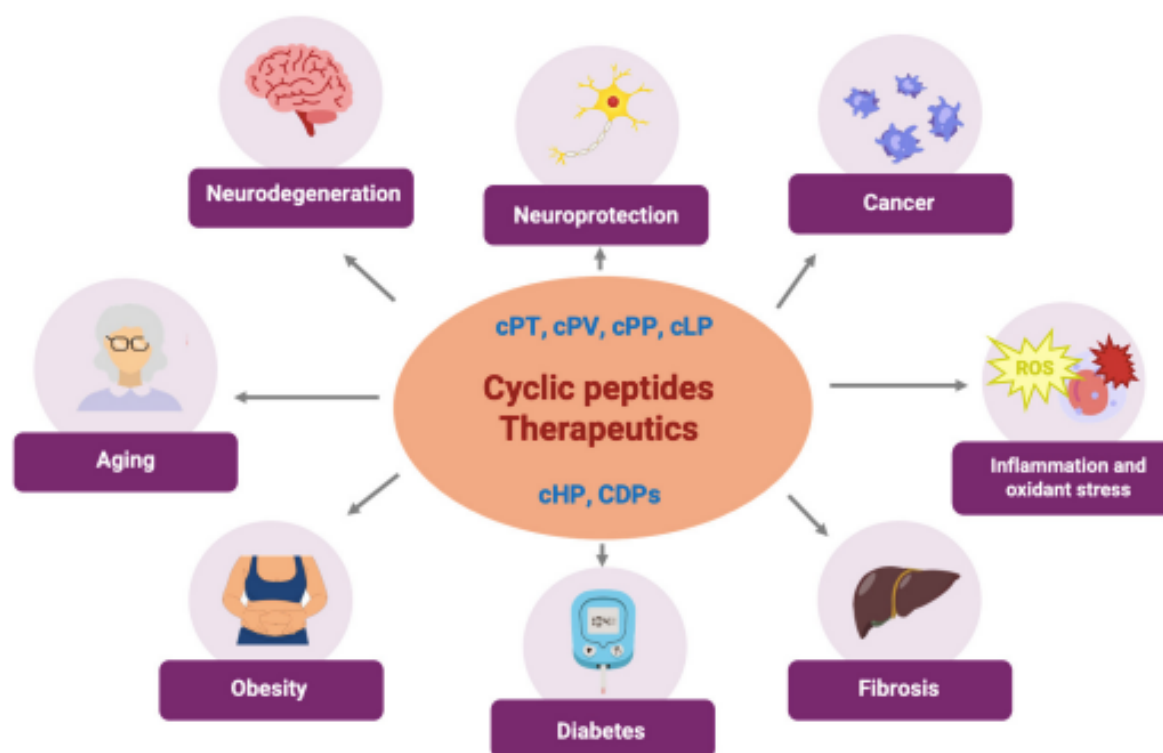


Figura 10. Péptidos cíclicos implicados en el tratamiento de diversos padecimientos humanos. (Martínez Alcántar *et al.*, 2025).

JUSTIFICACIÓN

El cáncer de mama es uno de los más prevalentes en México y en el mundo, y su heterogeneidad clínica y molecular representa un importante reto para lograr un tratamiento exitoso. Sumado a esto, hasta un 60 % de las neoplasias en nuestro país se detectan en etapas avanzadas (metástasis), lo que conduce a la resistencia al tratamiento y a un pronóstico desfavorable para los pacientes. Los ciclodipéptidos de *P. aeruginosa* PAO1 han demostrado tener efecto antineoplásico en diferentes tipos de cáncer *in vitro*, como el cervicouterino, el melanoma, las leucemias y el colorrectal, así como

una disminución significativa de los melanomas en un modelo in vivo. También se ha reportado que los CDPs afectan las vías de señalización implicadas en la proliferación, la resistencia a la apoptosis, el metabolismo energético, el metabolismo de lípidos y las metástasis.

Por lo anterior, es de nuestro interés estudiar el potencial anticancerígeno de los CDPs en modelos *in vitro* e *in vivo* de cáncer de mama metastásico.

HIPÓTESIS

Los ciclodipéptidos de *P. aeruginosa* PAO1 tienen un efecto antimetástico en un modelo *in vitro* de cáncer de mama y en un modelo *in vivo*.

OBJETIVO GENERAL

Determinar el efecto de los ciclodipéptidos de *P. aeruginosa* PAO1 en un modelo de cáncer de mama metastásico.

OBJETIVOS ESPECIFICOS

- 1.- Caracterizar el efecto de los ciclodipéptidos producidos por *P. aeruginosa* PAO1 en un modelo *in vitro* de cáncer de mama metastásico.
- 2.- Evaluar el efecto de los ciclodipéptidos en un modelo murino de cáncer de mama metastásico.
- 3.- Dilucidar las vías de señalización impactadas por los ciclodipéptidos involucradas en el proceso de metástasis en un modelo murino de cáncer de mama.

CAPÍTULO I. Ciclodipéptidos bacterianos inhiben la progresión de la invasión y metástasis en un modelo murino de cáncer de mama triple negativo MDA-MB-231

Article

Bacterial Cyclodipeptides Inhibit Invasiveness and Metastasis Progression in the Triple-Negative Breast Cancer MDA-MB-231 Mouse Model

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Abstract

Triple-negative breast cancer (TNBC) is a highly aggressive subtype linked to a high rate of metastasis and low survival rates worldwide. Bacterial cyclodipeptides (CDPs) demonstrate anticancer properties by targeting multiple signaling pathways. The impact of CDPs on TNBC metastasis was evaluated both in vitro and in advanced-stage tumors in immunosuppressed female mice. CDPs significantly decreased the migratory and invasive capabilities of the MDA-MB-231 cell line, outperforming methotrexate (MTX). This effect was associated with the inhibition of Akt/mTOR/S6K phosphorylation, as well as Gab1, Vimentin, and FOXO1. Mice bearing MDA-MB-231 xenografts treated with CDPs alone or in combination with MTX showed near-complete suppression of primary tumors and metastatic sites in organs; notably, the combined treatment displayed a synergistic effect. Consequently, key proteins involved in tumor progression and metastasis, including p-Akt, p-Gab1, and FOXO1, were markedly inhibited in tumors from CDP-treated mice. Additionally, genes related to EMT, invasiveness, and immune modulation—including PTEN, SNAIL, CXCL1, BRCA1, GADD45A, and PD-L1—were dysregulated in the livers of TNBC-bearing mice; however, CDP treatment restored their expression more effectively than MTX. These findings suggest that the anti-metastatic effects of CDPs in the TNBC xenograft model involve modulation of the Akt/mTOR/S6K pathway, EMT, invasiveness, and immune modulation, highlighting their potential for further preclinical development.

Keywords: cyclodipeptides; anti-metastatic; triple-negative breast cancer



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1. Introduction

Breast cancer is one of the leading causes of cancer-related mortality among women worldwide and represents a clinically heterogeneous disease. Approximately 10–15% of patients develop highly aggressive tumors with an increased propensity for metastasis [1]. Triple-negative breast cancer (TNBC) comprises a heterogeneous group of tumors with distinct histological, genomic, and immunological characteristics and is defined by the absence of estrogen and progesterone hormone receptors, as well as human epidermal growth factor receptor 2 (HER2). Several risk factors, including obesity, are associated with poor prognosis, as the interaction between adipocytes and tumor cells creates a permissive

tumor microenvironment that promotes cancer cell proliferation, invasion, metastatic dissemination, and resistance to cell death [2].

In the cancer context, liver metastasis represents one of the most challenging complications in TNBC and is associated with high rates of morbidity and mortality. The liver is among the most frequent sites of breast cancer dissemination and constitutes the initial metastatic organ in approximately 30% of individuals with metastatic breast cancer. The development of liver metastases is associated with an unfavorable prognosis and reduced overall survival, even in the context of advances in diagnostic and therapeutic strategies achieved in recent decades. Notably, TNBC patients with liver involvement exhibit a poorer clinical outcome compared with those affected by other breast cancer subtypes. Consequently, the identification of novel therapeutic strategies capable of improving outcomes in patients with metastatic breast cancer remains a critical unmet clinical need.

The FDA has approved several drugs with therapeutic uses targeting members of the protein kinase superfamily (PKs) associated with various diseases. Because the ATP-binding site is conserved across the human kinome, ATP-mimicking compounds often interact with multiple kinases. Drugs such as Abemaciclib, Palbociclib, Ribociclib, and Trilaciclib target the CDK4/6 proteins at the ATP-binding site; therefore, kinase-like proteins such as CDK4/6, AKT, HER2, ErbB1/2/4, and FKBP12/mTOR are considered therapeutic targets for breast cancer treatment [3]. In contrast, inhibitors designed for a single target, like the kinase inhibitor Capivasertib, which targets the AKT oncogene—a member of the PI3K/Akt/mTOR signaling pathway—have become key in treating cancer, diabetes, obesity, neurodegenerative diseases, aging, and autoimmunity [3]. Among the primary treatment options for metastatic breast cancer are taxanes, anthracyclines, methotrexate (MTX), carboplatin, immune checkpoint inhibitors targeting PD-1 or PD-L1, PARP inhibitors, and combinations of these agents with immunotherapy, radiotherapy, or surgery [4]. However, a significant number of patients respond poorly to these treatments, leading to low survival rates and a reduced quality of life. As a result, the pursuit of new therapeutic strategies that can improve the prognosis for patients with metastatic breast cancer remains a vital priority.

Cyclodipeptides (CDPs) are a class of molecules of the diketopiperazine family, produced by a wide range of organisms that exhibit notable biological activities, including cytotoxic effects against multiple cancer cell lines [5–8]. Previously, we reported that CDPs, such as cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Leu), and cyclo(L-Pro-L-Phe), isolated from the *Pseudomonas aeruginosa* PAO1 bacterium, induce apoptosis in cervical, colon, leukemia, and melanoma cancer cells. In HeLa cells, CDPs inhibit cell proliferation, including arresting cells at the G0–G1 stage, and induce apoptosis via an intrinsic pathway that relies on caspase-9 and -3 activation. Mechanistically, the antiproliferative effects of CDPs involve modulation of multiple signaling networks associated with tumor growth, survival, and plasticity, including the PI3K/Akt/mTOR, Ras/Raf/MEK/ERK1/2, PI3K/JNK/PKA, p27Kip1/CDK1/survivin, MAPK, HIF-1, Wnt/ β -catenin, HSP27, epithelial–mesenchymal transition (EMT), and cancer stem cell (CSCs) pathways [3,6,7]. Transcriptomic profiling of HeLa cells treated with CDPs revealed 151 differentially expressed genes distributed across 15 major biological processes, including regulation, signaling, development, locomotion, adhesion, metabolism, growth, proliferation, and immune response. Pathway enrichment analysis suggested that CDPs modulate multiple signaling networks, encompassing the insulin/IGF/MAP kinase cascade, P38/MAPK signaling, interleukin-mediated pathways, epidermal growth factor receptor (EGFR), PI3K signaling, platelet-derived growth factor (PDGF), Notch, cadherin, p53, Hedgehog, transforming growth factor- β (TGF- β), fibroblast growth factor (FGF), FAS-mediated signaling, angiogenesis, cholesterol biosynthesis, and pathways associated with inflammation, oxidative stress responses, apoptosis, cytoskeletal organization, and T- and B-cell activation [9]. In a murine melanoma model, CDP treatment

reduced tumor growth by decreasing the expression of proteins that mediate pathways involved in energy metabolism, lipid synthesis, EMT, invasion, and metastasis [10]. In this context, cyclic peptides have emerged as promising therapeutic agents for kinase inhibition, as dysregulated kinases play central roles in cancer cell survival, proliferation, metastasis, and drug resistance. Given this background, we sought to determine whether bacterial CDPs have a promising effect on metastatic breast cancer, including advanced stages of tumorigenesis using a xenograft model of triple-negative breast cancer established with MDA-MB-231 (ER-, PR-, HER2-) cells.

2. Results

2.1. Bacterial CDPs Reduce Migration and Invasiveness in the MDA-MB-231 Breast Cancer Cell Line

Previously, the cytotoxic and apoptotic properties of the CDPs have been demonstrated in HeLa and CaCo-2 [8] and murine melanoma lines [10]; however, their anti-metastatic potential remains unknown. Therefore, the MDA-MB-231 cell line was used as a model to study triple-negative breast cancer and evaluate the anti-metastatic properties of CDPs.

In wound-healing assays, untreated MDA-MB-231 monolayers recovered about >90% of the original wound area after 48 h. Meanwhile, the chemotherapeutic compound methotrexate (MTX; 0.05 mg/mL), used as an anti-migration control, resulted in about 40–50% of the wound remaining unclosed during the same period. In contrast, CDP treatment (0.01 mg/mL), whether alone or combined with MTX, significantly inhibited cell migration, leaving approximately 80% and 90% of the wound area unclosed after 48 h, respectively (Figure 1a).

Furthermore, since chemokine-mediated macrophage signaling enhances tumor invasiveness, the invasive ability of MDA-MB-231 cells was assessed using a transwell assay. Co-culturing with RAW macrophages increased the number of invading cancer cells, confirming the pro-invasive role of the macrophage microenvironment (Figure 1b). CDP treatment significantly reduced invasion, lowering the number of invading cells by approximately 75% in monoculture and about 60% in macrophage co-culture conditions. In comparison, invasion under MTX (0.05 mg/mL) treatment was less suppressed than with CDPs alone (0.01 mg/mL), but was most effectively inhibited when CDPs were combined with MTX, resulting in up to a 90% reduction in invasiveness (Figure 1b).

Additionally, spheroids are cellular aggregates that represent three-dimensional cultures of cancer cells and serve as models for tumor development. Therefore, the effect of CDPs on the viability and integrity of spheroids from the MDA-MB-231 cell line was assessed (Figure 1c,d). A cyclodextrin suspension, consistent with previous *in vivo* findings, was used as the delivery vehicle [10]. The results indicated a significant decrease in both the number and size of spheroids in the MDA-MB-231 cell line when treated with CDPs alone (0.1 mg/mL) or combined with cyclodextrins (CDPs-cd). The CDPs at 0.1 mg/mL showed a strong ability to disrupt spheroid formation, similar to MTX (0.05 mg/mL). Notably, the most effective outcome was observed with the combination of CDPs and the anti-neoplastic drug MTX (CDPs-cd + MTX) (Figure 1c–e). Spheroid viability and apoptosis induction were further assessed using MTT assays and flow cytometry, respectively. These analyses identified a lethal dose (LD₅₀) of 0.25 mg/mL and an effective dose for apoptosis induction (ED₅₀) of 0.02 mg/mL for CDPs after 4 h of treatment (Figure 1e,f).

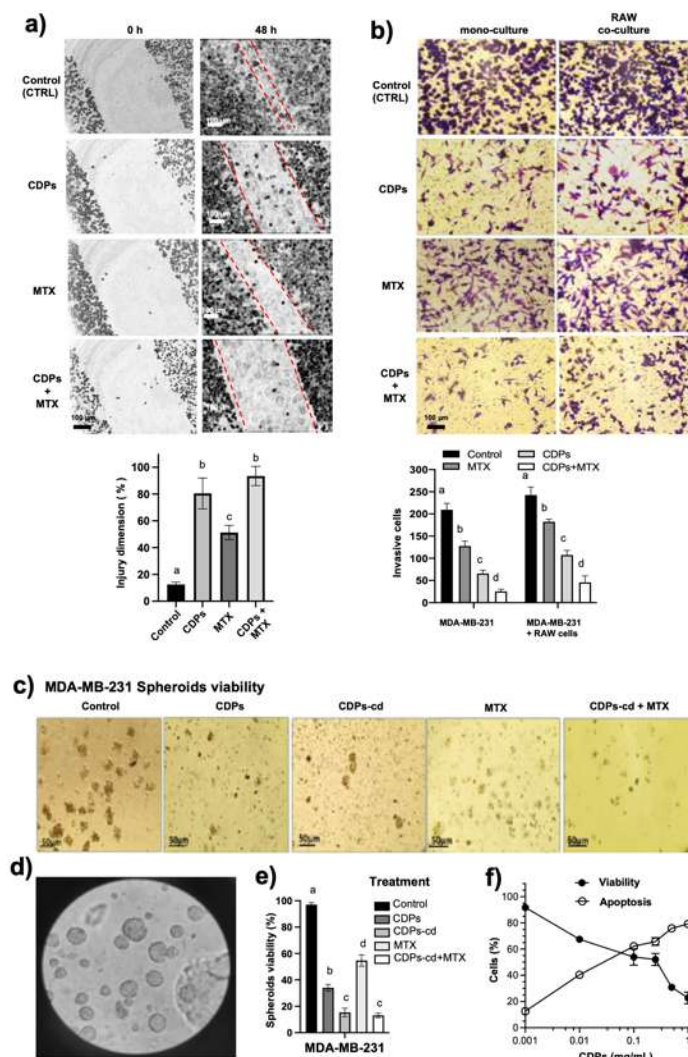


Figure 1. Effects of bacterial CDPs on the migratory and invasive properties of MDA-MB-231 breast cancer cells. **(a)** Representative photographs of wound closure migration assays taken at zero h and 48 h in MDA-MB-231 cell cultures treated with the saline solution (CTRL), CDPs (0.01 mg/mL), MTX (0.05 mg/mL), or combined CDP + MTX treatment (0.01 mg/mL + 0.05 mg/mL, respectively) **(b)** Representative photographs of the cancer cells that managed to invade the Matrigel-covered membrane, stained with crystal violet. The monoculture conditions and co-culture with RAW 264.7 macrophages are shown. Treatment were CDPs (0.01 mg/mL), MTX (0.05 mg/mL), or combined CDP + MTX treatment (0.01 mg/mL + 0.05 mg/mL, respectively) by 24 h. Below, the quantification of the wound dimension expressed in percentage **(a)** and the number of invasive cells **(b)** is done using ImageJ 1.53k Software. **(c,d)** Representative photographs of multicellular spheroids of the MDA-MB-231 line. **(e)** Cell viability of the spheroids treated with CDPs (0.1 mg/mL) plus MTX (0.05 mg/mL) determined by the MTT method after 24 h of incubation. **(f)** Cell viability and apoptosis induction of the MDA-MB-231 cell cultures submitted to CDP treatments for 4 h, determined by FACS. Bars represent means $\pm$ SE, $n = 3$. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. SE values are shown as bars; groups sharing the same lowercase letter are not significantly different from each other, while groups with different letters indicate statistically significant differences ($p < 0.05$). CDPs, cyclodipeptides; MTX, methotrexate; CDPs-cd, CDPs dissolved in β -cyclodextrins; CDPs-cd + MTX, combined treatment.

2.2. Effect of Bacterial CDPs on the Akt/mTOR/S6K Signaling Pathway in the MDA-MB-231 Cell Line

The signaling pathways related to cell survival and growth were examined to explore the anti-neoplastic effects of bacterial CDPs in the MDA-MB-231 cell line. Results showed that CDP treatment (0.1 mg/mL) did not change the overall levels of Akt, mTOR, and S6K proteins and had no time-dependent effects on their expression; however, their phosphorylation states—p-Akt, p-mTOR, and p-S6K—were significantly decreased over time after CDP treatment (Figure 2). Therefore, the ratio of phosphorylated forms to total protein was significantly reduced. Additionally, phosphorylation of p-Gab1 and the expression of Vimentin were notably decreased in MDA-MB-231 cells following CDP treatment (Figure 2).

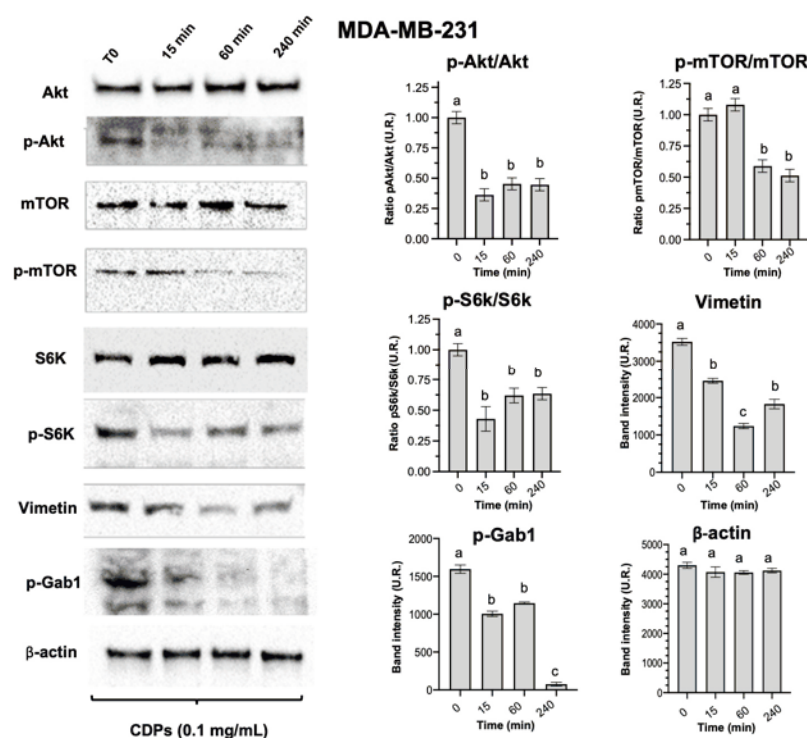


Figure 2. Effect of bacterial CDPs on proteins involved in proliferation, invasion, and metastasis in MDA-MB-231 triple-negative breast cancer cells. Protein extracts obtained from cell cultures treated with CDPs (0.1 mg/mL) over time were subjected to immunodetection. Protein expression for the total Akt, phosphorylated Akt, total mTOR, phosphorylated mTOR, total S6K, phosphorylated S6K, Vimentin, phosphorylated Gab1, and β -actin is shown. Densitometric analyses of the protein immunodetection results are presented on the right. The bars represent the means $\pm$ SE; $n = 3$ per group. Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test; SE values are shown as bars; groups sharing the same lowercase letter are not significantly different from each other, while groups with different letters indicate statistically significant differences ($p < 0.05$).

2.3. Effect of Bacterial CDPs in a Breast Cancer Mouse Model Through Implantation of the MDA-MB-231 Cell Line

An orthotopic TNBC xenograft model with metastatic dissemination was established by implantation of MDA-MB-231 cells into the mammary fat pad of immunosuppressed female mice, achieved 100% mammary tumor formation. Healthy mouse groups (procedure 1, Figure 3) and two treatment conditions were tested. In the second treatment regimen (procedure 2, Figure 3), mice were inoculated with MDA-MB-231 cells and treated simultaneously with CDPs, followed by a total of nine doses of CDPs (0.25 mg/kg) over

a 35-day therapeutic period. In the third regimen (procedure 3, Figure 3), tumors were permitted to develop for 35 days after implanting MDA-MB-231 cells; then, treatment with CDPs or MTX (0.05 mg/kg) was started and continued for another 35 days.

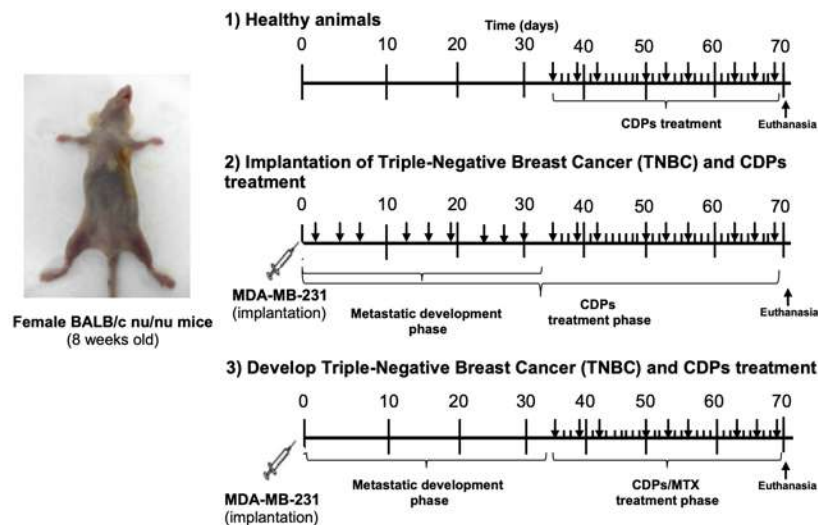


Figure 3. Therapeutic procedures in the MDA-MB-231 immunosuppressed mouse model of triple-negative breast cancer treated with bacterial CDPs. Female BALB/c nu/nu mice (8 weeks old) were used to establish an orthotopic triple negative breast cancer xenograft model. Three experimental treatment protocols were tested: (1) Healthy animals were treated or not with CDPs at 35 days after the start of the procedure (indicated by arrows), followed by nine doses of CDPs (0.25 mg/kg) over 35 days. (2) Mice were orthotopically inoculated with MDA-MB-231 cells and simultaneously treated with CDPs, followed by nine doses of CDPs (0.25 mg/kg) during 35 days (indicated by arrows). Treatment was then continued for an additional 35 days in the corresponding groups. (3) The third treatment protocol, mammary tumors were allowed to develop for 35 days following MDA-MB-231 cell implantation. After that, nine doses of CDPs (0.25 mg/kg), MTX (0.05 mg/kg), or both CDPs and MTX were administered during 35 days (arrows). Finally, at 70 days from the start of treatment, all animals were ethically euthanized.

Results of body weight monitoring showed that weight gain was similar across all mouse groups, except for the TNBC groups treated with CDPs (TNBC + CDPs) or with the combination of CDPs and MTX (TNBC + CDPs + MTX), which exhibited lower body weight gain slopes compared to untreated TNBC mice (Figure S1).

The tumor volume was monitored in all mouse groups throughout the therapeutic experiment. On day 70, tumors in the untreated TNBC group reached an average volume of approximately 300 mm³ (Figure 4a). In contrast, TNBC mice treated with CDPs showed a marked reduction in tumor volume, either from the time of MDA-MB-231 cell implantation or after the tumor establishment phase (procedures 2 and 3, respectively), with a significant reduction to approximately 10 mm³ and 3 mm³, respectively. This indicates that both CDP treatment regimens were more effective than MTX, in which tumors reached an average volume of about 90 mm³ (Figure 4a).

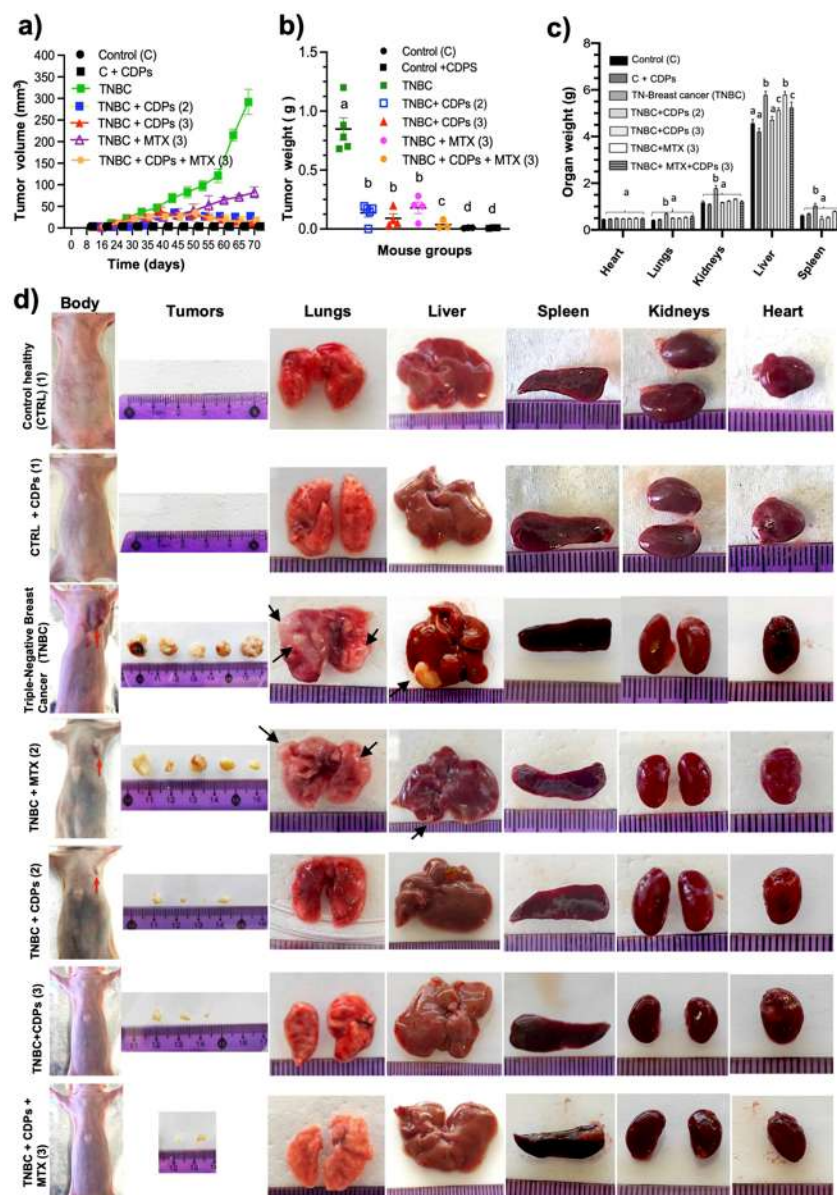


Figure 4. Antitumor effects of bacterial CDPs in an MDA-MB-231 xenograft mouse model of triple-negative breast cancer. (a) Tumor volume expressed in mm³ was monitored during the 70 days of the therapeutic procedure. (b) Tumor weight expressed in grams. (c) Weight of organs in grams. (d) Representative photographs of animals, tumors, and organs from each mouse group are shown, n = 5 per group. Arrows indicate metastatic foci in organs. Mouse groups: (CTRL), control healthy animals without treatment (procedure 1); CTRL + CDPs, healthy animals administered with CDPs (procedure 1); TNBC, animals that developed breast cancer without treatment (procedure 2); TNBC + CDPs (2), TNBC animals administered with CDPs (procedure 2); TNBC + CDPs (3), TNBC animals administered with CDPs (procedure 3); TNBC + MTX (3), TNBC animals administered with methotrexate (MTX) as procedure 3; TNBC + CDPs + MTX (3), TNBC animals co-administered with CDPs + MTX as procedure 3. Graphs show means $\pm$ SE; n = 5. Statistical analysis was performed using one-way ANOVA, followed by Tukey's post hoc test. SE values are shown as bars; groups sharing the same lowercase letter are not significantly different from each other, while groups with different letters indicate statistically significant differences ($p < 0.05$).

After euthanasia, primary tumors and organs were dissected for detailed analysis (Figure 4b,c). Tumors from the untreated TNBC group weighed about 0.9 g on average. In contrast, the TNBC mice treated with CDPs showed significantly lower tumor weights, averaging around 0.15 g in the preventive treatment regimen (procedure 2) and approximately 0.05 g in the therapeutic regimen (procedure 3) (Figure 4b). Similarly, the MTX-treated group had a mean tumor weight of roughly 0.15 g. Notably, in the TNBC + CDPs and TNBC + CDPs + MTX groups, tumor weights were greatly reduced to about 0.05 g, and in 40–60% of these groups, no tumors were observed (Figure 4d).

In the orthotopic xenograft TNBC model, mice with mammary tumors that received no treatment (TNBC) showed a significant increase in the weights of the lungs, kidneys, liver, and spleen (Figure 4c,d), along with metastatic foci in the lungs and liver. In contrast, the groups treated with CDPs and the combination of CDPs plus MTX exhibited normalized organ weights and no visible metastatic foci (Figure 4c,d).

2.4. Effect of Bacterial CDPs on Biochemical and Hematological Parameters in the Breast Cancer Mouse Model with MDA-MB-231 Cell Line Implantation

The enzymes alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH), used as markers of hepatocellular injury and systemic tissue damage, were measured in the blood serum of animals implanted with breast tumors. The untreated TNBC mice group showed a significant increase in AST and LDH enzyme activities compared with healthy controls (Figure 5a), which was reversed by treatment with CDPs, MTX, or their combination. Importantly, treatment with CDPs in the healthy control mice group (C + CDPs) did not affect AST or LDH levels, as seen in the control group.

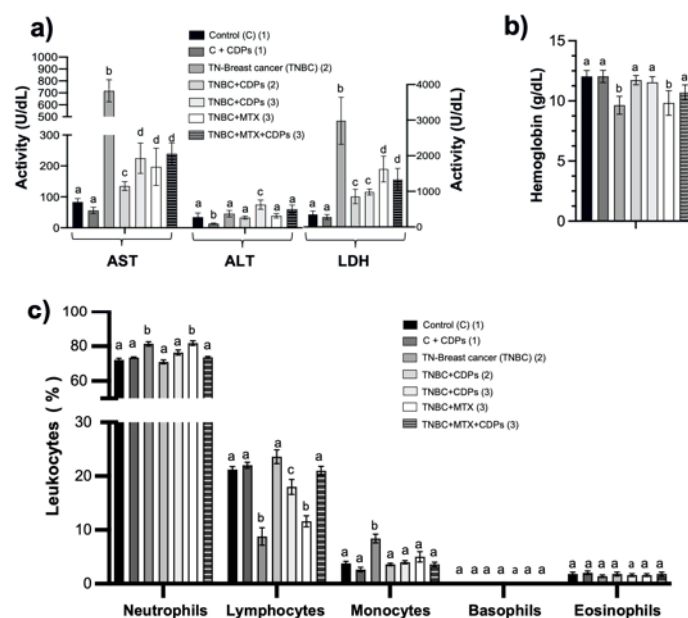


Figure 5. Hematological parameters in xenografted triple-negative breast cancer MDA-MB-231 cells in immunosuppressed mice treated with bacterial CDPs. (a) Measurement of enzyme activity of AST, ALT, and LDH in the serum of the mouse groups. (b) Hemoglobin levels. (c) Hematological profile of leukocyte counts. Group nomenclature is consistent with that described in Figure 4. Graphs display means $\pm$ SE; $n = 5$. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's post hoc test. SE values are shown as bars; groups sharing the same lowercase letter are not significantly different from each other, while groups with different letters indicate statistically significant differences ($p < 0.05$).

The hematological analysis indicated that blood hemoglobin levels were significantly lower in mice with TNBC tumors and in the TNBC + MTX group; however, hemoglobin levels were restored in mice with tumors treated with CDPs, nearly reaching the levels of the healthy control group (Figure 5b). Additionally, leukocyte profiling revealed changes in neutrophil, lymphocyte, and monocyte counts in the TNBC and TNBC + MTX groups (Figure 5c). These changes were normalized in the groups treated with CDPs, which showed leukocyte distributions similar to healthy controls. Furthermore, healthy mice treated with CDPs did not exhibit any changes in these hematological parameters (Figure 5c).

2.5. Histological and Protein Expression in Tumors from the Xenografted TNBC MDA-MB-231 Line Treated with Bacterial CDPs

Histological analyses of tumors were conducted using hematoxylin and eosin (H&E) staining. Tumors from untreated TNBC mouse groups displayed nuclear hyperchromasia and infiltration of inflammatory cells (Figure 6). Notably, tumors from TNBC mouse groups treated with MTX or CDPs showed reduced nuclear hyperchromasia. In these treated groups, focal areas of necrosis and inflammatory cell infiltration were observed, indicating treatment-related histopathological changes (Figure 6).

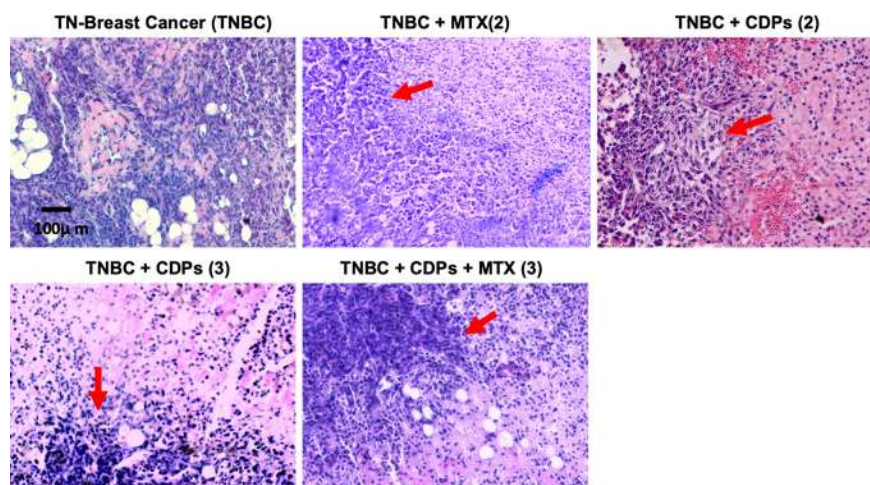


Figure 6. Histopathological features of tumors in an MDA-MB-231 xenograft mouse model of triple-negative breast cancer treated with bacterial CDPs. Female BALB/c nu/nu mice (8 weeks old) were subjected to antitumor treatments and analyzed at experimental endpoints corresponding to 35 or 70 days ($n = 5$ per group). Tumor sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Representative images of tissues from the animal groups are shown. Regions of tumor necrosis and inflammatory cell infiltration are indicated by red arrows.

Protein immunodetection analysis was carried out on crude tumor extracts from the xenograft model. Results indicated that CDPs and MTX treatments significantly decreased Akt phosphorylation; notably, the ratio of the phosphorylated form to total Akt protein levels was greatly influenced by these treatments (Figure 7). The Gab1 protein expression ratio between the phosphorylated form and the total protein was significantly reduced in the TNBC + MTX(3) and TNBC + CDPs + MTX(3) groups. Similarly, the levels of FOXO1 and its phosphorylated form (p-FOXO1) were considerably lower in tumors from mice treated with CDPs, MTX, or both in combination. However, the ratio of phosphorylated p-FOXO1 to total protein was significantly lower in the TNBC + MTX(2) and TNBC + MTX(3) groups but was restored in the TNBC + MTX(3) and TNBC + CDPs + MTX(3) groups (Figure 7).

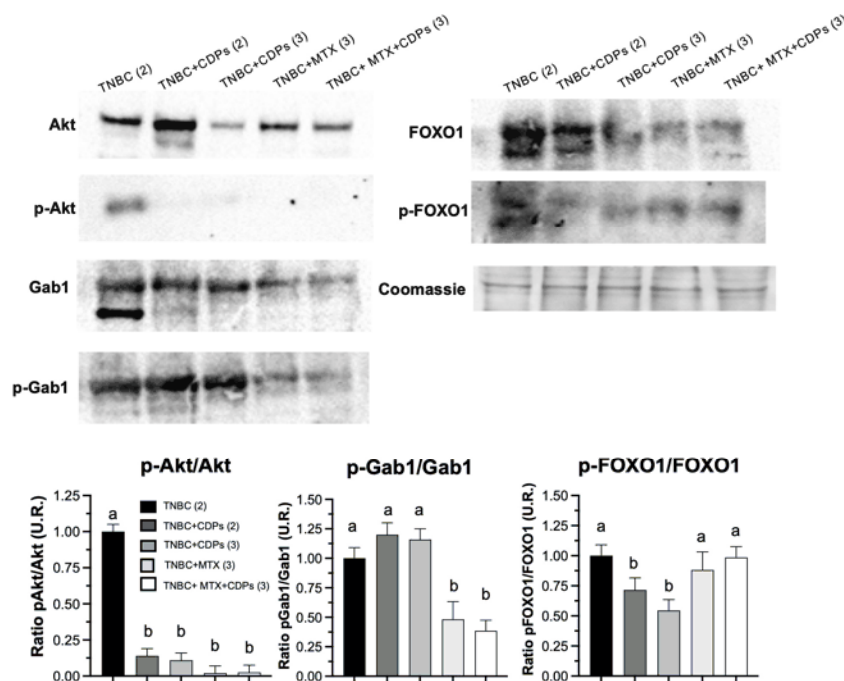


Figure 7. Effects of bacterial CDPs on signaling proteins in tumors grafted with MDA-MB-231 triple-negative breast cancer cells in immunosuppressed mice. Protein extracts from the tumors excised from the mouse groups (administered with CDPs, MTX, and co-administered CDPs + MTX) were subjected to immunodetection. Group nomenclature is consistent with that described in Figure 4. Representative immunoblots show total and phosphorylated forms of Akt, Gab1, and FOXO1. Densitometric analyses of the immunodetection results are presented below. The bars represent the means $\pm$ SE; $n = 3$ per group. Statistical analysis was conducted using one-way ANOVA, followed by Tukey's post hoc test. SE values are shown as bars; groups sharing the same lowercase letter are not significantly different from each other, while groups with different letters indicate statistically significant differences ($p < 0.05$).

2.6. Histological of Organs from Xenografted TNBC MDA-MB-231 Mice Treated with Bacterial CDPs

Histological examination of the liver sections showed reduced nuclear hyperchromasia in mice treated with CDPs, either alone or combined with methotrexate (MTX), compared to untreated TNBC mice, with no signs of hepatocellular or tissue hypertrophy (Figure 8). In lung sections, untreated TNBC mice exhibited significant disruption of normal alveolar architecture, while healthy control mice (C) and healthy mice treated with CDPs (C + CDPs) maintained well-preserved alveolar structures. Notably, in the tumor groups treated with CDPs and MTX, the alveolar structure was restored to resemble that of the healthy groups (see yellow arrows in Figure 8). Additionally, spleen histology revealed disintegration of structural features in the tumor-bearing animals (TNBC), which was reversed in the TNBC groups treated with CDPs, MTX, or both (see dashed circles in Figure 8).

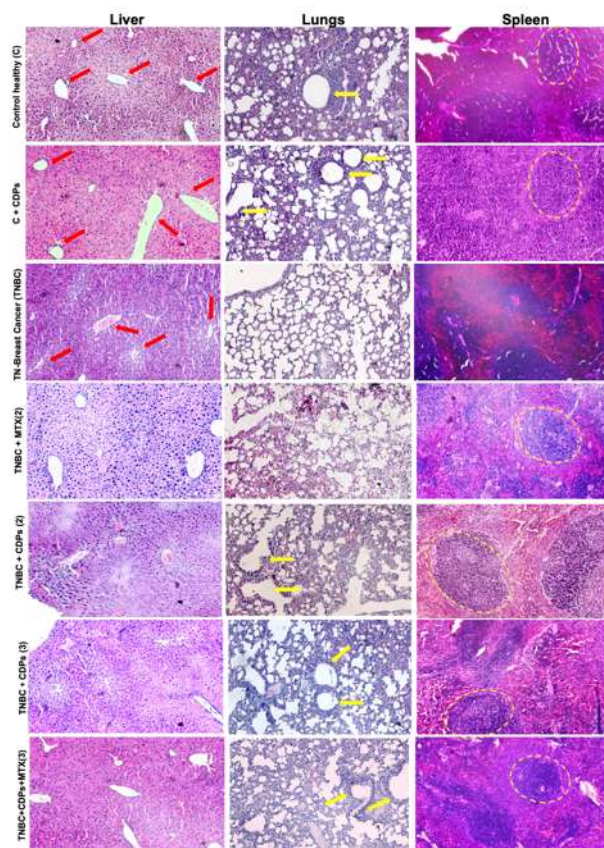


Figure 8. Histological features of the liver, lungs, and spleen of xenografted triple-negative breast cancer MDA-MB-231 cells in immunosuppressed mice treated with bacterial CDPs. Female BALB/c nu/nu mice (8 weeks old) were subjected to an anti-tumor study and analyzed at experimental endpoints of 35 or 70 days ($n = 5$ per group). Organ tissue sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Representative images of tissues dissected from the animal groups are shown. The blood conductors (red arrows), alveolar structures (yellow arrows), and splenic cellular structures (dashed circles) are indicated in the histology images.

2.7. Gene Expression in the Liver of Xenografted TNBC MDA-MB-231 Line Treated with Bacterial CDPs

The PI3K/Akt/mTOR and FOXO1 signaling pathways play crucial roles in fundamental cellular processes such as development, metabolism, and cell survival. Their dysregulation contributes to systemic changes associated with tumors. Based on our *in vitro* and *in vivo* results showing that CDP treatment modulates Akt, Gab1, and FOXO1 in the orthotopic TNBC xenograft mouse model, we aimed to further investigate the molecular mechanism behind CDP-mediated tumor inhibition. To do this, we examined the regulation of genes involved in pathways like cell proliferation, metastasis, apoptosis, immune response, and drug resistance. Gene expression analysis was carried out using RT-qPCR on liver tissues from healthy mice, untreated TNBC mice, and TNBC mice treated with CDPs, methotrexate (MTX), or their combination (Figure 9). The tumor suppressor genes PTEN and CDKN1A (p21), along with the chemokine CXCL12, were significantly upregulated in the livers of TNBC-bearing mice compared to healthy controls. Notably, their expression was substantially higher with CDP treatment, especially in combination with MTX (Figure 9). Conversely, ZEB1 showed no significant differences among the experimental

groups. Interestingly, genes such as BRCA1, GADD45A, PD-L1, and SNAIL were markedly elevated in the livers of TNBC mice compared to healthy controls, but their expression decreased notably with CDP and MTX treatments, sometimes reaching levels similar to those in healthy mice (Figure 9).

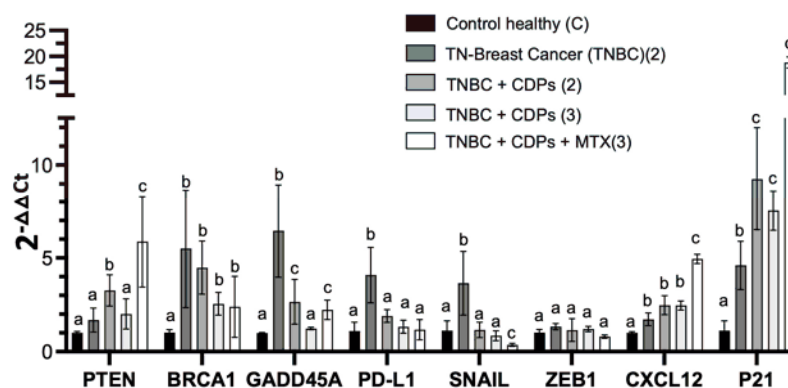


Figure 9. Hepatic gene expression analysis in an MDA-MB-231 xenograft mouse model of triple-negative breast cancer treated with bacterial CDPs. Relative expression levels of PTEN, BRCA1, GADD45A, PD-L1, SNAIL, ZEB1, CXCL12, and CDKN1A (p21) were determined in liver tissue by RT-qPCR. Gene expression was normalized to the housekeeping gene ACTB (β -actin), and relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Experimental groups are defined as described in Figure 4. The bars indicate the means $\pm$ SE; $n = 3$ per group. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. SE values are shown as bars; within each gene, groups sharing the same lowercase letter are not significantly different from each other, whereas groups with different letters indicate statistically significant differences ($p < 0.05$).

3. Discussion

CDPs have become promising options for cancer treatment due to their stability, high selectivity, and ability to block key cancer signaling pathways [11]. Their cytotoxic and pro-apoptotic effects have been shown in multiple cancer cell lines [3,5–8]. Beyond their direct anti-proliferative activity, several diketopiperazines have been shown to interfere with mechanisms associated with tumor aggressiveness and therapeutic resistance. For example, fumitremorgin C analogs can overcome multidrug resistance by inhibiting the breast cancer resistance protein (BCRP), a key efflux transporter that limits intracellular drug accumulation. Similarly, the HLY838 diketopiperazine functions as an O-GlcNAc transferase (OGT) inhibitor, enhancing the anti-tumor effects of CDK9 inhibitors by downregulating c-Myc and E2F1 [12].

Additionally, Verticillin A inhibits c-Met phosphorylation and the downstream Ras/Raf/MEK/ERK signaling pathway, resulting in reduced metastatic potential in colon cancer models [13]. Together, these studies highlight the potential of CDPs and related diketopiperazines to serve as multitarget anticancer and anti-metastatic agents. Consistent with this, earlier research in mouse melanoma showed that CDPs from *Pseudomonas aeruginosa* significantly decreased key EMT markers, including MMP-1, E-cadherin, N-cadherin, HIF-1 α , Vimentin, and CK-1, supporting their ability to inhibit tumor cell migration and invasion [6,10]. These observations support investigating the anti-invasive and metastasis-related effects of bacterial CDPs in aggressive breast cancer subtypes like triple-negative breast cancer.

In this study, functional assays further confirm the ability of bacterial CDPs to suppress invasion and metastasis-associated behaviors in the TNBC model. Wound-healing assays demonstrated that CDPs inhibit the migratory capacity of MDA-MB-231 cells more

effectively than methotrexate (MTX) (Figure 1a). Similarly, transwell invasion assays show that CDPs significantly reduce the invasive ability of MDA-MB-231 cells under both mono-culture and macrophage co-culture conditions compared to MTX, with their combination being even more effective. Tumor-associated macrophages (TAMs) play a key role in tumor progression by secreting chemokines and growth factors that enhance cancer cell proliferation, migration, and invasiveness [14]. Our data indicate that CDPs interfere with TAM–cancer cell interactions in the MDA-MB-231 cell line, highlighting their dual role in promoting apoptosis and reducing metastasis-related processes. This supports their function as inhibitors of tumor cell migration and invasiveness. The data are particularly relevant because the interaction between cancer cells and macrophages, along with the release of chemokines, enhances malignancy and the invasive capacity of cancer cells. The greatest inhibition observed with the combination of CDPs and MTX (up to 90% reduction in invasiveness) suggests a synergistic effect between these agents.

Three-dimensional spheroid models further demonstrated the anti-metastatic and anti-tumor effects of CDPs. Spheroid cultures are enriched with cancer stem cell (CSC)-like populations that exhibit increased self-renewal and resistance to therapy [14]. Therefore, the spheroid structure in MDA-MB-231 cells promotes resistance to cytotoxic compounds. Our results showed that CDPs significantly reduced spheroid size and number, lowered cell viability, and increased apoptosis in MDA-MB-231 spheroids compared to MTX-treated cultures. The effects were more pronounced when using the CDPs + MTX combination or cyclodextrins (Figure 1c–e). Additionally, the spheroid integration assay, which mimics three-dimensional tumor growth and plasticity, supports the ability of CDPs to disrupt key stages of tumor progression and stemness-related properties. These findings highlight the potential of CDPs to overcome CSC-associated resistance mechanisms in aggressive breast cancer models.

Mechanistically, our results further support previous findings that indicate CDPs act through the PI3K/Akt/mTOR pathway [3,5,6,10]. In MDA-MB-231 cells, CDPs significantly decreased phosphorylation of Akt, mTOR, and S6K (Figure 2), as well as reducing levels of Vimentin and Gab1—two proteins closely linked to EMT-related and metastasis-associated signaling [15]. Thus, CDPs effectively target the PI3K/Akt/mTOR signaling pathway, a key regulator of cell survival, proliferation, and growth. This aligns with the observed suppression of p-S6K phosphorylation and the decrease in Vimentin and p-Gab1 expression. Vimentin is an essential marker of EMT, a process crucial for invasiveness and metastasis. Therefore, the downregulation of Vimentin and inhibition of the PI3K/Akt/mTOR pathway are primary molecular mechanisms responsible for the reduced migration and invasiveness observed *in vitro* in MDA-MB-231 cells.

Beyond protein-coding genes, growing evidence highlights the role of non-coding RNAs (ncRNAs), including lncRNAs and miRNAs, as key regulators of EMT, metastasis, and therapeutic resistance in breast cancer. Our previous transcriptomic analysis of bacterial cyclodipeptides (CDPs) in the HeLa cell line revealed modulation of transcription factors and regulatory genes involved in epigenetic control and the regulation of miRNAs, circRNAs, and lncRNAs, suggesting that ncRNA-mediated mechanisms may contribute to the anticancer effects of CDPs [9]. Although ncRNAs were not directly examined in this study, the observed suppression of the PI3K/Akt/mTOR/FOXO1 pathway and EMT-related markers aligns with signaling pathways known to be regulated by metastasis-associated lncRNAs. Notably, MALAT1 has been shown to promote EMT, activate PI3K/Akt/mTOR signaling, and inhibit FOXO1-mediated tumor suppressive functions in breast cancer [16]. In this context, the coordinated suppression of Akt phosphorylation, EMT markers, and FOXO1 inactivation after CDP treatment may indicate the involvement of molecular pathways controlled by MALAT1 and related ncRNAs.

Meanwhile, emerging therapies focus on targeting metastasis-related non-coding RNAs with advanced delivery systems. Notably, exosome-mediated siRNA delivery has been shown to inhibit postoperative breast cancer metastasis by disrupting pro-metastatic signaling and pre-metastatic niche formation in triple-negative breast cancer models [17]. Compared to earlier research, the current findings indicate that CDPs might have anti-metastatic effects by indirectly affecting signaling pathways often regulated by ncRNAs, such as the PI3K/Akt/mTOR/FOXO1 pathway, without requiring complex delivery methods.

In the orthotopic TNBC xenograft model, implanting MDA-MB-231 cells directly into the mammary gland of animals creates a tumor microenvironment that exhibits key features of aggressive breast cancer progression. Importantly, before assessing antitumor efficacy, histopathological evaluation of major organs combined with continuous monitoring of body weight showed no evidence of systemic toxicity related to CDP treatment, supporting a favorable *in vivo* safety profile (Figures 8 and S1). Treatment with CDPs significantly reduced tumor volume and weight, especially when administered after the tumor was established, and many treated animals showed no detectable tumors at the end of the experiment (Figure 4). Besides suppressing primary tumor growth, CDP treatment also alleviated tumor-associated systemic changes. Untreated TNBC-bearing mice had increased weights of the lungs, kidneys, liver, and spleen, along with histological changes such as metastatic foci indicating organ damage caused by the tumor (Figure 4). Conversely, groups treated with CDPs and the combination of CDPs plus MTX showed normalization of organ weights and no visible metastatic foci. Histopathological analysis of primary tumors revealed that untreated TNBC displayed extensive hyperchromasia. In contrast, CDP-treated tumors showed less hyperchromasia, with areas of cell aggregation observed (Figure 6). These findings suggest that in tumors from animals treated with CDPs and MTX, cell proliferation is inhibited, as indicated by the size and weight of the tumors dissected from each group (Figure 4).

Metastatic progression in breast cancer patients is often linked to systemic changes, including increased levels of serum ALT, AST, gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), and LDH [18]. In our TNBC model, untreated mice showed significant increases in AST and LDH (Figure 5). Treatment with CDPs normalized these enzyme levels and reduced organ weight gains. Changes in leukocyte profiles, especially a higher neutrophil-to-lymphocyte ratio (NLR), are recognized markers of poor prognosis in breast cancer [19]. In our study, TNBC mice exhibited the highest NLR, which was reduced in the CDP-treated groups, approaching control levels (Figure 5c), indicating decreased tumor aggressiveness. Anemia, another common cancer complication, was also improved with CDP treatment. These findings suggest that CDPs mitigate tumor-related systemic and liver dysfunction, supporting a reduction in tumor aggressiveness and metastatic potential.

It is important to emphasize the safety profile and systemic benefits. CDP treatment did not change the activity of liver enzymes ALT, AST, and LDH in healthy control mice (C + CDPs). Conversely, CDPs reversed the significant increase in AST and LDH activity observed in the TNBC group. These results, along with the restoration of histological structures in the lung and spleen, show that CDPs not only target the tumor locally but also help normalize tumor-related systemic changes. Histological analysis revealed a clear reduction in vascular structures in the liver of the TNBC group. In contrast, structural damage was observed in the lung and spleen tissues, which was significantly reduced after CDP treatment (Figure 8). These findings further suggest that, in our model, metastasis of the MDA-MB-231 line occurred in the animals following implantation into target organs such as the liver, lungs, and spleen. The ability of CDPs to restore normal tissue architecture supports their role in reducing tumor-related organ dysfunction and decreasing metastatic risk.

At the molecular level, protein and gene expression analyses revealed coordinated modulation of signaling pathways involved in metastasis, immune evasion, and DNA damage response. To further explore tumor-associated signaling in TNBC, we assess metastasis in the tumor of the xenografted TNBC model. Molecular analyses also showed reduced p-Akt levels in tumors from CDP-treated animals, along with decreased total Akt expression (Figure 7), indicating suppression of the PI3K/Akt/mTOR signaling pathway. FOXO1, a forkhead transcription factor that regulates apoptosis, autophagy, and cell cycle arrest, is negatively regulated by Akt-mediated phosphorylation [20]. In our study, CDP-treated animals showed decreased FOXO1 phosphorylation. Although total FOXO1 protein levels were reduced, the decrease in FOXO1 phosphorylation corresponds with increased FOXO1 activity, allowing its nuclear functions and the activation of growth-suppressive and stress-response pathways [21]. This activation aligns with the induction of apoptotic pathways. These findings match our previous transcriptomic data in HeLa cells, showing CDP-mediated upregulation of FOXO1-related genes like GADD45A and SGK1 [9].

Gab1, an adaptor protein that mediates signals for proliferation, angiogenesis, and invasion [22], was overexpressed and phosphorylated in TNBC tumors but was down-regulated by CDPs, especially when combined with MTX (Figure 7). This indicates that inhibiting Gab1 is a key mechanism behind the suppression of invasion- and metastasis-related processes caused by CDPs. Protein analysis in the xenografted tumors confirmed the inhibition of the Akt pathway. Consistent with the *in vitro* results, the expression of phosphorylated Akt protein was reduced in the CDP-treated groups, as Gab1, p-Gab1, FOXO1, and its phosphorylated form p-FOXO1 were markedly decreased in tumors from mice treated with CDPs and MTX.

The study of gene expression in the liver provided crucial data on tumor-related systemic changes influenced by the CDPs. To further explore the metastasis-related signaling pathways targeted by the CDPs, we analyzed the hepatic expression of gene markers associated with multiple pathways involved in tumor progression, immune regulation, and cellular plasticity (Figure 9). RT-qPCR analysis showed that CDP treatment significantly reversed the overexpression of key genes seen in the TNBC group, including SNAIL, which is linked to promoting EMT, invasiveness, metastasis, and drug resistance. Among immune checkpoint components, such as PD-L1, which contributes to immune evasion, overexpression was observed in the TNBC group and returned to normal levels after CDP treatment, approaching those seen in healthy controls. Regarding genes involved in DNA damage/repair and the cell cycle—such as BRCA1 and GADD45A, which exhibited a marked increase in the TNBC group consistent with tumor-induced systemic stress and genomic instability—these were normalized by CDP treatment. Conversely, genes like PTEN, CXCL12, and CDKN1A (p21) were overexpressed in the livers of animals treated with both CDPs and MTX compared to untreated healthy mice, indicating activation of compensatory tumor-suppressive and cell cycle-regulatory mechanisms related to EMT, invasiveness, metastasis, and drug resistance, reflecting an adaptive hepatic response aimed at reinforcing immune regulation, growth suppression, and homeostasis despite tumor burden. Overall, these findings suggest that bacterial CDPs in the TNBC model modulate tumor-associated systemic gene expression programs related to DNA damage responses, immune regulation, and cellular stress, thereby affecting molecular mechanisms connected to tumor aggressiveness and metastatic progression. This modulation targets both tumor cell-intrinsic signaling pathways and systemic responses linked to the tumor. The inhibition of Akt/mTOR/S6K signaling, suppression of EMT markers, modulation of immune-related genes, and the favorable safety profile collectively position CDPs as promising therapeutic agents. Moreover, the enhanced efficacy observed when combining

CDPs with MTX supports the potential of combination strategies to improve treatment outcomes in aggressive breast cancer subtypes.

In summary, in the TNBC mouse model, CDP administration significantly reversed primary tumor growth in the mammary glands, with greater effectiveness when combined with MTX. Beyond local tumor control, CDP treatment reduced systemic changes associated with tumors in distant organs, such as the liver, which are commonly involved in metastatic progression. The treatment with CDPs reduced metastatic foci in organs such as the liver. At the molecular level, CDPs modulated the expression of genes linked to tumor aggressiveness, immune regulation, and stress responses, such as SNAIL, GADD45A, and PD-L1, while increasing the expression of growth-suppressing and homeostatic regulators, including PTEN, CXCL12, and CDKN1A (p21). The data provide new insights into the molecular mechanisms behind the anti-metastatic and anti-invasive effects of CDPs, showing that these effects are connected to genes involved in EMT, invasiveness, and metastasis. This indicates that the signal transduction mechanism involves inhibition of phosphorylation in the Akt/mTOR/S6K pathway and of metastasis markers, such as Vimentin, Gab1, and FOXO1. The findings suggest that combining CDPs with MTX enhances the anti-metastatic effect in a TNBC xenograft model using the MDA-MB-231 line, indicating that bacterial CDPs could be a promising therapeutic agent. In conclusion, bacterial cyclic peptides represent a promising multi-target therapy for TNBC, showing greater efficacy than MTX *in vivo*. Their mechanism involves blocking the PI3K/Akt/mTOR/FOXO1 pathway and suppressing EMT, resulting in strong anti-tumor and anti-metastatic effects with a good safety profile.

4. Materials and Methods

4.1. Chemicals, Reagents, and Cell Culture

The chemicals and reagents used include Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), fetal bovine serum (FBS; Gibco Life Technologies, Grand Island, NY, USA), and trypsin solution (Sigma-Aldrich). A mixture mainly composed of cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Leu), and cyclo(L-Pro-L-Phe) is isolated from the cell-free supernatant of *Pseudomonas aeruginosa* PAO1 bacteria, with a purity of over 95% [23,24]. This mixture is dissolved in a DMSO-water ratio of 1:3 to prepare stock solutions (100 mg/mL). The MDA-MB-231 breast tumor cell line was obtained from invasive ductal carcinoma (ATCC, Manassas, VA, USA) and cultured in complete media [DMEM supplemented with 10% (*v/v*) FBS, 100 units/mL penicillin, 40 µg/mL streptomycin, and 1 µg/mL amphotericin B (Sigma-Aldrich)], with 1.6 g/L glucose added. Cell culture media were changed twice a week, with cells incubated at 37 °C, 80% humidity, and 5% CO₂. Cells were then trypsinized and counted using a hemocytometer. All cell cultures and procedures were performed in class II biological safety cabinets.

4.2. Cell Viability and Apoptosis Determination

Cell viability was determined with the MTT method. Briefly, cell cultures were grown in 96-well flat-bottomed plates in DMEM medium containing FBS for 24 h, then incubated with CDPs for 24 h at 37 °C with 5% CO₂. MTT (50 mg/mL) in PBS was added to each well, and the mixture was incubated for 4 h at 37 °C. Finally, 100 µL of 2-propanol/1M HCl (19:1, *v/v*) was added to dissolve the formazan crystals, and the absorbance was measured at 595 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA).

Necrosis and apoptosis were evaluated in cell cultures incubated in DMEM medium with FBS for 4 h with CDP treatment. Following incubation, cells were collected by centrifugation at 2000× *g* for 10 min. The pellet was suspended in 20 µL and incubated with annexin V and propidium iodide (PI) (Molecular Probes, Invitrogen, Life Technologies,

Carlsbad, CA, USA). Fluorescence was immediately quantified by FACS using an Accuri-C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA). At least 20,000 cellular events were used for calculations.

4.3. Wound Closure Migration Assay

The MDA-MB-231 cell line was grown to 95% confluence, and three wounds were created per plate using a sterile pipette tip. All assays were performed in triplicate. After wounding, plates were washed twice with phosphate-buffered saline (PBS) and replenished with fresh complete medium. Cells were then treated with CDPs (0.01 mg/mL), methotrexate (MTX; 0.05 mg/mL), or their combination for 48 h. Representative images were taken every 24 h, and wound dimensions were quantified using ImageJ 1.53k software (NIH, Bethesda, MA, USA).

4.4. Invasion Assay

The invasive capacity of the MDA-MB-231 cell line was evaluated using a transwell chamber pre-coated with Matrigel (Corning Life Sciences, Union City, CA, USA). Cells were seeded in the upper chamber in complete DMEM medium. For co-culture experiments, RAW 264.7 macrophages were seeded in the lower chamber containing complete DMEM medium. Cells were treated with CDPs (0.01 mg/mL) or methotrexate (MTX; 0.05 mg/mL) incubated by 24 h at 37 °C with 5% CO₂. Inserts were then briefly washed with PBS, and cells that had invaded through the Matrigel and adhered to the lower surface of the membrane were fixed with 70% ethanol for 15 min at room temperature. Fixed cells were stained with 0.2% (w/v) crystal violet for 5 min, washed thoroughly with PBS to remove excess dye, and air-dried. Invaded cells were visualized by optical microscopy [25].

4.5. Multicellular Spheroids

MDA-MB-231 cell line (2×10^5 cells) was cultured under non-adherent conditions using 0.6% agarose in complete DMEM medium to generate multicellular spheroids. Cultures were maintained for 14 days with periodic medium replacement until the spheroids reached a diameter of 40–50 µm [14]. Spheroids were then treated with CDPs (0.1 mg/mL) or methotrexate (MTX; 0.05 mg/mL) and incubated for 4 h, a time point selected to correspond with the determination of lethal (LD₅₀) and apoptotic effective (ED₅₀) doses. Following treatment, spheroids were visualized by optical microscopy, and cell viability was assessed using the MTT assay.

4.6. Western Blot Assays

Confluent MDA-MB-231 cells were treated with CDPs (0.1 mg/mL) and incubated for 15 min, 1 h, and 4 h. Cells were then harvested by trypsinization, washed twice with PBS, and centrifuged at $5000 \times g$ for 10 min at 4 °C. Cells pellets or tumor tissue were resuspended in RIPA lysis buffer, and lysed by three cycles of low-intensity sonication (20 kHz, 5 W, 30 s each) at 4 °C using an Hielscher LS24 ultrasonic processor. The protein extract free of cellular debris was obtained by centrifugation at $7500 \times g$ for 15 min, and the protein concentration was determined by the Bradford method (BioRad, Hercules, CA, USA). Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA) using a Bio-Rad transfer system at 15 volts for 45 min. Membranes were blocked with TBS-T (Tris-HCL 10 mM; NaCl 0.9%; tween-20 0.1%, dry milk 5%, pH 7.8), washed three times for 6 min each, and incubated overnight at 4 °C with primary antibodies diluted in TBS-T (1:5000). The following primary antibodies were used: anti-Akt (cat. 2938), anti-p-Akt (cat. 4060), anti-mTOR (cat. 2983), anti-p-mTOR (cat. 5536), anti-S6K (cat. 2708), anti-p-S6K (cat. 9234), anti-Vimentin (cat. 3932); anti-Gab1 (cat. 3232), anti-p-Gab1 (cat. 3234), anti-FOXO1 (cat. 2880), anti-p-FOXO1

(cat. 9464), and anti- β -actin (cat. 4967) (Cell Signaling Technology, Boston, MA, USA and Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washes with TBS-T (6 min each), membranes were incubated with HRP-conjugated secondary antibody (BioRad) diluted 1:10,000 in TBS-T for 2 h at room temperature. Immunoreactive bands were detected using SuperSignal™ West Pico chemiluminescent substrate (Pierce; Thermo Fisher Scientific, Waltham, MA, USA) and visualized with a ChemiDoc™ MP imaging system (Bio-Rad). Assays were conducted at least three times, and representative images are shown. ImageJ (NIH) was used to quantify image band intensities.

4.7. Xenografted Metastatic Breast Cancer Model

Immunosuppressed female BALB/c nu/nu mice (8 weeks old) were acclimated for 15 days in a pathogen-free environment with a standard diet and water provided *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) from the Universidad Michoacana de San Nicolás de Hidalgo (IIIQB-UMSNH-IACUC-2022-35). Experiments complied with standard guidelines for the welfare of animals following the Institutional Committee and recommendations of the Mexican Official Regulations for the Use and Care of Animals (NOM 062-ZOO-1999; Ministry of Agriculture, Mexico).

Once the acclimation period concluded with 100% of animals survival, mice were orthotopically implanted with 1×10^5 MDA-MB-231 metastatic breast cancer cells suspended in Matrigel and injected into the mammary fat pad adjacent to the right axillary region. Animals were anesthetized by intraperitoneal administration of ketamine (80 mg/kg) and xylazine (10–15 mg/kg). Body weight was monitored throughout the experiment, and tumor dimensions were measured with a caliper. Tumor volume (TV) was calculated using the formula $TV = (0.4)(ab^2)$, where “a” represents the longest tumor diameter and “b” the shortest diameter. In addition, a pilot study was carried out to select the doses of CDPs to be tested in the TNBC mice model; thus, we decided to use 0.25 mg/kg of mouse body weight. Treatments were administered intraperitoneally and consisted of CDPs (0.25 mg/kg), methotrexate (MTX; 0.05 mg/kg), or a combination of CDPs and MTX (0.25 mg/kg CDPs + 0.05 mg/kg MTX). The CDP treatments involved three rounds of administration, each consisting of three doses given every 3 days (with a 2-day rest between doses), and a week of rest between rounds, totaling 9 doses per mouse. The animal groups, each consisting of five mice ($n = 5$) as follows (Figure 3): healthy mice without treatment (C)—procedure 1; healthy mice treated with CDPs (C + CDPs)—procedure 1; mice with TNBC tumors without treatment (TNBC)—procedure 2; mice with tumors treated with CDPs starting from cell inoculation in early stage (TNBC + CDPs (2))—procedure 2; mice with tumors treated with CDPs at the 35th day post-inoculation in advanced stage (TNBC + CDPs (3))—procedure 3; mice with advanced-stage tumors treated with MTX (TNBC + MTX (3))—procedure 3; and mice with tumors treated with a combination of CDPs and MTX (TNBC + CDPs + MTX (3))—procedure 3.

4.8. Animal Euthanasia and Biological Sample Collection

All mice were euthanized by intraperitoneal administration of a lethal dose of sodium pentobarbital (100–150 mg/Kg body weight), as recommended by IIQB/UMSNH-IACUC and NOM 062-ZOO-1999. Following confirmation of death, tumors were excised, and whole blood was collected by cardiac puncture into microtubes containing EDTA as an anticoagulant. Major organs were subsequently harvested and weighed.

Blood hemoglobin was determined using heparinized capillary tubes by centrifugation at 3500 rpm for 5 min to separate red blood cells from plasma. Subsequently, serum biochemical parameters, including aspartate aminotransferase (AST), alanine aminotrans-

ferase (ALT), and lactate dehydrogenase (LDH), were quantified using a Fuji Dry-Chem NX700 (Fujifilm, Santa Ana, CA, USA).

4.9. Histological Analysis of Tissues

After euthanasia, tumors, liver, lungs, and spleen were excised and processed for histological evaluation. Tissues were fixed in 4% paraformaldehyde and remained until dehydration. The samples were subjected to a 4 h dehydration process at varying ethanol and xylene concentrations, then embedded in paraffin. Paraffin-embedded tissues were sectioned at a thickness of 4 μ m, mounted on glass slides, and stained with hematoxylin and eosin (H&E). Photographs were taken under an optical microscope and recorded using an Accu-Scope EXC-120 LED microscope camera at magnifications of 4 $\times$, 10 $\times$, and 40 $\times$.

4.10. RNA Extraction and RT-qPCR

Total RNA from liver samples of each animal group was pooled, homogenized using a tissue homogenizer (Biospec Products, Bartlesville, OK, USA), and extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's protocol. The RNA concentration and quality were assessed using a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific). According to the manufacturer's instructions, complementary DNA (cDNA) was synthesized using a QuantiNova Reverse Transcription Kit (Bio-Rad). Quantitative PCR (qPCR) was performed using the Quantinova SYBR Green PCR Kit (Bio-Rad) in a reaction volume of 10 μ L. The thermocycling conditions were set as follows: initial denaturation at 95 $^{\circ}$ C for 10 min, followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 5 s and annealing/extension at 60 $^{\circ}$ C for 30 s and extension to 68 $^{\circ}$ C for 30 s using a thermocycler (QuantStudio 3; Applied Biosystems by Thermo Fisher Scientific). Relative gene expression levels was calculated using the comparative $2^{-\Delta\Delta C_t}$ method, normalizing to the internal reference gene *ACTB* (β -actin). All experiments were conducted in triplicate. The sequences of the gene-specific primers are provided in Table S1.

4.11. Statistical Analysis

All quantitative data are presented as mean $\pm$ standard error (SE). Statistical analyses were performed using one-way analysis of variance (ANOVA) when comparing more than two experimental groups. When ANOVA indicated significant differences, Tukey's post hoc multiple comparison test was applied to identify statistically significant pairwise differences between groups. A p -value < 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism version 6.0.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules31030543/s1>, Figure S1: The mouse body weight was monitored during the therapeutic experimental outline study; Table S1: Oligonucleotides used in this study.

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CAPÍTULO II. ARTÍCULO EN COLABORACIÓN: El receptor HGF/Met media el efecto citotóxico de ciclodipéptidos bacterianos en células humanas de cáncer cervical

The HGF/Met Receptor Mediates Cytotoxic Effect of Bacterial Cyclodipeptides in Human Cervical Cancer Cells

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Abstract: Background: Human cervix adenocarcinoma (CC) caused by papillomavirus is the third most common cancer among female malignant tumors. Bioactive compounds such as cyclodipeptides (CDPs) possess cytotoxic effects in human cervical cancer HeLa cells mainly by blocking the PI3K/Akt/mTOR pathway and subsequently inducing gene expression by countless transcription regulators. However, the upstream elements of signaling pathways have not been well studied.

Methods: To elucidate the cytotoxic and antiproliferative responses of the HeLa cell line to CDPs by a transcriptomic analysis previously carried out, we identified by immunochemical analyses, differential expression of genes related to the hepatocyte growth factor/mesenchymal-epithelial transition factor (HGF/MET) receptors. Furthermore, molecular docking was carried out to evaluate the interactions of CDPs with the EGF and MET substrate binding sites.

Results: Immunochemical and molecular docking analyses suggest that the HGF/MET receptor participation in CDPs cytotoxic effect was independent of the protein expression levels. However, protein modulation of downstream Met-targets occurred due to the inhibition of phosphorylation of the HGF/MET receptor. Results suggest that the antiproliferative and cytotoxicity of CDPs in HeLa cells involve the HGF/MET receptor upstream of PI3K/Akt/mTOR pathway; assays with the human breast cancer MCF-7 and MDA-MB-231 cell lines supported the finding.

Conclusion: Data provide new insights into the molecular mechanisms involved in CDPs cytotoxicity and antiproliferative effects, suggesting that the signal transduction mechanism may be related to the inhibition of the phosphorylation of the EGF/MET receptor at the level of substrate binding site by an inhibition mechanism similar to that of Gefitinib and Foretinib anti-neoplastic drugs.

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1. INTRODUCTION

The incidence of human cervical cancer (CC) is the fourth leading cause of cancer death among women worldwide, just below breast, lung, and colorectal cancers [1, 2]. Furthermore, CC is the cause of death most common among female malignant tumors and second in America [3, 4]. In normal cervical epithelium, CC arises through the progression of the disease for years, where the human papillomavirus (HPV) infection occurs in the host genome, silencing tumor-suppressor factors and induction of the tumor-

promoting factors [5-7]. These oncogenic factors drive neoplastic progression and severity towards the development of CC, which is dependent on HPV subtypes [8]. The HPV oncoproteins E5, E6, and E7 are important drivers of oncogenesis in normal cervical epithelium [9, 10].

In the search for novel natural molecules with anticancer activity, bacterial cyclodipeptides (CDPs) have attracted interest due to their antiproliferative and cytotoxic effects on cancerous cell lines [11-17]. CDPs have intrinsic physicochemical advantages such as higher stability, protease resistance, and conformational rigidity compared to other biomolecules. All these factors increase their ability to interact with biological targets associated with the disease, turning them into therapeutic candidates than their linear counterparts [18, 19]. Our research group reported that CDPs isolated from *Pseudomonas aeruginosa* PAO1 bacterium com-

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posed mainly of cyclo (L-Pro-L-Tyr), cyclo (L-Pro-L-Val), and cyclo (L-Pro-L-Phe) [20], suppress the proliferation of human adenocarcinoma cell lines: HeLa and CaCo-2 [17]; these CDPs were proposed to have a robust pharmaceutical potential for cancer treatment [12]. In HeLa cells, CDPs arrest the cell cycle at the G0–G1 transition by blocking the PI3K/Akt/mTOR pathway, inhibiting the mTORC1/mTORC2 complex in a TSC1/TSC2-dependent manner, leading to inhibit the phosphorylation of both Akt-S473 and S6k-T389 protein kinases [11, 21–23]. In addition, CDPs blocked protein kinases from multiple signaling pathways involved in survival, proliferation, invasiveness, apoptosis, autophagy, and energy metabolism, such as Ras/Raf/MEK/ERK1/2, PI3K/JNK/PKA, p27Kip1/CDK1/survivin, MAPK, HIF-1, Wnt/ β -catenin, EMT, and CSCs [11, 24]. Thus, the antiproliferative effects of the bacterial CDPs aided in identifying the crosstalk of the signaling pathways involved in HeLa cell malignancy, suggesting that they may be considered as potential anti-neoplastic drugs in human cervical adenocarcinoma therapy [11, 24]. However, there is scarce knowledge of the PI3K/Akt/mTOR upper pathway's participation in the cytotoxic effect of CDPs, such as the recognition path by external receptors, *i.e.*, hepatocyte growth factor/mesenchymal-epithelial transition factor (HGF/MET) receptor, epidermal growth factor receptor (EGFR), or transforming growth factor receptor (TGF α).

The HGF/MET pathway is constituted by the Met tyrosine kinase receptor (MET) and hepatocyte growth factor (HGF), which govern the epithelial-mesenchymal transition (EMT) process [25, 26]. HGF/MET mutations and upregulation have been associated with diverse types of cancers, suggesting these components as therapeutic targets for cancer management protocols [27–29]; in addition, high expression of EGFR in tumors is correlated with poor prognosis of CC patients [26, 30–32]. Met signaling is usually activated by the binding of its HGF ligand, which results in HGF/MET dimerization and phosphorylation of two tyrosine residues (Tyr1234 and Tyr1235) located in the kinase catalytic domain [27, 33]. Subsequently, the signal is transduced into the binding site residues (Tyr1349 and Tyr1356), leading to the activation of phosphoinositide 3-kinase (PI3K), phospholipase C1 (PLC γ 1), growth factor receptor-bound protein 2 (GrB2), GrB2-associated binding protein 1 (GaB1), and signal transducer and activator of transcription 3 (STAT3) [33–35]. Significantly, MET mediates the activation of several intracellular signaling pathways, such as PI3K/Akt, STAT3, SRC/FAK, and MAPK/ERK. The ubiquitination of the phosphorylation residues (Tyr1003) in the JM domain of the Met receptor attenuates or finishes the activation of the pathway as one of the control mechanisms involved in survival, proliferation, migration, invasion, and metastasis [26, 36]. In this regard, hypoxia upregulation of HIF-1 α and VEGF-A expression has been described to promote invasion by enhancing tumor angiogenesis *via* dysfunctional HGF/c-MET signaling in HCC [30, 37].

Thus, the EGF/PI3K/AKT signaling pathway plays crucial roles both in normal cellular processes and in a wide

range of cancer cells, including carcinogenesis, proliferation, survival, metastasis, tumor microenvironment (TME) regulation, metabolism reprogramming, EMT, stemness, and drug resistance [38, 39]. Hence, overexpression of MET signaling is negatively associated with the clinical prognosis of numerous cancers such as CC [40, 41]. Collectively, HGF/MET-targeted therapy has led to breakthroughs in some cancers. Current and future efforts are underway to explore more options for HGF/MET-targeted therapy in cancer [30, 42]. Several lines of research in the past few decades have provided strong evidence for the essential role of MET in human cancers, including breast and cervical cancer [42–44].

Despite the biological effects presented by the bacterial CDPs on cancer cells, it is interesting to elucidate the mechanisms involved in these drugs' cytotoxic and antiproliferative effects. The present study aimed to evaluate the participation of upstream elements of the PI3K/Akt/mTOR signaling pathway in the cytotoxic effect of bacterial CDPs using mainly the HeLa cell line and two lines of breast cancer as study models.

2. MATERIALS AND METHODS

2.1. Chemicals, Reagents, and Cell Culture

Chemicals and reagents included are Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich), fetal bovine serum (FBS; Gibco Life Technology), and trypsin solution (Sigma Life Science). Cyclodipeptides were purified from *P. aeruginosa* PAO1 cells-free supernatant as previously described [45]. CDPs were dissolved in a DMSO-water ratio of 1:3 to prepare stock solutions (100 mg/mL). The HeLa cancer line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) [46]. MCF-7 is a human metastatic breast cancer cell line (adenocarcinoma) dependent on estrogen, progesterone, and glucocorticoid receptors (ATCC, Manassas, VA, USA) [47]. The MDA-MB-231 cell line isolated from a patient with invasive ductal carcinoma is commonly used to model late-stage breast cancer (ATCC, Manassas, VA, USA). This cell line is ER, PR, and E-cadherin negative and expresses mutated p53, lacking the growth factor receptor HER2, representing a good model of triple-negative breast cancer [48]. MDA-MB-231 cells, when implanted in xenografts, spontaneously metastasize to lymph nodes. Cells were cultured in complete media [DMEM supplemented with 10% (v/v) FBS, 100 units/mL of penicillin, 40 μ g/mL of streptomycin, and 1 μ g/mL of amphotericin B (Sigma-Aldrich), for MDA-MB-231 and MCF-7 cells cultures was supplemented with 1.6 g/L of glucose]. Cell culture media were changed twice a week, maintained at 37 °C under 80% humidity, and incubated in an atmosphere of 5% CO $_2$ to confluency. Cells were then trypsin-treated, counted using a hemocytometer chamber, and used for subsequent assays. Cell cultures and other procedures were performed in class II biological safety cabinets.

2.2. Cell Viability, Necrosis, and Apoptosis

Cell viability was determined colorimetrically with the MTT method [49]. Briefly, cells were seeded in 96-well flat-bottomed plates at a density of 3×10^4 cells per well in 200 μ L of CM medium and incubated for 24 h at 37°C with 5% CO₂ as described above. Then, cell culture media were removed and replaced with serum-free DMEM medium (DMEM). Following incubation in DMEM medium with FBS for 24 h, cells were incubated in the presence or the absence of the indicated amounts of CDPs for 24 h at 37°C with 5% CO₂. To determine cell viability, MTT, 50 mg/mL in PBS, was added to each well and incubated for 4 h at 37°C. Finally, 100 μ L of 2-propanol/1M HCl (19:1, v/v) was added to dissolve the formazan crystals, and the absorbance was measured at 595 nm using a microplate reader (BioTek Instruments).

Necrosis and apoptosis were evaluated in cell cultures incubated in DMEM medium with FBS for 4 h before treatment with CDPs. DMSO was used as a control at the same concentration used to dissolve the CDPs. Following incubation, cells were collected by centrifugation at $2,000 \times g$ for 10 min. The pellet was suspended in 20 μ L and incubated with annexin V and propidium iodide (PI) (Dead Cell Apoptosis Kit; Molecular Probes, Invitrogen Life Technologies). Fluorescence was immediately quantified by FACS using an Accuri-C6 Flow Cytometer (BD Biosciences). The percentages of fluorescent cells (PFC) and median fluorescence intensity (MFI) were determined from histograms of the fluorescence emission in the plots, labeled as PFC or relative fluorescence units. For apoptosis and necrosis assays, annexin V fluorescence was measured with the fluorescence channel FL1 at 495/519 nm and for propidium iodide in the FL2 channel at 535/617 nm [50]. At least 20,000 cellular events were used for calculations.

2.3. Western Blot Analysis

Proteins from HeLa, MCF-7, and MDA-MB-231 culture extracts were separated under denaturing conditions using polyacrylamide gel electrophoresis at 10–12% (SDS-PAGE) [51]. 30 μ g of protein extracts were typically loaded per lane. Protein samples were mixed with 10 μ L of denaturing buffer (Tris-HCl 0.06 M, pH 6.8, 5% glycerol, 4% SDS, 4% 2-mercaptoethanol and 0.0025% bromophenol blue) and incubated for 5 min at 95°C. Gels were stained with Coomassie blue, and proteins from replicate gels were transferred to polyvinylidene difluoride (PVDF, Millipore) membranes for immunodetection assays [52, 53]. Briefly, PVDF membranes were incubated with TBS-T (Tris-HCl 10 mM; NaCl 0.9%; tween-20 0.1%, dry milk 5%, pH 7.8). PVDF membranes were cut according to the range of molecular weight markers and incubated with the indicated antibodies at the concentration suggested by the manufacturer: anti-Met, anti-Met-phosphorylated (T-1003), anti-Met-phos-

phorylated (T-1234/T1235), anti-Met-phosphorylated (T-1349), anti-Gab1, anti-Gab1-phosphorylated (T-307), anti-EGF, anti-EGF (T-992), anti-EGF (T-1068), anti-EGF (T-1045), and anti- β -actin (all from Cell Signaling Technology). Following 12 h of incubation (4 °C) with the primary antibody, the membranes were washed and incubated with Goat anti-rabbit IgG HRP-conjugated (1:10,000) in a blocking solution for 4 h at 4 °C; the membranes were washed twice with TBS-T buffer and developed using hydrogen peroxide and Supersignal West Pico Luminol (Pierce, Thermo Fisher Scientific). Then, images were captured using a ChemiDoc™ MP System (Bio-Rad). Assays were conducted at least three times, and representative images were shown. The Image J software (NIH Image) was used to quantify band intensities in gel images.

2.4. Transcription Factor Enrichment Analysis using ChEA3 FT

The tool ChIP-X Enrichment Analysis 3 (ChEA3) consists of a transcription factor enrichment tool that ranks TFs associated with user-submitted gene sets. The ChEA3 background database includes a collection of gene set libraries generated from multiple sources, including TF–gene co-expression from RNAseq studies, TF–target associations from ChIP-seq experiments, and TF–gene co-occurrence computed from crowd-submitted gene lists. How the tool works: the first step is to conduct the Fisher's test to compute the exact p-value of the input list of differentially expressed genes with each target gene list in the ChEA3 database; the Fisher's test is a statistical overrepresentation test. Then, the resulting p values list is ranked, and ChEA3 outputs the transcription factor experiments with the lowest p value. Sequence enrichment results from these distinct sources are integrated to generate a composite rank that improves the prediction of the correct upstream TF compared to ranks produced by individual libraries [54].

2.5. Docking and Statistical Analysis

Data on human protein structures were obtained from the protein data bank [cMET (PDB: 6SD9) and EGF (PDB: 4WKQ)]. The three-dimensional models of CDPs used in the study were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>) and as previously described [45]. Docking analysis was performed using the software Autodock 4.2.5.1 (available at: <http://autodock.scripps.edu/>) [55]. After docking, one hundred conformations for each compound were obtained and then clustered for analysis using ADT 1.5.2 software. The conformations selected were within the most represented cluster and corresponded to those showing the lowest values of binding energy and *K_i*. Model analyses and figure drawing were done with PYMOL (The PyMOL Molecular Graphics System, Version 2.1.0, Schrödinger, LLC; <https://sourceforge.net/p/pymol>) [56].

Data were analyzed using GraphPad Prism 5 (GraphPad).

3. RESULTS

3.1. Network Analysis Revealed DEG Associations with the Neoplastic Phenotype

Our group previously described the connection of transcriptome changes, biological processes, and pathways, showing the global list of co-modified DEGs in the HeLa cell cultures with CDPs exposure [24]. Network analysis revealed that 125 DEGs have TF/target-gene associations, and the more enriched genes are related to the neoplastic phenotype. Crucial cancer-related processes, including cell cycle, apoptosis control, DNA damage, proliferation, transcriptional dysregulation of oncogenes, and tumor suppressors, are regulated by the TFs found [24].

The heatmap and STRING networks revealed a group of DEGs in CDPs-exposed HeLa cells, showing high frequency and significant differences in expression levels associated with the HGF/MET signaling pathway (Figs. 1a and b). In the constructed STRING networks, the HGF/MET-associated group contained DEGs, such as FOS, FOSB, BTG2, MAFF, SGK1, EGR3, GADD45A, DUSP1, DUSP8, BTG2, DDIT3, BCL2, TXNIP, BCL6, ID2, SMAD6, HES1, CTGF, AMOTL2, CCDC88A, COL6A1, *etc.* Upon deepening the identification of components of pathways and DEGs of different cancer-associated pathways, a refined heat map showed 41 DEGs in HeLa cells exposed to CDPs (Fig. 1b), especially DEGs involving the EMT pathway were revealed. In this context, biomarkers for CC, including APC, BCL2, FOS, FRAT2, FZD4, GADD45A, GSTA2, and HES1 were identified [57, 58].

Analysis of the differentially expressed genes using an established computational ChEA3 TF tool [54], that offers a better understanding of gene regulatory networks provided an interactive clustergram overlapping the top 20 DEGs; in addition, the STRING network [59] showed the top prioritized DEGs along the overlapping genes modified by CDPs exposure related with the HGF/Met signaling pathway (Figs. 1c and d). These findings led us to study the participation of upper elements, such as the HGF/MET receptor, in the transduction of signals and gene expression modifications associated with bacterial CDPs effects in HeLa cells.

3.2. The Participation of MET/EGF Receptors in the Activation Mechanism and Modulation of Gene Expression in Cancer Cells by CDPs.

Immunoblotting analysis showed the expression of both Met and EGF proteins in HeLa cells, but the expression was not modified by CDPs-exposure (Fig. 2). However, phosphorylation on the Met-Tyr¹⁰⁰³, Met-Tyr^{1234/1235}, and Met-Tyr¹³⁴⁹ were significantly decreased by CDPs-exposure (0.1 mg/mL) in a time-dependent manner. In addition, the Gab1-Tyr³⁰⁷ also showed a significant decrease in signal phosphorylation, but no change was observed in the total protein expres-

sion of Gab1 (Fig. 2).

Concerning the EGF receptor, we found that CDPs-exposure in HeLa cells decreased the phosphorylation of residues Tyr⁹⁹² and Tyr¹⁰⁶⁸, but no changes were observed in residue Tyr¹⁰⁴⁵ or in the EGF total protein expression (Fig. 2). These results evidenced the participation of MET/EGF receptors in the activation mechanism and modulation of gene expression of downstream elements as a result of the cytotoxic effect of CDPs on HeLa cells, whose modulation was at the level of tyrosine residues phosphorylation.

Additionally, the cytotoxic and apoptotic effects of CDPs from *P. aeruginosa* PAO1 were tested in other cancer cell lines. The impact of exposure to these compounds on the MCF-7 line of human adenocarcinoma and the MDA-MB-231 line of human triple-negative breast cancer was evaluated. As described previously, in the HeLa cell line, the CDPs exposure (0.1 mg/mL) was cytotoxic for both the MCF-7 and MDA-MB-231 lines in a time-dependent manner (Fig. 3a). MCF-7 and MDA-MB-231 cell cultures showed 60% and 75% dead cells with CDPs at 1 mg/mL after 4 h of treatment, rendering an LD₅₀ of 0.05 mg/mL and 0.01 mg/mL, respectively. To further support the CDP effect on the MCF-7 and MDA-MB-231 lines, annexin V and propidium iodide were used to determine apoptotic and necrotic cells by FACS analysis (Fig. 3b). Findings showed that for both MCF-7 and MDA-MB-231 lines, apoptosis was induced in a concentration-dependent manner, leading to an EC₅₀ value for apoptosis induction of ~0.05 mg/mL at 4 h of CDPs treatment (Fig. 3b). These data indicated that CDPs from *P. aeruginosa* PAO1 were cytotoxic, inducing apoptosis in HeLa, MCF-7, and MDA-MB-231 human cancer cell lines.

Once the HGF/MET receptor participation in the transduction of signals and modification of expression of genes associated with bacterial CDPs effects in HeLa cells was established, additional upregulation of Met and EGF proteins in tumors of xenografted melanoma mice was observed [12]. Two other human cancer lines were utilized to further confirm the findings. Immunoblotting results showed a similar behavior to HeLa cells in both CDPs-treated MCF-7 and MDA-MB-231 cell lines (Fig. 4). The protein expression of both Met and EGF receptors in the MCF-7 cell line did not change with the CDPs-treatment (Figs. 4a and b). However, phosphorylation on the Met-Tyr¹⁰⁰³ and EGF-Tyr¹⁰⁶⁸ and the Gab1-Tyr³⁰⁷ factor was significantly decreased by the CDPs exposure (0.1 mg/mL) at 4 h of exposure (Fig. 4a).

On the other hand, in the MDA-MB-231 line, protein expression levels of the EGF receptor and phosphorylation (EGF-Tyr¹⁰⁶⁸), as well as the Gab1-Tyr³⁰⁷ protein, showed similar behavior to HeLa and MCF-7 lines (upregulated). However, although the total protein expression of Met showed an identical behavior in HeLa and MCF-7 lines, in the MDA-MB-231 line, the Met-Tyr¹⁰⁰³ showed an opposite effect; the phosphorylation was significantly induced by CDPs-exposure (0.1 mg/mL) in a time-dependent manner (Fig. 4b).

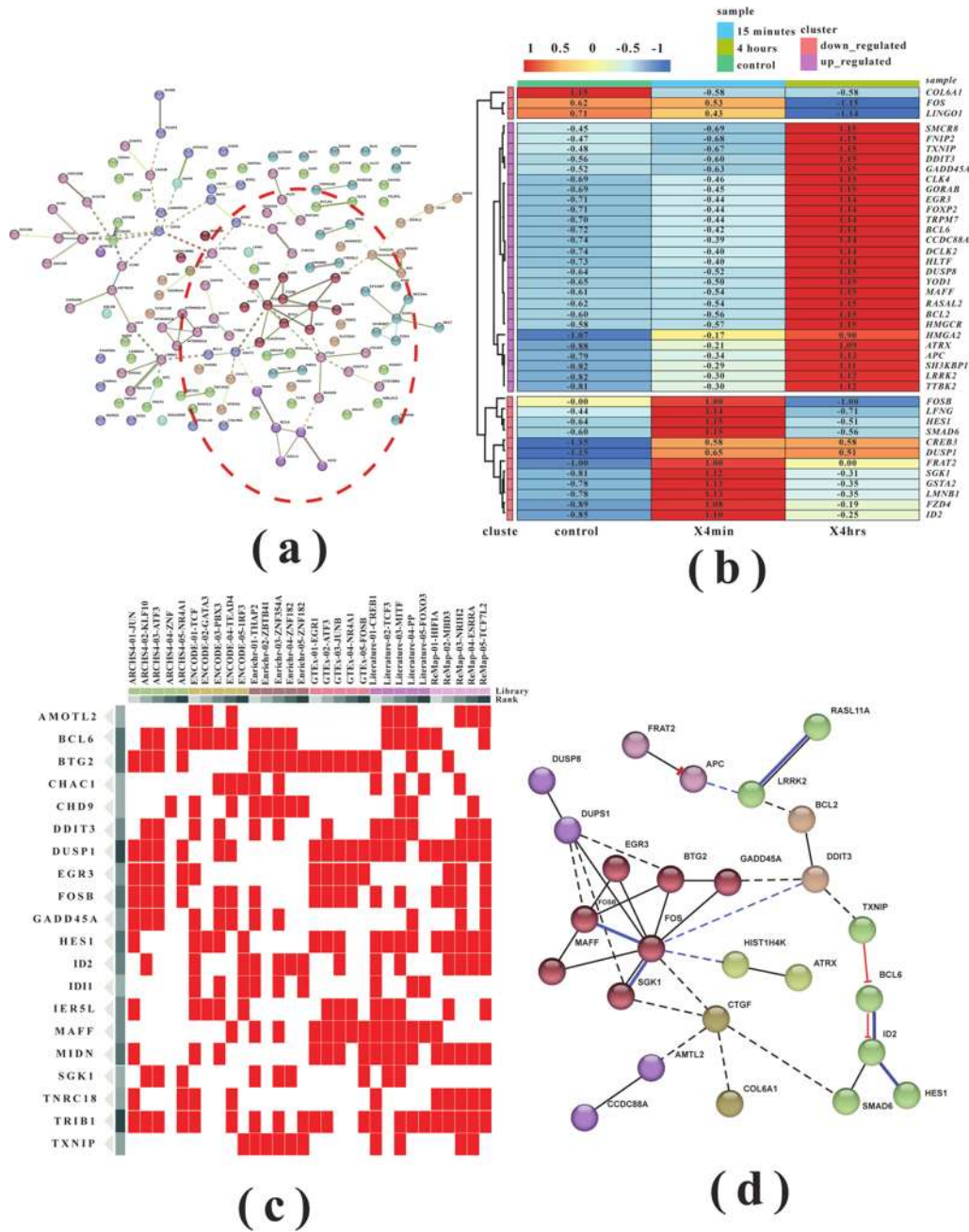


Fig (1). Identification of cancer-related genes and pathways in HeLa cells exposed to CDPs. **(a)** STRING network shows 150 cancer-related DEGs that modify their expression upon CDPs exposure. Each cluster represents a set of highly connected nodes which is illustrated by discrete color. The DEGs related to the MET pathway are shown in a red dashed circle. **(b)** Heat maps of 41 cancer-related DEGs commonly expressed in the comparative transcriptomes of HeLa cells exposed to CDPs at t = 0 (Control), 15 min, and 4 h. **(c)** The interactive clustergram overlaps the top 20 query-targeted genes related to the CDPs exposure among the entire cancer-related gene library. **(d)** ChEA3 analysis of DEGs related to MET pathway associated with CDPs-exposure. (A higher resolution/colour version of this figure is available in the electronic copy of the article).

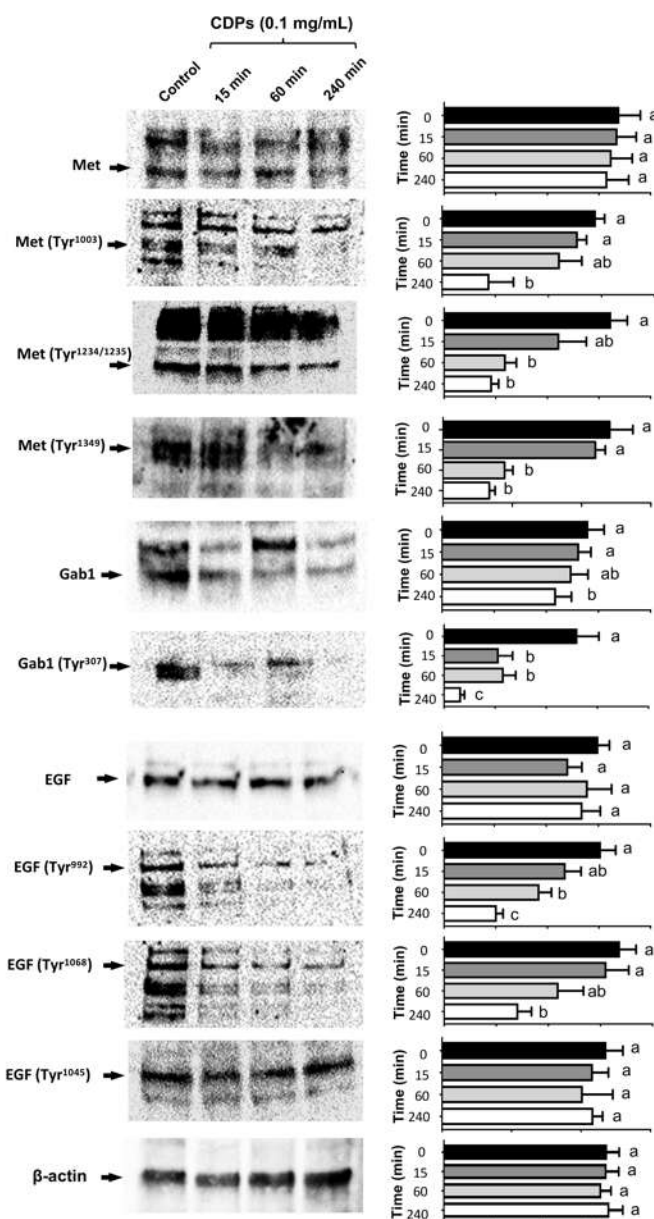


Fig. (2). Effects of CDPs on the phosphorylation of the MET/EGF receptor in HeLa cells. HeLa cells were homogenized to obtain protein extracts used for Western blot assays as described in Materials and Methods. Representative images corresponding to western blot analysis of protein extracts of at least three independent assays with the indicated antibodies. The antibodies used were as follows; anti-Met, anti-Met-phosphorylated (Tyr-1003), anti-Met-phosphorylated (Tyr-1234/T1235), anti-Met-phosphorylated (Tyr-1349), anti-Gab1, anti-Gab1-phosphorylated (Tyr-307), anti-EGF, anti-EGF (Tyr-992), anti-EGF (Tyr-1068), anti-EGF (Tyr-1045), and anti- β -actin. On the right, the graphs correspond to the determination of band intensity by densitometry analysis of the Western blot assays (left), which were analyzed using the Image J software. Data represent the means $\pm$ SE of densitometry determinations using protein extracts from at least three independent assays. One-way ANOVA with the Bonferroni post hoc test was used to compare treatment times concerning the control (time 0). Significant differences ($P < 0.05$) vs. control are denoted by lower-case letters.

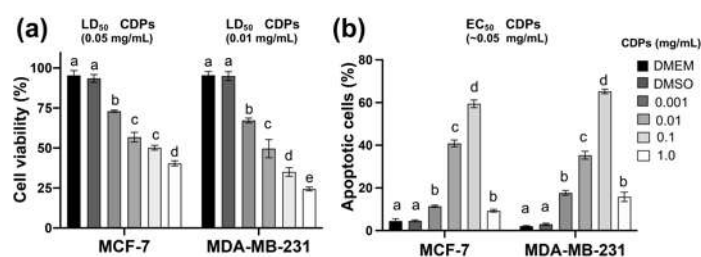


Fig. (3). Effects of CDPs on the viability and induction of apoptosis of the MCF-7 and MDA-MB-231 lines. Cancer cell cultures were incubated with CDPs to determine cell viability and apoptosis as described in Materials and Methods. (a) Determination of the viability of MCF-7 and MDA-MB-231 cells treated with different concentrations of CDPs from *P. aeruginosa* PAO1 by MTT method, the LD₅₀ of CDPs are shown. (b) Measurements of apoptosis using annexin V binding and necrosis by propidium iodide were conducted by flow cytometry using different concentrations of CDPs; the EC₅₀ is shown. Bars represent the means $\pm$ SE of three independent assays, $n = 3$. One-way analysis of variance (ANOVA) was carried out with Tukey's post hoc test; statistical significance ($P < 0.05$) of differences between treatments is indicated with lower-case letters.

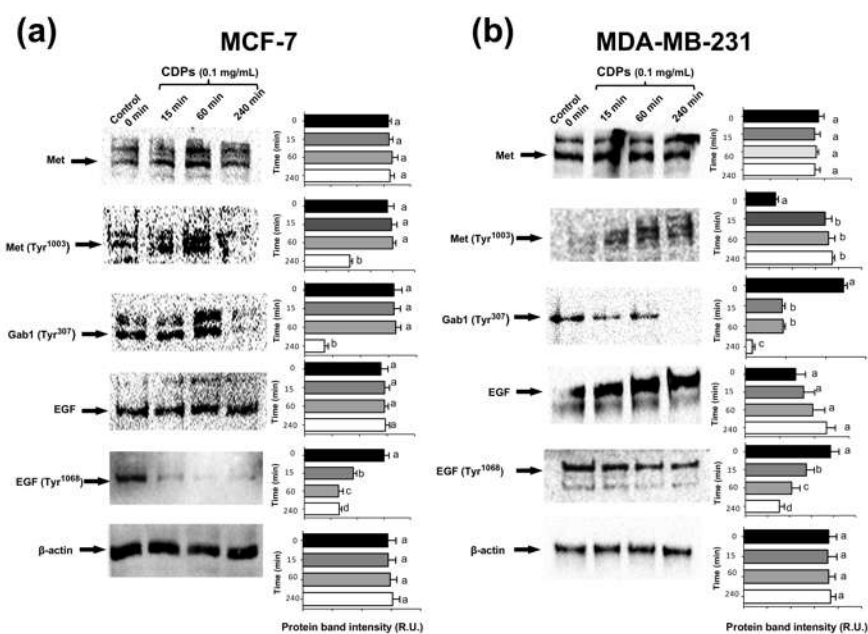


Fig. (4). Effects of CDPs on the phosphorylation of the MET/EGF receptor in MCF-7 and MDA-MB-231 lines. As described in Materials and Methods, MCF-7 and MDA-MB-231 cells were homogenized to obtain protein extracts used for Western blot assays. Representative images corresponding to western blot analysis of protein extracts of at least two independent assays with the indicated antibodies. The antibodies used were as follows: anti-Met, anti-Met-phosphorylated (T-1003), anti-Gab1-phosphorylated (T-307), anti-EGF, anti-EGF (T-1068), and anti- β -actin. On the right, the graphs correspond to the determination of band intensity by densitometry analysis of the Western blot assays (left), which were analyzed using the Image J software. Data represent the means $\pm$ SE of densitometry determinations using protein extracts from at least two independent assays. One-way ANOVA with Bonferroni post hoc test compared treatment times to control (time 0). Significant differences ($P < 0.05$) vs. control are denoted by lower-case letters.

3.3. The CDPs Inhibited the Phosphorylation of HGF/MET by Interactions in the Catalytic Site of the Serine-threonine Kinase Family

Although the mode of action of CDPs is not elucidated, we previously proposed that CDPs target multiple pathways and cellular processes involving oncogenes and tumor suppressors by blocking the catalytic site conserved in the serine-threonine kinase family. In this sense, the HGF/MET are proteins from the serine-threonine kinase family, and based on the fact that CDPs inhibited the phosphorylation of these receptors by interaction with CDPs, molecular docking was carried out to evaluate possible interactions. Docking results showed that CDPs were capable of interacting with different theoretical affinities in the binding site of the inhibitors Foretinib and Gefitinib in cMET (PDB:6SD) and EGFR (PDB:4WKQ), respectively (Table 1 and Fig. 5). As indicated in Fig. (5), cMET showed two binding sites for CDPs with different affinities, where the best ligand was cyclo (L-

Pro-L-Tyr) with theoretical values of binding energy (B. E.) = -7.92 kCal/mol and K_i = 1.57 μ M, followed by cyclo (L-Pro-L-Phe) B. E. = -7.27 kCal/mol and K_i = 4.71 μ M, and cyclo (L-Pro-L-Val) B. E. = -6.03 kCal/mol and K_i = 38.12 μ M; while for the inhibitor Foretinib the theoretical B. E. and K_i values were -8.66 kCal/mol and 0.446 μ M, respectively. The inhibition constants of CDPs for site 2 were in the same order of magnitude as site 1 (Table 1 and Fig. 5a). The binding energy and inhibition constants of the EGF receptor with the CDPs and its inhibitor gefitinib were also determined. Data of binding energy and K_i values indicated that the affinities for CDPs cyclo (L-Pro-L-Val) B. E. = -7.29 kCal/mol and K_i = 4.52 μ M and cyclo (L-Pro-L-Tyr) B. E. = -6.29 kCal/mol and K_i = 14.68 μ M, were higher than for cyclo (L-Pro-L-Phe) B. E. = -5.72 kCal/mol and K_i = 63.96 μ M; while for the inhibitor gefitinib B.E. and K_i values were -6.5 kCal/mol and 17.15 μ M, respectively (Table 1 and Fig. 5b).

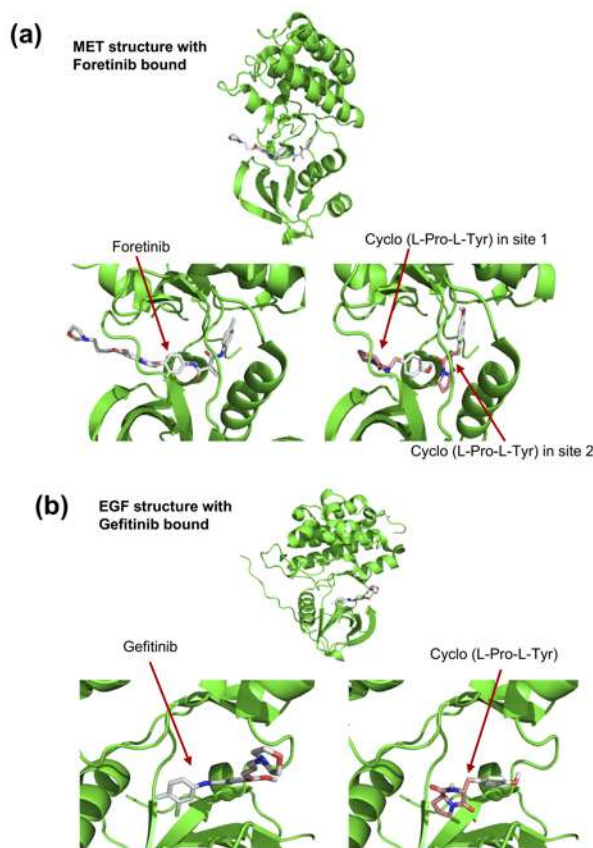


Fig. (5). Molecular docking of human cMET/EGF receptors with bacterial CDPs. **(a)** MET interaction with Foretinib (inhibitor) and cyclo (L-Pro-L-Tyr) in binding sites 1 and 2. **(b)** EGF interaction with Gefitinib (inhibitor) and cyclo (L-Pro-L-Tyr) in the binding site. The protein is shown as a surface with the interior in the cartoon representation; cyclo (L-Pro-L-Tyr) and other molecules are shown as sticks colored by elements. Models were carried out with PYMOL (The PyMOL Molecular Graphics System, Version 2.1.0, Schrödinger, LLC). (A higher resolution/colour version of this figure is available in the electronic copy of the article).

Table 1. Docking analysis of CDPs was conducted with the human cMET (PDB:6SD9) and EGFR (PDB:4WKQ) receptors, comparing with their inhibitors Foretinib and Gefitinib, respectively.

Drug	Site 1		Site 2	
	B.E. (kCal/mol)	K _i (μM)	B.E. (kCal/mol)	K _i (μM)
cMET				
Foretinib	-8.66	0.446	-	-
Cyclo (L-Pro-L-Tyr)	-7.92	1.57	-7.25	4.83
Cyclo (L-Pro-L-Val)	-6.03	38.12	-5.73	62.89
Cyclo (L-Pro-L-Phe)	-7.27	4.71	-7.22	5.09
EGFR				
Gefitinib	-6.5	17.15	-	-
Cyclo (L-Pro-L-Val)	-7.29	4.52	-	-
Cyclo (L-Pro-L-Phe)	-5.72	63.96	-	-
Cyclo (L-Pro-L-Tyr)	-6.59	14.68	-	-

4. DISCUSSION

Drug supply is the most common therapy for treating different human cancer types. Hence, unraveling the molecular mechanisms of drug action is crucial to improve anticancer therapies. Substances such as natural cyclodipeptides that impact cell proliferation have a high potential for cancer treatment [11]. The cyclodipeptides cyclo (L-Pro- L-Tyr), cyclo (L-Pro- L-Phe), and cyclo (L-Pro- L-Val) produced by *P. aeruginosa* PAO1 inhibit the proliferation of human HeLa cells line by arresting the cell cycle at the G0-G1 transition, involving inhibition of phosphorylation of protein-kinases, such as Akt-Ser473, mTOR-Ser2448, and p70S6K-Thr389 as one of the action mechanism elucidated [11, 13, 17].

The mechanisms of tumorigenesis associated with the HGF/MET pathway involve the Des-gamma-carboxy prothrombin (DCP), which induces cell proliferation *via* the c-MET-Janus kinase 1-STAT3 signaling pathway by binding to Tyr1234/1235 of c-MET, resulting in c-MET autophosphorylation in hepatocellular carcinoma cells [60]. Similarly, FER phosphorylates Tyr1349 of c-MET to maintain the recruitment of signaling molecules, such as GAB1 and SH-P2-ERK, to potentiate metastasis in ovarian cancer cells. Moreover, slug activates c-MET by phosphorylating Tyr1234/1235 to induce chemoresistance in metastatic ovarian cancer cells [30, 61].

Heatmap and STRING network from the transcriptomic analysis showed differential expression of essential biomarkers associated with CC and other cancer types, including BCL6, DDIT3, GADD45A, HMGA2, and ID2 [24]. Members of the pathway are also involved in controlling cell growth, proliferation, and cell death. Interestingly, B cell lymphoma 6 (BCL6) [62], a transcriptional suppressor of anti-apoptotic B cell lymphoma-2 (BCL2) [63], was targeted in CDPs-treated HeLa cells. The Dual-specificity phosphatase 1 (DUSP1) inhibitor of the MAPK pathway was also significantly upregulated in response to CDPs [24].

We also found three essential upregulated genes of response to ER stress involved in the apoptotic signaling, including the DNA damage-inducible transcript 3 (DDIT3,

called CHOP) [64, 65], growth arrest and DNA-damage-inducible beta (GADD45B) [66, 67], and the Glutathione-Specific Gamma-Glutamyl-cyclo-transferase, encoded by the CHAC1 gene, an inductor of glutathione depletion that is an essential factor for apoptosis initiation and execution [68]. Prolonged ER stress may lead to apoptosis by activating DDIT3/CHOP [69, 70], which agrees with our previous results [11].

The main signaling pathways that seem to be impacted by CDPs, also commonly activated in many physiological responses, are the growth factors of the tyrosine kinase type (RTKs; *e.g.*, EGFR), small GTPases (*e.g.*, RASL11A), serine/threonine kinases (*e.g.*, STK36, DCLK, and SGK1), the cytoplasmic tyrosine kinases (*e.g.*, TTBK2), the lipid kinases (*e.g.*, PI3Ks), as well as the nuclear receptors (*e.g.*, the estrogen receptor, ER), and the components of developmental signaling pathways, such as Wnt (*e.g.*, FZD4 and APC), TGF-β (*e.g.*, ID2 and SMAD6), bone morphogenetic protein, G protein-coupled receptor (*e.g.*, GPR176, CCDC88A/GIRDIN), Hedgehog (*e.g.*, GAS1), PI3K/Akt/mTOR (*e.g.*, SGK1), Hippo (*e.g.*, CCN2), and Notch (*e.g.*, HES1 and LFNG), since all of them have downstream nuclear target signaling pathways, including transcription factors (*e.g.*, HMGA2, FOS and EGR3), chromatin remodelers (*e.g.*, ATRX), and cell cycle motors (*e.g.*, CCNF cyclin) [24].

In this study, we found that CDPs in cell cultures inhibit the phosphorylation of c-MET in residues Tyr1003 (involved in regulation by calcineurin B-like protein, CBL) and in Tyr1234/Tyr1235 (engaged in the kinase-domain), which crosslink with STAT3, GAB1/PI3K/AKT, or RAS/RAF/MEK/ERK pathways. In addition, the inhibition of phosphorylation of Tyr1349 modulates the signaling to PTPN1/2 in proteasomal regulation [71]. Concerning the EGF receptor, our findings showed inhibition of phosphorylation in residues Tyr992 and Tyr1045, which have been described to be involved in the transduction of signals to RAS/RAF/MEK/ERK in proliferation and survival pathways, which can be inhibited by the Erlotinib and Gefitinib drugs (Fig. 6) [36, 72]. The results indicate MET's participation in the modulation of downstream master regulators and

gene expression in the antiproliferative and cytotoxic effects of CDPs on HeLa cells [12]. In addition, Met regulates various cancer types through crosstalk mechanisms and activation of downstream signals, which depend on anti-oncogenic and oncogenic non-coding RNAs [73, 74], and the targeting of HGF/MET receptor pathway has been widely proposed as an opportunity for cancer therapy [42]. CDPs may be considered for cancer patients that could potentially benefit from HGF/MET targeted therapies, which have been categorized into the following groups: selective MET tyrosine kinase inhibitors (TKIs), non-selective MET TKIs, anti-MET monoclonal antibodies, and anti-HGF antibody [30].

The cytotoxic and apoptotic effects of the CDPs from *P. aeruginosa* PAO1 were tested on the MCF-7 line of human adenocarcinoma and the MDA-MB-231 line of human triple-negative breast cancer. The CDPs exposure was a cyto-

toxic and apoptotic inducer in both the MCF-7 and MDA-MB-231 lines in a time-dependent manner, showing LD₅₀ around 0.01-0.05 mg/mL and EC₅₀ values of apoptosis induction ~0.05 mg/mL, respectively (Figs. 3a and b). These data indicated that CDPs from *P. aeruginosa* PAO1 were cytotoxic, inducing apoptosis in HeLa, MCF-7, and MDA-MB-231 human cancer cell lines. In agreement with HeLa line findings, immunoblotting results showed that in both the MCF-7 and MDA-MB-231 lines CDPs-treated, similar behavior was observed (Fig. 4). The protein expression of both Met and EGF receptors in the MCF-7 line was upregulated, but the CDPs treatment did not show any effect in expression levels as a function of the exposure time. However, phosphorylation on the Met-Tyr¹⁰⁰³ and EGF-Tyr¹⁰⁶⁸, as well as in the Gab1-Tyr³⁰⁷ factor, was significantly decreased by the CDPs exposure (Fig. 4a).

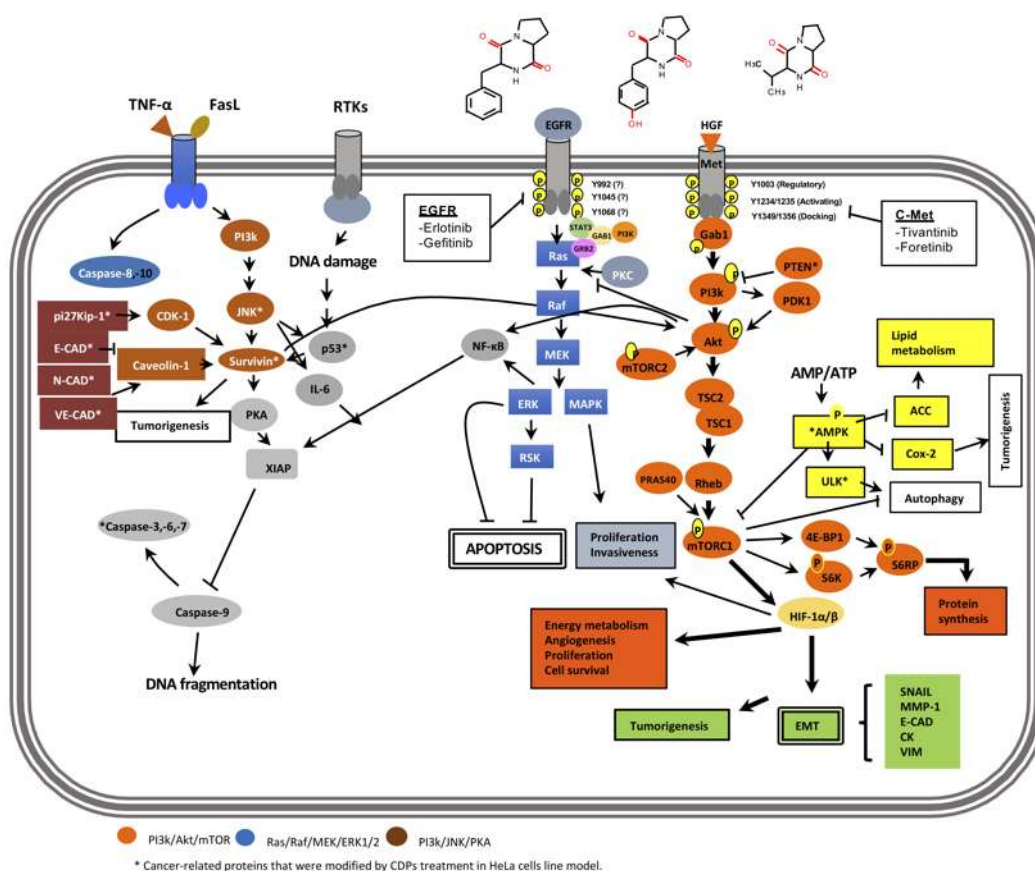


Fig. (6). Scheme of the proposed mechanisms involved in interacting bacterial CDPs with cMET/EGF receptors in HeLa cells. Different colors for protein elements of each signaling pathway are used. (A higher resolution/colour version of this figure is available in the electronic copy of the article).

Upon HGF binding, Met receptors undergo dimerization and autophosphorylation of Tyr¹²³⁴ and Tyr¹²³⁵, leading to phosphorylation of Tyr¹³⁴⁹ and Tyr¹³⁵⁶ of the C-terminal regulatory tail. When phosphorylated, the C-terminal regulatory domain acts as a docking site for intracellular adaptors of molecules, including growth factor receptor-bound protein 2, growth factor receptor-bound protein 2-associated binder 1, and Shc, activating downstream signaling, such as Rac1/Cdc42, PI3k/Akt, and Erk/MAPK. These pathways regulate cellular proliferation, motility, migration, invasion, and tubulogenic [44]. In addition, Met can be directed by c-Src and the p85 subunit of PI3K or by scaffolding protein Gab1 to prevent apoptosis *via* Akt-activation and JAK/STAT pathways. Thus, CDPs primarily induce apoptosis in the MCF-7 and MDA-MB-231 cell lines through the MET/EGF pathway inhibition.

The prominent Y1003 in the DpYR motif of MET protein encoded by exon 14 is required to efficiently recruit the ubiquitin ligase CBL for its ubiquitin-mediated degradation [75]. In breast cancer patients, studies have shown that high levels of MET expression are associated with a favorable prognosis, demonstrating that overexpression of the HGF/MET pathway has an antiproliferative effect and good prognosis in breast cancer patients [25, 26, 41, 42, 76]. Thus, CDPs treatment could induce the ubiquitination of MET protein through the Y1003 residue, causing the inactivation of the translation signal by phosphorylating downstream protein targets.

On the other hand, expression of the EGF receptor, its total protein expression levels, and phosphorylation (EGF-Tyr¹⁰⁶⁸), as well as the Gab1-Tyr³⁰⁷ protein, showed similar behavior to HeLa and MCF-7 lines (upregulated). Although the total protein expression of Met showed an identical behavior in HeLa and MCF-7 lines upon CDPs (0.1 mg/mL) exposure, in the MDA-MB-231 line, the Met-Tyr¹⁰⁰³ showed the opposite behavior, inducing significant levels of phosphorylation in a time-dependent manner (Fig. 4b).

CONCLUSION

The results found here suggest the participation of the MET/EGF receptor pathway in the activation mechanism and gene modulation of some of the downstream elements in the cytotoxic effect of CDPs on HeLa, MCF-7, and MDA-MB-231 lines. These findings suggest that the mechanism of inhibition of phosphorylation by CDPs on the EGF/MET receptors occurs at the level of the substrate binding site by an inhibition mechanism similar to those brought about by the gefitinib and foretinib anti-neoplastic drugs.

AUTHORS' CONTRIBUTIONS

Conceptualization, J. C-G; methodology, L. H-P, M.X. D-M, L M-A; software, J.S. R-Z; writing—original draft preparation, J. C-G, J.S. R-Z; project administration, J. C-G; funding acquisition, J. C-G. All authors have read and agreed to the published version of the manuscript.

LIST OF ABBREVIATIONS

CC	= Cervical Cancer
CDPs	= Cyclodipeptides
GrB2	= Growth Factor Receptor-bound Protein 2
HGF/MET	= Hepatocyte Growth Factor/Mesenchymal-epithelial Transition Factor
PI3K	= Phosphoinositide 3-kinase
PLCγ1	= Phospholipase C1
TME	= Tumor Microenvironment
GaB1	= GrB2-Associated Binding Protein 1

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data sets used and/or analysed during this study are available from the corresponding author upon request.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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CAPÍTULO III. ARTÍCULO DE REVISIÓN: Péptidos cíclicos como moduladores de proteínas cinasas y su implicación en el tratamiento de diversas enfermedades humanas.



Review

Cyclic Peptides as Protein Kinase Modulators and Their Involvement in the Treatment of Diverse Human Diseases

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Abstract: Protein kinases (PKs) are an important and very popular family of enzymes that play a vital role in regulating cellular processes via the phosphorylation of targets. Nevertheless, modifications in the expression due to mutations or their dysregulation can lead to diseases, including autoimmune disorders, cardiovascular problems, diabetes, neurological diseases, and cancers. Cyclic ultra-short peptides are amazing structures with unique properties. The cyclicity of cyclic peptides (CPs) can mimic the interactions between PKs and natural substrates, influencing the enzyme activity essential in health and disease physiology. Our review summarized that interference in the signal transduction mechanism of the PKs by CPs implies the inhibition of substrate phosphorylation at the level of the active site, similar to anti-neoplastic drugs. The remarkable capacity of CPs to interact with targets positions them as promising candidates for developing protein kinase inhibitors in treating diseases. This review offers new insights for CPs in molecular mechanisms, cytotoxicity, target selectivity, and the possibility of designing more effective and safe therapeutic agents.

Keywords: protein kinases; protein kinase inhibitors; cyclic peptides; cyclodipeptides; anti-neoplastic drugs; therapeutic targets; neuroprotective; aged and diabetes

1. Protein Kinases as Therapeutic Targets

The reversible addition of a phosphate group to a protein is perhaps one of the significant critical post-translational modifications in the cell since most of the signaling pathways in eukaryotes are regulated at the post-translational level. Processes such as metabolism, transport, mobility, division, differentiation, and cell death are controlled by signal transduction mediated by phosphorylation/dephosphorylation involving molecules of carbohydrates, nucleic acids, proteins, lipids, etc. In this context, regulation can activate various pathologies, including metabolic disorders, diabetes, cancer, cardiovascular, and neurodegenerative and immune diseases, among others, making them key pharmacological targets for therapeutic treatment [1–5].

Since Burnnet and Kennedy (1954) first described a protein kinase and obtained the initial crystallized structure of the protein kinase PKA [6], extensive research on this protein family has been underway (Figure 1A). Today, advancements such as “the complement of protein kinases of the human genome (kinome)” [7] and new technological tools enable more precise insights into the structure, functions, and interactions of protein kinases [8].

By bioinformatics, Manning et al. (2002) found 518 genes encoding protein kinases, representing 2.5% of the human genome. Of these, 478 contain the eukaryotic catalytic domain (ePK), and 40 were considered atypical in having kinase activity but lacking the kinase catalytic domain; moreover, they expanded the classification into ten large groups and more than 100 subgroups [7,9]. Moreover, over 5000 interactions within kinase networks have been identified, along with numerous kinase–disease associations that span from genetic disorders to complex conditions like cancer [8,10].

Generally, protein kinases can be categorized based on the type of residue they phosphorylate or the nature of the protein–OH groups, resulting in 90 protein–tyrosine kinases, 43 protein–tyrosine kinase-like enzymes, and 385 protein–serine/threonine kinases [11–13]. Some of the most studied and clinically relevant groups of protein kinases include receptor tyrosine kinases (RTKs). Genetic changes or abnormalities in RTKs can disrupt the activity, cellular distribution, or regulation of these proteins. For instance, various cancer types are frequently overexpressed or continuously activated [14]. In addition, the mRNAs of different RTKs may undergo alternative splicing [15], such as EGFR and VEGFR, the variants of which are involved in tumor progression [14,16], or the compensatory activation of mechanisms after the inhibition of these RTKs is carried out.

On the other hand, non-receptor protein kinases also participate as second messengers in signaling pathways. Src kinase is one of the most studied, but the other family members are BTK, JAK, BCR-Abl, and TYK2. Well-studied Ser/Thr kinases typically recognize specific amino acid residues around the phosphorylation site at several positions. However, until 2022, sequence motifs for phosphorylation sites have been identified for only a portion of the human protein Ser/Thr kinome. Nevertheless, Johnson et al. (2023) published their work about the substrate specificities for the human serine/threonine kinome, providing the link between phosphorylation events and biological pathways [17]. Some Ser/Thr kinase family members with extensive research as therapeutic targets are CDK4/6, BRAF, FKBP, and ROCK [13]. However, many Ser/Thr kinases are involved in diverse pathologies proposed as promising targets with non-specific approved inhibitors. For example, the PI3K/AKT/mTOR pathway is frequently overexpressed in various cancers. Although numerous Akt antagonists have been in development, it was not until 2023 that the FDA approved Capivasertib, a specific inhibitor for this protein kinase, used in combination with Fulvestrant for the treating of hormone-receptor-positive, HER2-negative advanced breast cancer [18].

Protein kinases catalyze the transfer of phosphate groups to tyrosine, serine, and threonine residues of different protein substrates, functioning as molecular switches that are typically in an “off” state and switch to “on” when triggered by a signal [19]. They can exist in various conformational states that dictate their activation or inactivation. PKs possess a primary catalytic site, but around 83 domains with different functions have also been found in multiple kinases [7]. Most protein kinases possess additional domains that regulate kinase activity, the recruitment of binding partners, or the oligomerization state. Some of the most well-characterized domains in protein kinases include the catalytic domain or ATP-binding domain, which features a pocket formed between the N-terminal domain and C-terminal domains, connected by a hinge sequence. The ATP-binding site is located at the junction of the two essential regulatory domains and the A loop, which is also part of the protein substrate binding site [20]. The SH2 domain is also relevant in studying kinases since it binds to phosphorylated tyrosine sequences [21]. For example, it is known that the C helix is recognized as a critical structural component influencing the allosteric behavior of protein kinases [22] (Figure 1).

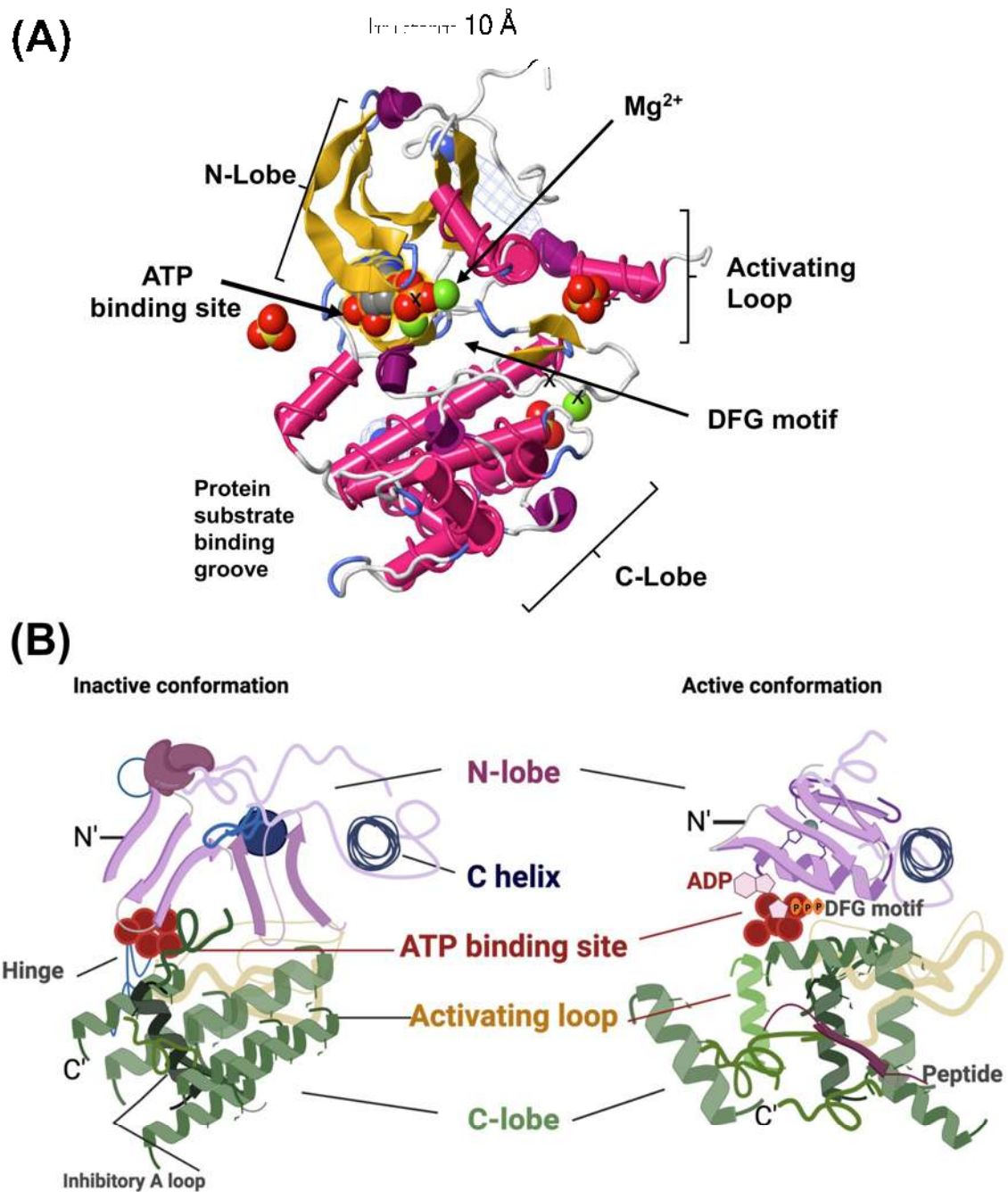


Figure 1. The general structure and principal components of kinases. **(A)** Cartoon of the secondary structure of the crystallized Aurora-A (1OL5) PK, an oncogenic serine–threonine kinase modeled using FirstGlance in Jmol (<http://firstglance.jmol.org>, accessed on 30 November 2024). **(B)** Cartoon illustrating the location of the principal structural regions on the inactive and active state of the tyrosine kinase domain of the epidermal growth factor receptor (EGFR). In the inactive state, kinases adopt a conformation restricting substrate access to the active site. Upon activation, kinases undergo structural changes that enable the phosphorylation of specific substrates (cartoons adapted from [23]).

On the other hand, it has been described that the highly conserved Asp-Phe-Gly (DFG) motif in kinases is relevant in the allosteric regulation of these enzymes by presenting “in” or “out” conformational states that define the binding of ATP at the catalytic site [24] (Figure 1B). Recently, small specific sequences of amino acid residues have been identified as playing a crucial role in the interaction between protein kinases and their specific substrates. These sequences are called KLIFS (kinase–ligand interaction fingerprint and structure). They are housed in a database that gathers all kinase structures, aligning, decomposing, annotating, curating, and enriching them with related information. This significant contribution from research groups reflects the growing interest in developing new kinase inhibitors for various human diseases [25,26]. The domains identified in these protein kinases and insights into their roles in folding and interactions with other scaffold or substrate proteins have enabled the advancement of multiple classes of inhibitors currently under approval or in clinical trials for treating numerous diseases.

1.1. Protein Kinase Inhibitors

The role of kinases in disease pathophysiology is highlighted by evidence that genetic alterations in kinases can disrupt cellular signaling. Kinase translocations, mutations, and overexpression can lead to dysregulated protein kinase activity, which plays a significant role in developing various diseases. Pharmacological and pathological evidence has established that kinases are promising targets for drug development. All catalytic domains of kinases exhibit homologous structures for ATP binding, with the binding strength and specificity varying based on the compound (Figure 1A). This standard feature among kinases facilitates the design of synthetic inhibitors aimed at the ATP-binding site, resulting in improved selectivity and favorable drug-like characteristics, such as enhanced solubility and bioavailability. The presence of electron donor and acceptor sites is a crucial criterion for predicting the therapeutic efficacy of small-molecule kinase inhibitors. The sequence and nature of amino acids influence interactions with pharmacological sites, potentially modifying binding sites through weak interactions [13].

Since the 1980s, protein kinases have been identified as promising drug targets, primarily due to advances in the molecular understanding of cancer [27]. Developing protein kinase inhibitors that target the ATP site proved challenging. However, by the late 1980s, Sun and colleagues successfully identified and optimized synthetic small molecule kinase inhibitors aimed at the ATP-binding site, achieving desirable drug-like properties, selectivity and potency. Afterward, in 1991 and 1992, the natural compounds Sirolimus and Imatinib were recognized as significant milestones in developing kinase inhibitors. Sirolimus became the first allosteric kinase inhibitor, playing a crucial role in the discovery of the mechanistic target of rapamycin (mTOR) [28] and was approved by the FDA for preventing organ rejection [29]. Imatinib, which potently inhibits several tyrosine kinases, was initially approved for treating chronic myelogenous leukemia (CML) driven by the BCR–ABL oncogene, and, later, for other indications, including treating gastrointestinal tumors [30].

The number of approved kinase inhibitors globally has risen to 103 drugs, with 82 of these being small protein kinase inhibitors approved by the FDA (September 2024). Additionally, 21 small-molecule protein kinase inhibitors have been approved in China, Japan, Europe, and South Korea. Among these 21 agents, 11 target receptor protein–tyrosine kinases, 8 inhibit receptor protein–tyrosine kinases, and 2 block protein–serine/threonine kinases [31]. All 21 drugs are orally bioavailable or topically effective. Robert Roskoski also compiled a small set of 80 FDA-approved protein kinase inhibitors [13]. However, these information sources typically concentrate only approved inhibitors (compounds that have completed phase 4 clinical trials) and lack long-term insights into the next generation of protein kinase inhibitors. Expanding the scope to include protein kinase inhibitors currently in clinical trials (compounds in development from phase 0 to phase 3) would provide valuable information on the state of the art and current trends in kinase inhibitor design. Notably, the Kinase Profiling Inhibitor Database lists 367 oral protein kinase

inhibitors currently undergoing clinical trials worldwide, and this comprehensive catalog is continuously updated (www.icoa.fr/pkidb/, accessed on 3 September 2024).

1.2. Small Protein Kinase Inhibitors Approved by FDA

Targeted therapies generally are divided into two types: small molecules (<1000 Da) and macromolecules (ranging from 1500 to 150,000 Da), including monoclonal antibodies, polypeptides, antibody–drug conjugates, and nucleic acids [32]. Compared to macromolecule medications, small-molecule targeted drugs offer advantages in pharmacokinetic properties, cost-effectiveness, patient compliance, and drug storage and transport [33].

Small-molecule kinase inhibitors are named according to the specific kinase family they target, with each family associated with a distinctive suffix. “-inib” is used for tyrosine kinase (TK) inhibitors, “-limus” for mTOR inhibitors, “-rafenib” for RAF inhibitors, “-anib” for VEGFR inhibitors, “-metinib” for MEK inhibitors, “-lisib” for PI3K inhibitors, “-clib” for CDK inhibitors, and “-mab” for monoclonal antibodies that target kinases. The mechanism of action for small-molecule protein kinase inhibitors involves reversible and irreversible interactions. Reversible inhibitors partially occupy the ATP-binding pocket and form hydrogen bonds with the hinge region linking the enzyme’s small and large lobes. This category includes types I, I½ A, I½ B, II A/B, II A, and II B. Additionally, reversible inhibitors can bind to an allosteric site in the cleft between the kinase’s small and large lobes near the ATP-binding pocket, as seen in types III and IV.

Moreover, type V inhibitors extend across two separate regions of the kinase domain, limiting kinase activity and exhibiting diverse residence times, indicating the duration they stay bound to the enzyme. Irreversible interactions occur when type VI inhibitors form covalent bonds with the kinase’s active site. Furthermore, most protein kinases contain extra domains that regulate kinase activity, oligomerization, and the recruitment of binding partners.

From 518 members of the protein kinase superfamily, FDA approved only 26 drugs with therapeutic applications that targeted PKs associated with different diseases (Table 1). Due to the conserved ATP-binding site across the human kinome, ATP-mimicking compounds often interact with multiple kinases, resulting in drugs with broad, sometimes promiscuous, activity profiles. The promiscuous ATP-binding site applies to drugs such as Abemaciclib, Palbociclib, Ribociclib, and Trilaciclib, all targeting the CDK4/6 protein. Kinases like CDK4/6, AKT, HER2, ErbB1/2/4, and FKBP12/mTOR are regarded as therapeutic targets for breast cancer treatment [34] (Figure 2; Table 1).

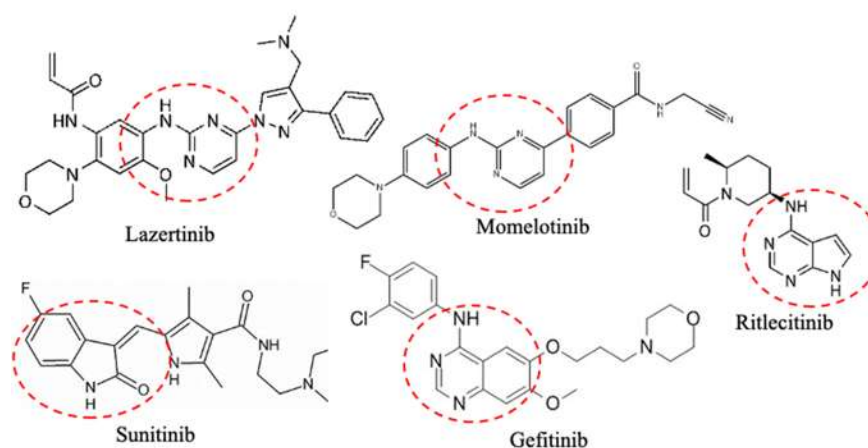


Figure 2. Molecular structures of small protein kinase inhibitors. Discontinuous circles show the adenine pocket in the therapeutic agent. Structures loaded from PubChem database (<https://pubchem.ncbi.nlm.nih.gov>, accessed on 22 September 2024).

In contrast, some inhibitors are designed for a single target, such as the kinase inhibitor Capivasertiv, which targets the AKT protein and is considered an oncogene. The AKT protein is part of the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway, which has become a key target in cancer, diabetes, obesity, neurodegenerative diseases, aging, and autoimmunity. That is due to its central role in controlling essential cellular processes like cell growth, proliferation, survival, and metabolism [35].

Additionally, the VEGFR protein serves as a broad-spectrum therapeutic target, with ten drugs known to inhibit it and treat various cancers (Table 1). The regulation of protein kinases is critical from a therapeutic perspective, as some protein kinase inhibitors can directly block multiple proteins or therapeutic targets, as seen with Afatinib, Lapatinib, Neratinib, and Tucatinib (Table 1). Some inhibitors are promiscuous, targeting more than two proteins, as in the case of Ponatinib and Regorafenib, each with nearly 11 targets (Table 1). Ponatinib is specifically indicated for treating a single disease (leukemia-CML or ALL).

Approximately 28 approved drugs act as multi-kinase inhibitors (Table 1), and the simultaneous inhibition of multiple protein kinases offers potential benefits, as blocking multiple targets may enhance the therapeutic effectiveness of these agents. Due to the inherent genetic alterations in cancer cells, resistance to protein kinase inhibitors nearly always develops [36]. This challenge led to the creation of second-, third-, and later-generation inhibitors designed to target the original enzyme and the associated malignancy. Gatekeeper mutations often drive the acquired drug resistance in the initial protein kinase target [37]. A prime example is the gatekeeper mutation in BCR-Abl, which spurred the development of first-, second-, and third-generation drugs that have significantly extended the life expectancy of leukemia patients. First-generation Imatinib, second-generation Bosutinib, Dasatinib, and Nilotinib are FDA-approved for frontline therapy. Ponatinib, a third-generation drug, is approved for resistant cases with the BCR::ABL fusion protein (T315I mutation) or after failure with at least two other protein-tyrosine kinase inhibitors [38].

In 2024, the FDA approved two new small-molecule protein kinase inhibitors, Lazertinib and Tovorafenib. Lazertinib is an epidermal growth factor receptor (EGFR) kinase inhibitor targeting EGFR exon 19 deletions and exon 21 L858R substitution mutations at lower concentrations than wild-type EGFR. In non-small-cell lung cancer (NSCLC) and mouse xenograft models with EGFR exon 19 deletions or EGFR L858R substitution mutations, Lazertinib showed anti-tumor effects. When combined with Amivantamab, Lazertinib demonstrated enhanced anti-tumor activity in vivo compared to either treatment alone in a mouse xenograft model of human NSCLC with an EGFR L858R mutation [39]. Tovorafenib is a Type II RAF kinase inhibitor that demonstrated anti-tumor activity in cultured cells and xenograft melanoma tumor models with BRAF V600E and V600D mutations [40].

Table 1. List of protein targets by FDA-approved small-molecule protein kinase inhibitors.

Protein Targets	Drug	Disease	References
CDK4/6	Abemaciclib Palbociclib Ribociclib Trilaciclib	Breast cancer and myelosuppression	[41–44]
AKT	Capivasertib	Breast cancer	[45]
HER2	Lapatinib # Neratinib # Tucatinib #	Breast cancer	[46–48]

Table 1. Cont.

Protein Targets	Drug	Disease	References
ErbB1/2/4	Afatinib Lapatinib Neratinib Tucatinib	Non-small-cell lung cancer (NSCLC), breast cancer, and colon cancer	[47,49–51]
FKBP12/mTOR	Sirolimus Everolimus *	Breast cancer and * lymphangioleiomyomatosis	[52,53]
MEK1/2	Binimetinib Cobimetinib Selumetinib	Melanoma and neurofibromatosis	[54–56]
BCR-Abl	Dasatinib # Imatinib # Nilotinib # Ponatinib # Asciminib Bosutinib #	Chronic myeloid leukemia (CML), and acute lymphocytic leukemia (ALL)	[57–59]
Flt3	Gilteritinib Midostaurin # Quizatinib	Leukemia	[60–62]
BRAF	Dabrafenib Encorafenib Vemurafenib Tovorafenib **	Melanoma	[63]
JAK1/2/3	Abrocitinib * Mometinib Pacritinib Baricitinib * Fedratinib Ritlecitinib * Tofacitinib * Upadacitinib *	* Atopic dermatitis, myelofibrosis, * rheumatoid arthritis, * alopecia areata, and * ulcerative colitis	[64–69]
ALK	Lorlatinib Alectinib Brigatinib # Ceritinib # Crizotinib # Entrectinib #	ALK-positive NSCLC, and inflammatory myofibroblastic tumor	[70,71]
PDGFR	Midostaurin # Ripretinib # Avapritinib #	Acute myeloid leukemia, and gastrointestinal stromal tumors	[72–74]
VEGFR 1/2/3	Axitinib Cabozantinib # Fruquintinib Lenvatinib # Pazopanib # Regorafenib # Sorafenib # Sunitinib # Tivozanib # Vandetanib #	Advanced renal cell carcinoma, advanced medullary thyroid cancer, renal cell and hepatocellular carcinoma, metastatic colorectal cancer, differentiated thyroid cancer, gastrointestinal stromal, and pancreatic neuroendocrine tumors	[75–79]
ROCK2	Belumosudil *	* Graft vs. host disease	[80]

Table 1. Cont.

Protein Targets	Drug	Disease	References
Kit	Pexidartinib # Ripretinib # Avapritinib #	Tenosynovial giant cell tumors, and gastrointestinal stromal tumors	[81,82]
MET	Capmatinib Crizotinib # Tepotinib	NSCLC, anaplastic large cell lymphoma, inflammatory myofibroblastic tumor, and MET mutant NSCLC	[83]
EGFR	Dacomitinib # Erlotinib Gefitinib Mobocertinib Osimertinib Lazertinib **	EGFR-mutant NSCLC, NSCLC, and pancreatic cancer	[84,85]
TYK2	Deucravacitinib *	* Psoriasis	[86]
TRKA/B/C	Entrectinib Larotrectinib	Solid tumors with NTRK fusion proteins	[87]
ROS1	Repotrectinib Entrectinib #	ROS1-positive NSCLC	[88]
FGFR1/2/3/4	Erdafitinib Futibatinib Infigratinib Nintedanib Pemigatinib	Urothelial bladder cancer, bile duct cancer, and idiopathic pulmonary fibrosis	[89–92]
BTK	Ibrutinib Pirtobrutinib Zanubrutinib	Chronic lymphocytic leukemia (CLL), and small lymphocytic lymphoma	[93]
SYK	Fostamatinib	Chronic immune thrombocytopenia	[94]
T970M	Osimertinib #	NSCLC with exon 19 deletions or exon 21 substitutions	[95]
CSF1R	Pexidartinib #	Tenosynovial giant cell tumors	[96]
Axl	Sunitinib Cabozantinib	Renal cell carcinoma	[97,98]

* Non-oncological therapeutic applications. ** New approved drugs by FDA in 2024. # Compounds that display more than one target.

Among the 82 approved small-molecule protein kinase inhibitors, 14 target protein-serine/threonine protein kinases, 4 act on dual specificity protein kinases (MEK1/2), 20 inhibit nonreceptor protein-tyrosine kinases, and 44 block receptor protein-tyrosine kinases. While most FDA-approved kinase inhibitors are anti-neoplastic, others serve primarily as immunomodulators. Only a select group, including Abrocitinib, Baricitinib, Belumosudil, Deucravacitinib, Ritlecitinib, Tofacitinib, Upadacitinib, and Everolimus, are approved for the treatment of inflammatory conditions, such as atopic dermatitis, rheumatoid arthritis, graft vs. host disease, psoriasis, alopecia areata, ulcerative colitis, and lymphangioleiomyomatosis (non-neoplastic disease) [13].

Development efforts for inhibitors have stalled or have been abandoned for at least 11 kinase families and about 20 kinase targets, of which no agents are currently in active trials [99]. The Aurora kinase family (Aurora kinase A, Aurora kinase B, and Aurora kinase C) remains the most targeted kinase family without an approved agent despite 22 compounds having entered clinical trials. Aurora kinases, known for their role in cell division and overexpression in numerous human tumors, have long been therapeutic targets. However, more than 80% of Aurora kinase inhibitors that reached clinical trials have since been discontinued [100].

2. Cyclic Peptides as Small-Molecule Therapies Through Human Protein Kinase Inhibitors

Peptide-based medications may pose less risk, as their degradation yields only amino acids, which are harmless once they bind to their target molecules [101]. Compared to small molecules used in conventional treatments, peptides exhibit several advantages, including reduced tissue accumulation, high selectivity, exceptional potency, and specific biotarget interaction. In this sense, ultra-short peptides, defined as sequences of up to 7 amino acids, typically containing no more than 45 amino acids, stand out for their unique structural and functional properties. Ultra-short peptides present strengths and limitations; their immense potential as versatile scaffolds for diagnostics and therapeutic applications is undeniable [102]. A key strategy for enhancing the performance of these peptides is cyclization, which stabilizes their active conformation. Cyclic ultra-short peptides not only retain the advantages of their linear counterparts, as it is, exhibiting enhanced mechanical stability, efficient tissue penetration, and reduced immunogenicity, along with bio- and cytocompatibility, characterized by non-hemolytic and non-cytotoxic behavior [103]. Moreover, the cyclic ultra-short peptides *in vivo* improve stability, resistance to protease, and plasma degradation [103], and lead to a high bioavailability [104], excellent membrane permeability [105], strong binding affinity [106], and limited conformational flexibility [107]. These attributes make cyclic ultra-short peptides exceptionally versatile and position them as up-and-coming candidates for advancing therapeutic and diagnostic innovations. Unlike small molecules in traditional therapies, peptides offer several advantages, including minimal tissue accumulation, high selectivity, potency, and biotarget specificity [108]. Cyclic peptides (CPs) are polypeptides composed of two or more amino acids, canonical or non-canonical, linked by amide bonds in a circular sequence [104]. The ring structure can be formed by joining one end of the peptide to another through amide bonds or other chemically stable bonds like lactone, ether, thioether, or disulfide. Many cyclic peptides gain their ring structure through amide bond formation between the amino and carboxyl termini, a method called N-to-C (or head-to-tail) cyclization [109]. CPs can naturally be synthesized post-ribosomally (via post-translational modifications) or through non-ribosomal pathways, with non-ribosomal synthesis primarily involving amide bonds. In contrast, post-ribosomal synthesis commonly uses disulfide bonds [110].

Cyclic peptides are approved for various therapeutic uses, including antibiotic, antifungal, anti-cancer, and immunosuppressive activities [111]. These peptides are sourced from terrestrial and marine environments [112], animal toxins [113], plants [9], and microorganisms [114], and are even found as endogenous compounds in humans. Another way to obtain cyclic peptides is through chemical synthesis, which allows for sufficient quantities for large-scale biological testing. Although science has advanced to the point where cyclic peptides can be modified and synthesized to achieve specific biological properties, most cyclic peptides used in approved drugs are still of natural origin [115].

Compared to linear peptides, cyclic peptides identified through library screening exhibit enhanced selectivity, potency, bioavailability, and metabolic stability. Their rigid structure reduces the entropy of Gibbs free energy, enabling the stronger binding to target molecules or receptors. The absence of carboxyl and amino termini in cyclic peptides also makes them resistant to hydrolysis by exopeptidases and, in some cases, endopeptidases [116]. CPs with structures of 5 to 10 residues can bind with antibody-like specificity and affinity [106]; this structural stability in the CPs directly contributes to their bioactivity, as a longer residence time increases their capacity to interact with various pharmacological targets.

The membrane permeability of drugs often depends on their physicochemical characteristics, such as molecular weight, hydrogen bonding, and polar surface area [117]. Peptides are generally large and polar and cannot diffuse via passive transport across the cell membrane [118]. The cyclization of peptides can help increase membrane permeability by promoting intramolecular hydrogen bonds that mask the polar regions; however, permeability tends to decrease with larger molecules (over ten amino acids and >1 kDa). Synthetic peptides are usually designed to target extracellular receptors [104]. Naturally

occurring CPs with a high membrane permeability often have N-methylamide bonds, ester bonds, or both in their leading chains [105,119].

The potential of CPs to mimic the binding of endogenous molecules, including receptors, transcription factors, and enzymes, is crucial for their biological effects. Kinases naturally interact with endogenous proteins and peptides, which can serve as potent inhibitors or activators. The peptide-based and cyclic structure of the CPs allows them to replicate interactions between kinases and their natural substrates, thereby influencing kinase activity, a critical factor in health and disease. Reversible protein phosphorylation, mediated by kinases and phosphatases, is vital for biological and pathological processes. By transferring the γ -phosphate group from ATP to serine (Ser), threonine (Thr), and tyrosine (Tyr) residues, protein kinases regulate core cellular functions like metabolism, cell cycle progression, and cytoskeletal organization. CPs have shown significant effectiveness in disrupting protein–protein interactions [120].

Cyclic Peptides as Therapeutic Tools: Kinase Inhibition and Biomedical Applications

Cyclic peptides can selectively target microbial proteins that differ from human proteins, making them suitable for developing antimicrobial drugs with minimal toxicity to patients [121]. Recently, the FDA approved several CPs for treating bacterial and fungal infections; for example, Telavancin and Daptomycin are effective against Gram-positive bacteria, mainly by disrupting cell membrane homeostasis through depolarization [122,123]; Polymyxin B targets Gram-negative bacteria by altering the lipid A component of lipopolysaccharide in the outer membrane [124]. Antifungal agents such as Anidulafungin inhibit fungal cell wall synthesis, leading to osmotic cell lysis [125]. Research is also underway on CPs for viral infections, as their affinity for viral fusion proteins shows antiviral potential [126]. For instance, CPs have been studied in SARS-CoV-2, where they block receptor binding in cultured cells in a dose-dependent manner [127]. A similar effect is seen in human immunodeficiency virus (HIV), where CPs interfere with the specific interaction between the virus and host proteins, preventing viral replication [128]. CPs can also function as inhibitors of RNA–protein interactions and enzymes, effectively targeting proteases, phosphatases, the proteasome, HIV integrase, and dam methyltransferase, among others [129].

Cyclic peptides emerged as promising therapeutic agents for the inhibition of kinases; deregulated kinases are often oncogenic and vital for cancer cell survival and proliferation (Table 1). The HGF-MET pathway drives essential processes like invasion, metabolic reprogramming, angiogenesis, metastasis, immune evasion, and drug resistance within the tumor microenvironment, all of which contribute to malignant transformation [130,131]. Although no selective HGF-MET inhibitors are yet to be approved as cancer therapies, the selective inhibition of MET activation is anticipated to be an effective molecularly targeted cancer treatment. HGF is synthesized and secreted as single-chain HGF (scHGF), which becomes active two-chain HGF (tcHGF) after cleavage at Arg494-Val495 [132]. Only tcHGF can activate MET, as scHGF is an inactive precursor that cannot activate the MET receptor. The cyclic peptide HiP-8 inhibits MET activation/phosphorylation in cultured human cells, binding specifically to tcHGF with a dissociation constant (KD) of 0.4 nM and a slow dissociation rate ($k_{\text{off}} = 0.4 \times 10^{-3} \text{ s}^{-1}$). HiP-8 exhibits an IC₅₀ of 0.9 nM, indicating the potent inhibition of the HGF-MET interaction. Notably, HiP-8 inhibits only tcHGF and not scHGF, suggesting its potential as cancer therapy [133]. The proteolytic conversion of scHGF to its active form by serine endopeptidases like matriptase, hepsin, and the HGF activator is the rate-limiting step in HGF/MET signaling. A novel class of macrocyclic peptide inhibitors, such as compound VD2173, potently inhibits matriptase and hepsin, blocks scHGF activation in lung cancer models, halts HGF-mediated wound healing, and overcomes the resistance to EGFR- and MET-targeted treatments [134].

Inhibiting angiogenesis has become a key strategy in cancer therapy. The new proapoptotic peptide CIGB-300 (P15-Tat) has demonstrated antiangiogenic effects on endothelial cells by disrupting the CK2-mediated phosphorylation. CPs are widely used as scaffolds

in drug design due to their notable stability and favorable biopharmaceutical properties. Two highly stable cyclic trypsin inhibitors, MCoTI-II and SFTI-1, serve as frameworks to integrate the bioactive epitope P15 into various backbone loops. This approach enhances the antiangiogenic activity and stability of P15 when integrated into cyclic trypsin inhibitor scaffolds [135].

The human epidermal growth factor receptor 2 (HER2) is overexpressed in numerous cancers, making it an important therapeutic target [136]. Trastuzumab is an FDA-approved monoclonal antibody that targets HER2 and is used to treat HER2-positive cancers [137]. The cyclic peptide Cyclo-GCGPep1 was designed in silico, based on the binding interaction between an antibody and the HER2 protein to create peptide–drug conjugates with Camptothecin. Biological assessments show that the resulting conjugate has antiproliferative solid effects on SK-BR-3 and NCI-N87 cells, retaining Camptothecin’s pro-apoptotic and Topo I inhibitory actions. Simultaneously, Cyclo-GCGPep1 enables the effective targeting of HER2-positive cells.

Compared to Camptothecin alone, the conjugated peptide shows superior permeability in tumor spheroid models. Designing CPs based on antibody–protein target binding modes is crucial for developing new therapeutic strategies. The Cyclo-GCGPep1 and Camptothecin conjugate holds promise as a treatment for HER2-positive cancers [138].

The polo-box domain (PBD) of Polo-like kinase 1 (Plk1) is a promising target for cancer therapies. PBD plays a role in substrate targeting and Plk1’s subcellular localization and it regulates kinase activity through an auto-inhibitory interaction with the kinase domain. Plk1, a key serine/threonine kinase, is essential in various mitotic processes, including mitotic entry, centrosome maturation, bipolar spindle assembly, chromosome segregation, and cytokinesis [139]. Elevated Plk1 levels and activity are associated with tumor progression and a poor prognosis [140]. These findings indicate that targeting Plk1/PBD could be an effective strategy for developing new anti-cancer drugs. Innovative phosphorylated macrocyclic peptidomimetics, engineered based on the acyclic phosphopeptide PMQSpTPL, target PBD and demonstrated anti-tumor activity both in vitro and in vivo. The novel macrocyclic peptidomimetics could be powerful templates for developing potent Plk1-PBD inhibitors [141].

Cancer cells undergo an epithelial–mesenchymal transition (EMT), transforming into invasive forms that spread to other organs. A novel treatment strategy to inhibit cancer cell migration and invasion involves targeting molecules associated with the EMT process. The cyclic-pentapeptide (-Arg-Gly-Asp-D-Phe-Lys) (cRGDfK) has been recognized as an integrin $\alpha_v\beta_3$ inhibitor, functioning as a targeted peptide to enhance tumor cell sensitivity to drugs in anti-angiogenic cancer therapy. When used in conjunction with cRGDfK, Sunitinib increased its anti-EMT effects and decreased the expression levels of mesenchymal marker mRNA and protein compared to its use alone [142]. The use of cyclic pentapeptides (cRGDfK) containing the arginine-glycin-aspartic acid (RGD) motif enhances the target selectivity and drug delivery of anti-cancer medications [143,144]. Numerous studies have demonstrated the potential of RGD-containing peptides as delivery vehicles for anti-cancer drugs. Cyclic peptides, particularly cRGDfK and cRGDyK (cyclo Arg-Gly-Asp-D-Tyr-Lys), exhibit a strong affinity and selectivity for binding integrin $\alpha_v\beta_3$. Thus, CPs can deliver therapeutic small molecules such as doxorubicin and paclitaxel to cancer patients [145].

The epidermal growth factor receptor (EGFR) family comprises EGFR (ErbB1), ErbB2, ErbB3, and ErbB4, all exhibiting tyrosine kinase activity. These receptors can form homodimers or heterodimers upon binding to ligands, leading to the phosphorylation of specific tyrosine residues in their intracellular domains. These phosphorylated residues serve as docking sites that can activate several intracellular signaling pathways [146]. The activation of EGFR signaling is associated with various physiological and pathological processes, including embryonic development, cell proliferation, cell survival, and cancer [147]. Small-molecule inhibitors, such as Gefitinib and Erlotinib, have been clinically approved to target the tyrosine kinase domain of EGFR (TK-EGFR). Lazertinib, a recently developed third-generation EGFR tyrosine kinase inhibitor (TKI), is used for patients with advanced

EGFR T790M-positive non-small-cell lung cancer who have shown disease progression after treatment with first- and second-generation TKIs [148] (Figure 2; Table 1). Despite progress, significant concerns persist regarding the severity of side effects, off-target effects, and drug resistance. Among the CPs explored for novel drug development, NP1 has shown an inhibitory effect on TK-EGFR with an IC₅₀ in the nanomolar range. NP1 has a binding affinity (K_D) of 18.40 ± 5.50 μ M for EGFR. It has been shown to inhibit phosphorylated EGFR levels upon activation in cancer cells [149]. The EGFR gene can undergo several mutations, particularly in exons 18–21, which encode part of the EGFR kinase domain associated with certain lung cancer types [150]. The most common mutation is EGFR variant III (EGFRvIII), which involves the deletion of exons 2–7 and is linked to more aggressive diseases and a worse prognosis [151]. Two cyclic peptides, c(CHVPGSYIC) and c(CVNAMQSYC), effectively bind and internalize the EGFR and EGFRvIII cell lines. Docking simulations revealed that the peptides interact with the EGFR receptor near the EGF binding site, showing substantial overlap with EGF [151]. A novel cyclic peptide, M-2-5, demonstrates the robust targeting of the extracellular domain of EGFR, binding tightly and effectively antagonizing EGF stimulation; EGFR phosphorylation and the subsequent signal transduction, representing a potential therapeutic use for cancer treatment [152]. PAD-1, a cyclic peptide self-assembled into a scaffold, recognizes the membrane protein EGFR and arrests EGFR signaling by inducing aggregation through multivalent interactions. By promoting by the endocytosis of overexpressed EGFR on cancer cell membranes, PAD-1 forms nanofibers in the lysosome, resulting in lysosomal membrane permeabilization (LMP). This mechanism disrupts EGFR homeostasis and inhibits downstream signaling transduction, presenting a promising therapeutic approach [153].

Pathological angiogenesis begins when abnormally expressed vascular endothelial growth factors (VEGFs) bind to their receptors (VEGFRs). Inhibiting the interaction between VEGF and VEGFR has been clinically validated as an effective cancer treatment. Novel cyclic peptides A-cL1, B-cL1, and P-cL1 mimic Loop 1 of VEGF-A, VEGF-B, and PlGF. These peptides primarily bind to the D3 domain of VEGFR1 and VEGFR2, successfully blocking their activation. They exhibit antiangiogenic and anti-cancer effects by inhibiting the MAPK/ERK1/2 signaling pathway. The VEGFR-targeting peptide VEGF125–136 (QR) blocked VEGF signaling, promoting tumor vascular normalization. When conjugated with a lytic peptide (KLU), it forms a peptide–drug conjugate QR-KLU, inhibiting liver cancer cell growth and downregulating VEGF expression under normoxic and hypoxic conditions [154].

Renal fibrosis is a standard and irreversible process associated with chronic kidney disease (CKD), characterized by excessive extracellular matrix deposition. It is a common endpoint for nearly all forms of CKDs. Despite significant advancements in recent years, no approved antifibrotic therapies for kidney conditions [155].

The extracellular matrix (ECM) protein CCN2 regulates the fibrotic process by activating the EGFR signaling pathway. To create compelling and durable selective inhibitors of the CCN2/EGFR interaction, new peptides targeting CCN2 have been developed. Notably, the 7-mer cyclic peptide OK2 shows intense activity in inhibiting STAT3 phosphorylation and cellular ECM protein synthesis induced by CCN2/EGFR activation. In vivo experiments also indicate that OK2 significantly reduced renal fibrosis in a mouse model of unilateral ureteral obstruction, providing a promising new strategy for treating renal fibrosis [156].

The monomeric GTPases K-Ras, H-Ras, and N-Ras play critical roles in various signaling pathways, such as cell proliferation, differentiation cytoskeletal reorganization, membrane trafficking, and apoptosis, with mutations found in about 30% of cancer cases [157,158]. Genetic studies indicate that inhibiting the interaction between Ras and its effector proteins could be a potential strategy for treating cancer patients. However, developing effective drugs has proven challenging due to the lack of distinct binding pockets for small-molecule drugs on the Ras protein surface. Wild-type Ras cycles consisting of its inactive GDP-bound state (Ras-GDP) and its active GTP-bound state (Ras-GTP),

the Ras-GTP activating effector proteins like Raf, PI3K, and Ral-GDS, or the inhibitors designed to block Ras-effector protein interactions have typically exhibited deficiencies in potency, selectivity, and/or membrane permeability [159]. Cyclorasin 9A5 is an 11-residue cyclic peptide that can penetrate cells and selectively inhibits Ras-GTP. The simultaneous inhibition of the MEK and PI3K signaling pathways results in a combined decrease in cell viability and an increase in apoptosis in Ras mutant cancer cells. Cyclorasin 9A5 effectively disrupts protein–protein interactions (PPI) in H358 lung cancer cells and reduces Ras-dependent signaling in a dose-dependent manner, with an IC₅₀ of approximately 3 mM [160]. Structure–activity relationship studies found that Cyclorasin 9A54 exhibits even more potent PPI inhibition (IC₅₀ = 18 nM). Both peptides contain a cell-penetrating peptide (CPP)-like motif (Arg-Arg-dNal-Arg-Fpa) where dNal is d-b-naphthylalanine and Fpa is l-4-fluorophenylalanine. However, only Cyclorasin 9A5 effectively penetrates cells to demonstrate significant cell-based activity, suggesting that the conformational flexibility allowing the peptides to adopt an amphipathic structure is essential for their ability to permeate cells [161].

KS-58 is a bicyclic peptide advancing the development of new cancer therapeutics. It enters cells and blocks the interaction between K-Ras (G12D) and their effectors, restricting cell growth and showing considerable resistance to protease degradation [162]. P110 α , a family member of the phosphoinositide 3-kinase (PI3K), is critical in transducing signals downstream of RAS. RAS proteins facilitate the activation of p110 α through direct interaction with its RAS binding domain (RBD), thereby promoting various cellular functions, including growth, proliferation, and survival. Cyclo-CRVLIR is a novel kinase inhibitor that binds to the p110 α -RBD domain, blocking its interaction and exhibiting promising therapeutic potential in RAS-associated malignancies [163].

N-methyl-D-aspartate receptors (NMDARs), neuronal nitric oxide synthase (nNOS), and Postsynaptic Density Protein-95 (PSD-95) form a complex that is an attractive therapeutic target for the treatment of acute ischemic stroke. The complex is formed via the PDZ protein domains of PSD-95. Attempts to disrupt the complex often rely on C-terminal peptides sourced from the NMDAR; however, nNOS interacts with PSD-95 via the β -hairpin motif, presenting an alternate foundation for developing PSD-95 inhibitors. The inhibition strategy interrupts the nNOS/PSD-95 interaction using a cyclic nNOS β -hairpin mimetic peptide. Peptide macrocycles exhibit one of the highest affinities documented for a PDZ domain, providing insights for developing a treatment for acute ischemic stroke. [164]. Tropomyosin receptor kinases (Trks) are tyrosine kinases activated by neurotrophic factors called neurotrophins. TrkA engages with nerve growth factor (NGF), leading to pain induction. A macrocyclic peptide named Peptide 19 obstructs interactions between TrkA and NGF (IC₅₀ = 21 nM), resulting in an analgesic effect in a rat model of incisional pain [165].

Momelotinib is an ATP-competitive small-molecule type I inhibitor of Janus kinases JAK1 and JAK2, approved for use in anemic patients with high- or intermediate-risk myelofibrosis (MF) (Figure 2) [166]. Cytokines act as communicative agents for the immune system, serving critical roles in various biological responses and influencing the immunological response. When cytokine production or biological activity is disrupted, the homeostatic balance of the immune response is altered, resulting in many diseases, including autoimmune and inflammatory disorders. Cytokines attach to specific receptors on cells, activating intracellular enzymes known as Janus kinases (JAKs). JAK3 modulates a limited range of γ cytokines, rendering it a potentially optimal target. Ritlecitinib is the most advanced selective irreversible JAK3 type VI inhibitor, exhibiting no activity against other JAK isoforms, and is approved for the treatment of alopecia areata (Figure 2). Z583 is a new and effective inhibitor of JAK3 with significant therapeutic potential for autoimmune illnesses, particularly in rheumatoid arthritis (RA) [167]. The activation of the Janus kinase/signal transducers and activators of the transcription (JAK/STAT) pathway is primarily marked by the building and disassembly of protein complexes, which are strongly associated with the expression of inflammatory genes. Suppressors of cytokine signaling (SOCS) proteins negatively regulate the JAK/STAT pathway. SOCS1 possesses a small

kinase inhibitory region (KIR) responsible for inhibiting JAK kinases. A peptidomimetic of KIR SOCS1, designated PS5, diminishes JAK/STAT phosphorylation and the production of interferon regulatory factor-1 (IRF-1), leading to a significant decrease in inflammatory gene expression, demonstrating potential therapeutic effects in cardiovascular diseases [168].

PEP1, a cyclic peptide, efficiently transports into COS-1 cells and inhibits the phosphorylation of Gonadotropin-regulated testicular RNA helicase (GRTH) in a dose-dependent manner [169]. Rats, mice, and humans express GRTH, a constituent of the DEAD-box family of RNA helicases, in their testes, functioning as a post-transcriptional regulator of genes essential for the completion of spermatogenesis [170]. Prior research suggested that blocking the GRTH phospho-site or the interaction between GRTH and protein kinase A (PKA) might help create male contraceptives [171].

One tactic to potentially improve peptide therapies effectiveness, target selectivity, and metabolic regulation is the introduction of non-natural amino acids [172]. D/L-Hybrid peptides represent another exciting type of molecular modality due to their enhanced resistance to proteolysis and broader structural diversity compared to peptides composed only of proteinogenic L-amino acids. Macrocyclic D/L-hybrid peptides, featuring five types of D-amino acids (D-Tryptophan, D-Alanine, D-Serine, D-Histidine, and D-Tyrosine), demonstrate inhibitory activity against hEGF–hEGFR interaction and exhibited remarkable protease resistance after 24 h of incubation in human serum [173]. Moreover, the hEGF–hEGFR interaction can be inhibited by α/β^3 -macrocyclic peptides (in which β -homoglycine, β -homoproline, β -homophenylglycine, and β -homoglutamine are introduced by reprogramming the genetic code). Peptides composed of β -amino acids frequently exhibit distinctive and robust folding capabilities, thereby earning the designation of foldamers [174]. Peptides composed exclusively of β -amino acids (β -peptides) and those having a combination of α - and β -amino acids (α/β -peptides) exhibit superior folding capabilities relative to α -peptides. Due to the self-folding capability of these α/β -peptides, they can achieve a favorable entropic effect relative to α -peptides, resulting in an anticipated enhanced binding affinity to target proteins. Furthermore, α/β -peptides typically exhibit enhanced proteolytic stability compared to α -peptides due to the absence or limited efficacy of naturally occurring peptidases on peptide bonds containing β -amino acids [175]. The application of β^3 AAs (β^3 -amino acids) improves the pharmacological characteristics of peptides, offering a distinctive platform for developing macrocycles targeting a specific protein [176].

3. Cyclodipeptides, a Type of Cyclic Peptides with Potential Use in Human Diseases That Are Protein-Kinase-Associated

Cyclodipeptides (CDPs) or diketopiperazines are the simplest cyclic peptides found abundantly in nature [177] (Figure 3). CDPs that include proline exhibit distinctive characteristics such as an exceptionally rigid conformation, significant resistance, enhanced cellular permeability, and an augmented ability to bind various substrates with superior affinity. CDPs have emerged as crucial metabolic intermediates and a promising platform for therapeutic advancement. These molecules offer the structural stability and functional versatility characteristic of cyclic peptides, making them a unique class of biomolecules with vast potential [178,179]. They are often regarded as “diamonds in the rough”, presenting numerous opportunities for innovative therapies. Since their initial discovery in 1924, there has been growing interest in their applications, with multiple studies in recent years highlighting their broad-spectrum biological activities. This increasing attention underscores the potential of CDPs to play a pivotal role in future therapeutic advancements [180].

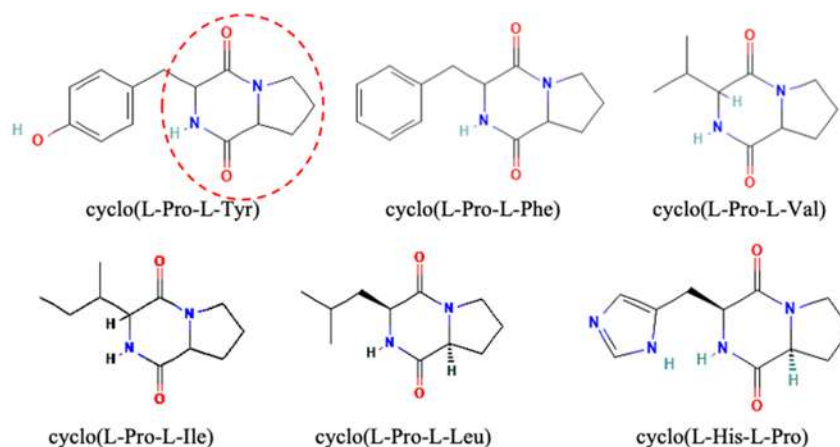


Figure 3. Molecular structures of bacterial cyclodipeptides (CDPs). Discontinuous circles show the adenine pocket in the therapeutic agent. Structures loaded from PubChem database (<https://pubchem.ncbi.nlm.nih.gov>, accessed on 22 September 2024).

CDPs have been gaining increased attention since they are biomolecules with structural diversity, an absence of charged termini, enhanced lipophilicity facilitating rapid membrane absorption, reduced conformational freedom, and distinctive structural rigidity, which imparts more excellent resistance against enzymatic degradation, and their cyclic nature contributes to increased stability [178,179]. Moreover, CDPs have broad-spectrum bioactive properties and can be functionalized, making them promising scaffolds for a new drug design [180,181]. Due to these notable chemical features and diverse pharmacological activities, including the involvement of signal transduction pathways, where protein kinases participate [182–187], CDPs can potentially become candidates for drug development as PKI [178]. Regarding safety, initial studies indicate that CDPs have a low toxic profile in preclinical systems, particularly in cell cytotoxicity tests [188–190]. This makes them attractive candidates for new drug development. However, challenges still relate to the specificity of their biological activity, stability in physiological media, and possible accumulation in tissues, which could influence their long-term safety. Therefore, more extensive studies, including in vivo assays and comprehensive toxicological analyses, are needed to ensure their safe use in humans.

3.1. Cyclodipeptide and Protein Kinase Interaction in Cancer

The cyclo(L-Pro-L-Tyr) (cPT), cyclo(L-Pro-L-Val) (cPV), and cyclo(L-Pro-L-Phe) (cPP) from *Pseudomonas aeruginosa* PAO1 (CDP-PA) [191] promoted cell death and apoptosis in a dose-dependent manner in cultures of cervical adenocarcinoma (HeLa cells), colorectal adenocarcinoma (Caco-2 cells) [189], melanoma (B16-F0 cells) [183], and human breast cancer (MCF-7 and MDA-MB-231 cells) [192] (Figure 4). The CDPs cPL, cPV, and cPP were applied in an isolated way in HeLa cells and also promoted cell death with an LD50 of 61, 37, and 15 µg/mL, respectively, and induced apoptosis with an EC50 of 0.32, 0.27, and 0.50 µg/mL respectively [188]. In the B16-F0 melanoma cell line, the CDP-PA mix showed an LD50 of 10.7 µg/mL and EC50 of 24 µg/mL [183]. In MCF-7 and MDA-MB-231 cell lines, the LD50 was 50 and 10 µg/mL, respectively, while the EC50 was 50 µg/mL [192].

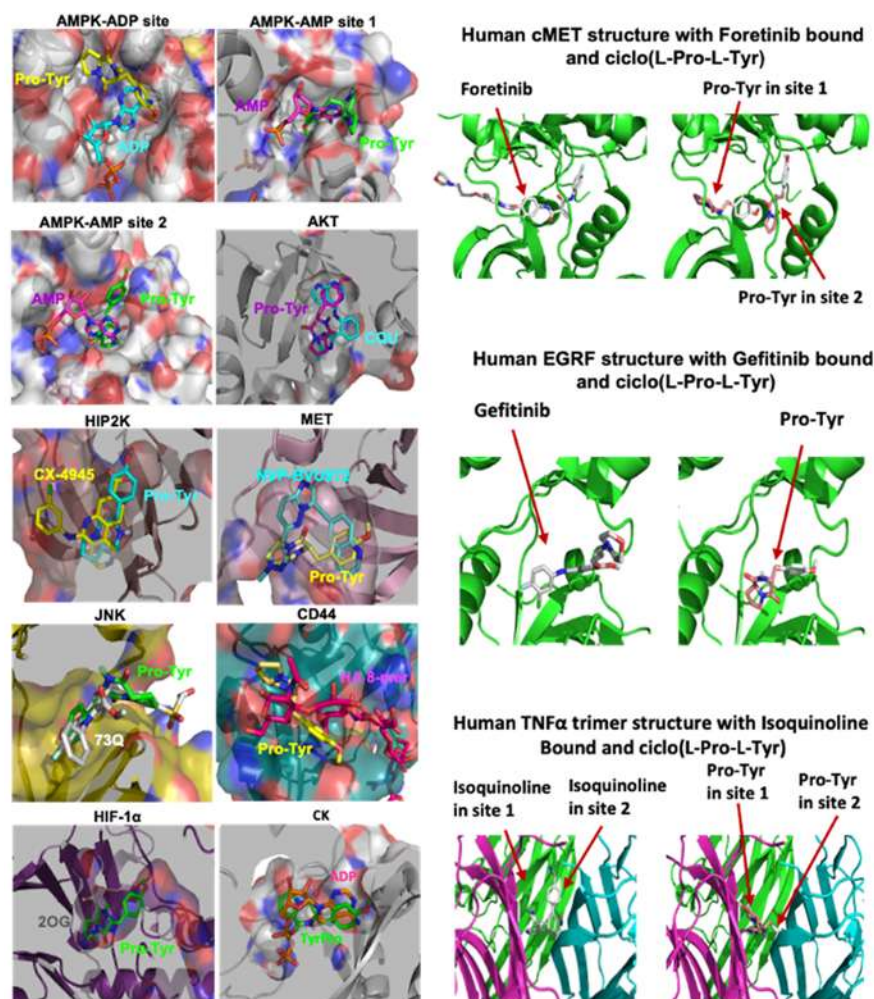


Figure 4. Molecular docking of human protein kinases interacting with substrates, inhibitors, and bacterial cyclodipeptides (CDPs). Docking analysis of the CDPs in the binding sites of tyrosine kinases. The protein is shown as a surface with the interior in the cartoons. Pro-Tyr, cyclo(L-Pro-L-Tyr); CQU, (N-[2-(5-methyl-4H-1,2,4-triazol-3-yl)phenyl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine; CX-4945, 5-[(3-chlorophenyl)amino]benzo[c][2,6]naphthyridine-8-carboxylic acid; NVP-BVU972, 6-[[6-(1-methyl-1-H-pyrazol-4-yl)imidazo[1,2-b]pyridazin-3-yl]methyl]quinolone; 73Q/DB07218, 6-chlor-9-hydroxy-1,3-dimethyl-1,9-dihydro-4H-pyrazolo[3,4-B]quinolin-4-one; HA 8-mer, hyaluronan octamer; 2OG, 2-oxoglutarate. AMPK, AMP-activated protein kinase; AKT, protein kinase B (PKB); HIP2K, homeodomain-interacting protein kinase 2; MET, mesenchymal-epithelial transition factor; JNK, c-Jun N-terminal kinases; CD44, homing cell adhesion molecule; HIF-1 α , hypoxia-inducible factor 1-alpha; CK, creatine kinase; cMET interaction with Foretinib (inhibitor) and cyclo(L-Pro-L-Tyr) in binding sites 1 and 2; human epidermal growth factor (EGF) interaction with Gefitinib (inhibitor) and cyclo(L-Pro-L-Tyr) in the binding site. Human TNF- α interaction with isoquinoline inhibitor on sites 1 and 2 and with the cyclo(L-Pro-L-Tyr) in both binding sites. Adapted from [183,192].

The xenotransplant melanoma tumor model caused a substantial reduction in both the weight and volume of the implanted tumor, with measurements of 5.5 g compared 2 g and 2100 mm³ versus 30 mm³ for untreated versus CDP-treated mice, respectively. Interestingly, mice injected with the B16-F0 tumor and concurrently treated with CDP-PA did not develop

tumors in 40% of the subjects. In B16-F0 melanoma tumor cells treated with CDPs, apoptosis was associated with an exacerbated generation of ROS. Immunoblotting analysis indicated the involvement of the PI3K–Akt–mTOR signaling pathway which occurred since the phosphorylation of Akt-S473 and S6k-T389 was strongly activated in the tumor mice group, which decreased in the CDP treatment group [183]. Met C-terminal regulatory domain tail phosphorylation acts as a docking site for intracellular adaptors of molecules, activating downstream signaling cascades, such as Rac1/Cdc42, PI3k/Akt, and Erk/MAPK. In breast cancer MCF-7 cells, phosphorylation on the Met-Tyr1003, EGF-Tyr1068, and the Gab1-Tyr307 factor was significantly decreased by the CDP-PA exposure; however, in MDA-MB-231 cells, the Met-Tyr1003 phosphorylation was induced considerably by the CDP treatment [192].

The mechanism by which CDPs inhibit cell proliferation in HeLa cells includes the blockade of DNA synthesis and the arrest of cells at the G0–G1 stage. This process induces apoptosis through an intrinsic pathway reliant on caspase-9 and caspase-3 activation, affecting the mitochondrial functionality before cytochrome *c* release and increasing ROS generation [188]. The inhibition of cell proliferation occurred through the PI3K/Akt/mTOR, Ras/Raf/MEK/ERK1/2, PI3K/JNK/PKA, p27Kip1/CDK1/survivin, MAPK, HIF-1, Wnt/ β -catenin, HSP27, EMT, and CSCs signaling pathways and receptors, such as EGF/ErbB2/HGF/Met, since the inhibition of the phosphorylation of Stat3-Tyr705, Akt-Thr308, Akt-Ser473, AMPK α -Thr172, S6-RP-Ser235/236, mTOR-Ser2448, HSP27-Ser78, Bad-Ser112, p70S6k-Thr389, PRAS40-Thr246, P53-Ser15, P38-Thr180/Tyr182, SAPK/JNK-Thr183/Tyr185, PARP-Asp214, Caspase 3-Asp175, GSK-3 β -Ser9, Gab1-Tyr307, Met-Tyr1003, Met-Tyr1234/1235, Met-Tyr1349, EGF-Tyr992 and EGF-Tyr1068 with a cyclic behavior in a time- and concentration-dependent manner was shown [188,192,193]. The further implication of the PI3K/AKT/mTOR pathway was confirmed by utilizing the AZD8055 mTOR inhibitor and LY294002 PI3K inhibitor [193].

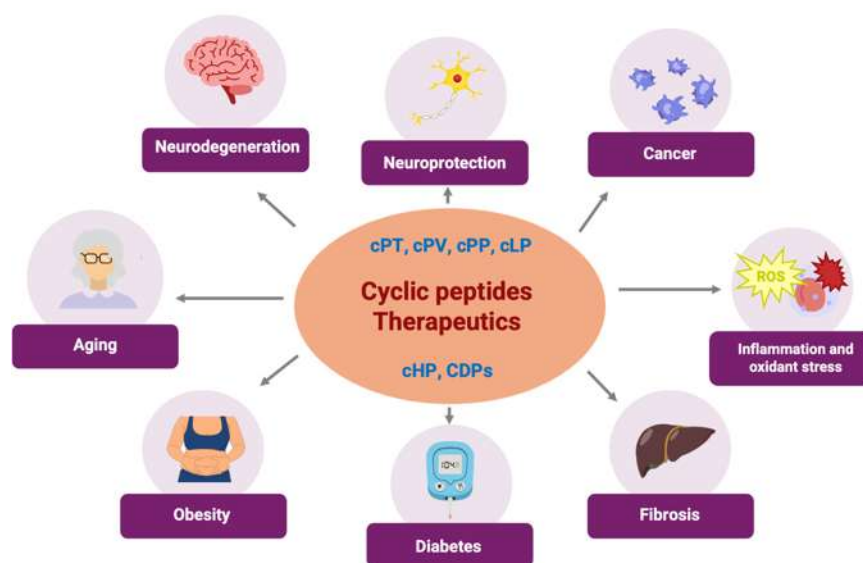
The transcriptomics analysis of untreated and CDP-PA HeLa cells exhibited 151 differentially expressed genes that were either up- or downregulated in response to CDP-PA across 15 biological processes. These processes included cellular component organization or biogenesis, cellular processes, localization, biological regulation, response to stimulus, signaling, developmental processes, rhythmic processes, multicellular organismal processes, locomotion, biological adhesion, metabolic processes, growth, cell population proliferation, and immune system processes. The biological pathways potentially targeted by CDP-PA in HeLa cells included the insulin/IGF/MAP kinase cascade, P38/MAPK, interleukins, EGF receptor, PI3K kinase, PDGF, Notch, cadherin, P53, the gonadotropin-releasing hormone receptor pathway, Hedgehog, TGF- β , FGF, the FAS signaling pathways, the Gonadotropin-releasing hormone receptor pathway, angiogenesis, cholesterol biosynthesis, and additional pathways related to inflammation, oxidative stress response, apoptosis, cytoskeleton, and T- and B-cell growth/activation [187]. This fact confirmed the role of CDP-PA in various signal transduction pathways, including PI3K-Akt, mTOR, FoxO, Wnt, MAPK, P53, and TGF- β .

A molecular docking analysis showed possible interactions between bacterial CDPs and protein kinases such as AKT, HIPK2, AMPK, MET, JNK, HIF-1 α , CD44, cMET, and EGFR [183,192]; thus, the CDP-PA mechanism for inhibiting or activating protein kinases in cancer models may involve obstructing the substrate binding site of these protein factors (Figure 4). The CDP-PA mix demonstrates a negligible impact on apoptosis induction in cultures of normal human mononuclear blood cells, as well as in cellular structures from the spleen, liver, heart, kidney, lung, and skin from treated mice; thus, CDP-PA treatments were safe in those models [183,188]. The findings suggest that CDP-PA has potential as PKI anti-neoplastic agents for treating human cervical adenocarcinoma and melanoma.

3.2. Cyclo(Pro-Tyr)

Cyclo(Pro-Tyr) is also produced by sponge *Callyspongia fistularis*, *Bacillus pumilus* [194], and *Bacillus* sp. [195] (Figure 3). This CDP demonstrated cytotoxic and apoptotic-inducing activity against the human hepatocellular carcinoma (liver cancer) HepG2 cell line, with an

IC50 value ranging from 42.98–140 µg/mL. In contrast, no toxic effects on non-cancerous cell lines were observed. Cyclo(Pro-Tyr)-treatments significantly increased the percentage of cells in the G2/M phase, induced ROS production, and caused nuclear fragmentation, chromatin condensation, and membrane blebbing. Additionally, the Bax/Bcl2 ratio protein expression was significantly amplified, as was the protein expression of cytochrome *c*, caspase-3, and cleaved PARP, indicating that apoptosis was promoted [194]. Hepatocellular carcinoma (HCC) generated by N-diethylnitrosamine (DEN) in male Swiss albino mice caused weight loss. Meanwhile, in cyclo(Pro-Tyr)-treated mice, the body weight of the animals was equal to that of the control group. In cyclo(Pro-Tyr)-treated mice, aspartate and alanine aminotransferase markers of liver damage showed decreased levels of both enzymes, cell proliferation was inhibited, and apoptosis was stimulated [196] (Scheme 1).



Scheme 1. Cyclic peptides play a pivotal role in future therapeutic advances. Due to their remarkable chemical characteristics and diverse pharmacological activities, cyclic peptides can potentially become candidates for drug development as protein kinase inhibitors, as they can interact with kinases of relevance in multiple diseases. The cyclic dipeptides cPT, cPV, cPP, and cLP (CDPs, Figure 3) modulate protein kinases to affect multiple cancer-related processes. At the same time, cHP targets protein kinases related to neurodegeneration, fibrosis, obesity, aging, diabetes, inflammation, and ROS production.

Like in the CDP-PA mix, the PI3K/AKT signaling pathway plays a vital role in cyclo(Pro-Tyr)-treated cells and animals since phosphorylated PI3K-Y607 and phosphorylated AKT-T308 levels were diminished. In contrast, the levels of PTEN (a negative pathway regulator), Bax, Caspase 3, and P53 increased [194–197]. Thus, cyclo(Pro-Tyr) has the potential to act as a PKI anti-cancer drug, suppressing HCC tumor development by targeting the PI3K/AKT pathway.

3.3. Cyclo(L-Leu-L-Pro)

The cyclo(L-Leu-L-Pro; cLP) has been isolated from *Lactobacillus coryniformis* BCH [198], *Staphylococcus* sp. MB30 has been observed to have anti-fungal, antimicrobial, and antioxidant activity. In addition, cyclo(L-Leu-L-Pro) has proven to have cytotoxic and antiproliferative activities against lung (A549) and cervical (HeLa) cancer cells [199] (Scheme 1).

In MDA-MB-231 and MDA-MB-468 Triple-Negative Breast Cancer (TNBC) cellular lines treated with cyclo(L-Leu-L-Pro) and with eribulin (a chemotherapeutic agent primarily for the treatment of metastatic breast cancer and liposarcoma), we observed cell cycle arrest

at the G2-M phase, inhibited proliferation and migration, and induced DNA strand breaks, DNA damage, and apoptosis; those effects were not detected in human breast healthy epithelial cell line (MCF-12A). A bioinformatics analysis predicted the cyclo(L-Leu-L-Pro) interaction with tetraspanin protein CD151 and EGFR, a member of the ErbB family of receptor tyrosine kinases. Immunoprecipitation assays showed that cyclo(L-Leu-L-Pro) reduces the interaction of CD151 with EGFR in MDA-MB 231 and MDA-MB 468 cell lines. Therefore, in TNBC cells, the cyclo(L-Leu-L-Pro) action mechanism could be through the disruption of CD151-EGFR crosstalk and the subsequent EGFR downstream signaling [200].

3.4. Cyclodipeptide-Protein Kinase Interaction Related to Antioxidant and Anti-Inflammatory Effects

Cyclo(His-Pro) (cHP) was discovered in the 1970s as an endogenous molecule produced by the cleavage of the hypothalamic thyrotropin-releasing hormone (TRH) [201,202]. cHP therapy offered cytoprotection, reduced apoptotic cell death, and decreased tissue damage in cells exposed to reactive oxygen species (ROS), pro-inflammatory or inflammation-induced tissue damage agents via transcription factor Nrf2 [182,185,186,190]. cHP stimulates Nrf2 phosphorylation and nuclear location and enhances ROS detoxification by the induction of gene expression [182,185,186,190,202,203]. Furthermore, cHP reduces inducible oxide nitric synthase (iNOS) gene expression with a consequent decrease in reactive oxygen-nitrogen species (ROSN) and ROS. Moreover, cHP inhibits the gene expression of the transcriptional factor involved in the inflammatory process, NF- κ B, which suppresses pro-inflammatory signaling pathways and reduces apoptosis activities such as caspase 3 and cytochrome *c* release [202] (Scheme 1).

In human LX-2 hepatic stellate cells, the cHP molecular mechanism has been associated with increased levels of cytosolic Ca^{2+} ; this fact may promote the phosphorylation of Nrf2 by protein kinase C (PKC) and the phosphorylated p38 MAPK prevented cHP-mediated Nrf2 activation [186,204].

3.5. Cyclodipeptide-Protein Kinase Interaction Related to Neuroprotective Effects

Following head trauma, metabolic changes are initiated, including alterations in ionic homeostasis, excitatory amino acids, free radicals, proteases, and inflammatory-immune responses. These changes ultimately cause neuronal cell death in adjacent areas of physical insult [205].

In paraquat-exposed hSOD-1G93A microglial cells, cyclo(His-Pro) treatment causes an early and sustained increase in glutathione (GSH), showing a protective effect against oxidant insults via nuclear translocation of Nrf2, an upregulation of Nrf2-driven genes, such as Gclc, Gclm, xCT, and Nqo1 by increased GSH. cHP inhibited ERK1/2 phosphorylation and delayed AKT inactivation. Furthermore, cHP enhances BDNF expression, which may lead to the proposal of this CDP as a neurotrophic agent [184] (Scheme 1).

The research has focused on novel drugs that target multiple injury factors. The CDP 1-ARA-35b (35b) derived from a modified thyrotropin-releasing hormone (TRH) analog showed neuroprotective characteristics in in vitro and in vivo models [205,206]. The 35b decreased cell death linked with necrosis, apoptosis, and mechanical injury in neuronal-glial cocultures. In a model of traumatic brain injury in rats, 35b improved motion recovery and led to a lower lesion volume [205].

Compounds with neuroprotective potential, CDPs related to cHP were synthesized by replacing the imidazole moiety from histidine residue with 3,5-di-tert-butyltyrosine. Two diastereomeric CDPs cyclo[(R)-30,50-di-tert-butyl-Tyr-L-Pro] and cyclo[(S)-30,50-di-tert-butyl-Tyr-L-Pro] were obtained. Both compounds showed neuroprotective activity by preventing Ca^{2+} -induced necrosis and cell death induced by FeSO_4 , a free radical generator [207]. Other derived CDPs from cHP, 35b, 144, 606, and 807, also produced motion and cognitive recovery and neuroprotective activity using in vitro models of neuronal injury and after a controlled cortical impact in mice. All CDPs were evaluated using models pertinent to necrotic cell death or caspase-dependent apoptosis, including mechanical injury and glutamate toxicity models that induce necrotic cell death. In contrast, trophic

withdrawal and β -amyloid models lead to caspase-dependent apoptosis [205,206]. The fact that the functional CDP, 35b, 144, 606, and 807, effects were similar to those of cHP indicates that they may share the same or similar underlying molecular mechanisms [206].

3.6. Cyclodipeptide–Protein Kinase Interaction Related to Fibrosis

Recently, the cyclo(His-Pro) was tested for its potential to prevent distinct-type fibrosis (Figure 3). Interestingly, cHP reduces fibrosis-related inflammatory molecules and immune cell infiltration, reducing inflammation [182,185,208] and attenuating steatohepatitis while reducing fibrosis and inflammation by reducing the infiltration of immune cells in liver tissue [208]. In human LX-2 hepatic stellate cells, a fibrinogenic cell type associated with fibrosis, cHP decreases fibrous extracellular matrix proteins, including COL1A [208]. The downregulation of COL1A protein was related to a reduction in ROS levels resulting from cHP treatment. In addition, by trying to silence Nrf2, the collagen-reducing effect of cHP was diminished, suggesting that the anti-fibrotic activity depends on Nrf2 signaling [186,208] (Scheme 1).

The liver transcriptomic analysis revealed that gene sets related to fibrosis, inflammation, and lipid metabolism were upregulated in mice with non-alcoholic fatty liver disease (NAFLD). Concurrently, cHP reduces the expression of essential genes involved in inflammation, fibrosis (extracellular matrix, hepatic stellate cells activation, and collagen production), lipid metabolism, oxidative stress, and apoptosis [208]. In NAFLD and CCl₄-induced liver fibrosis models, cHP significantly downregulated the ERK signaling cascade. This pathway is activated in two human datasets collected from patients with advanced non-alcoholic fatty liver disease and steatohepatitis (NASH) and fibrosis, as the ERK pathway is related to inflammation and fibrosis [208]. cHP treatment diminishes the phosphorylation levels of ERK1/2 within a short duration (4 h), with levels approaching baseline after 24 h. The inhibition of ERK1 has been related to a reduced proliferation of hepatic stellate cells, diminished fibrosis, and improved lipid homeostasis.

In contrast, the activation of ERK2 influences hepatocyte proliferation and contributes to inflammation and fibrosis following liver injury [208]. The CDPs cyclo(His-Ala) and cyclo(His-Gly) also exhibit anti-fibrotic properties since they inhibit fibrin formation [209]. However, the mechanism has not yet been elucidated, and it probably involves the inhibition of the ERK pathway as cHP.

3.7. Cyclodipeptide–Protein Kinase Interaction Related to Aging

During a study of the therapeutic effect of cyclo(His-Pro) on age-related muscle decline, few mice died in the control group. In contrast, no aged mice were lost in the treated group, indicating a beneficial effect of cHP on lifespan extension, like in *C. elegans* nematode [182]. The cHP lifespan extension mechanism probably involves the reduction in reactive oxygen species (ROS) and inhibiting the ERK pathway, as in the cHP anti-inflammatory and anti-fibrosis effect (Scheme 1).

Advanced (glycation) end products (AGEs) result from binding a sugar molecule to a protein and accumulating throughout the body over a lifetime. AGEs can bind to the receptor of advanced glycation end products (RAGEs), triggering the release of inflammatory mediators by the NF- κ B pathway [210]. AGEs are recognized as key contributors to various age-related diseases, including diabetic vascular complications, NAFLD, thyroid cancer progression, coronary artery disease, kidney pathologies, atherosclerosis, and Alzheimer's disease [179,210]. Twelve CDPs, cyclo(Dha-L-Leu), cyclo(L-Phe-L-Ala), cyclo(L-Leu-L-Ala), cyclo(L-Tyr-L-Val), cyclo(L-Tyr-L-Tyr), cyclo(L-Tyr-L-Leu), cyclo(L-Tyr-L-Pro), cyclo(L-Pro-L-Val), cyclo(L-Pro-L-Ile), cyclo(L-Leu-L-Leu), cyclo(L-Met-L-Met), and cyclo(L-Pro-L-Pro), derived from *Sphingobacterium* sp. possess detoxifying activity that effectively eliminates intracellular carboxymethyl lysine (CML). This final AGE product accumulates with age in human dermal fibroblast cells [210].

3.8. Cyclodipeptide–Protein Kinase Interaction Related to Diabetes

The cyclo(His-Pro) has been identified in the human blood and gastrointestinal tract, where it can be absorbed [201]. In humans, plasma levels of cHP increase following glucose ingestion, indicating its function as a gut peptide of the entero-insular axis [201]. Blood glucose metabolism is directly associated with cHP concentration, as cHP enhances intestinal zinc absorption and muscle glucose uptake (zinc is involved in glucose uptake), insulin synthesis and secretion, and insulin utilization in insulin-requiring cells [211,212]. The cHP influences leptin-type compounds, which are involved in appetite regulation and contribute to reduced food intake [204] (Scheme 1).

In a non-obese Type II diabetes mellitus model, cyclo-His-Pro and zinc (cycloZ) enhanced blood glucose metabolism by promoting glucose utilization in muscle cells. The anti-diabetic activity of cycloZ remains unclear; nonetheless, insulin-mediated signal transduction mechanisms are likely involved in muscle [212]. A similar effect was observed in genetically obese (ob/ob) Type II diabetic mice by cHP plus zinc and L-histidine treatment. The primary cause of Type II diabetes is insulin resistance, whereas a significant contributor to obesity is leptin resistance [213]. CycloZ combined with L-histidine may improve insulin and leptin resistance since both systems participate in signal transduction mechanisms through PI3K phosphorylation [212].

In insulin resistance diabetes, cycloZ treatment resulted in improved tissue condition alongside reduced inflammation [208,214]. The global acetyl-lysine level in the liver of cycloZ-treated mice was diminished, indicating that cycloZ influences the regulation of lysine acetylation, which is related to the activity of various metabolic enzymes and transcription factors. Non-histone transcription factors with decreased acetylation include p65, LKB1, and PGC-1 α , associated with mitochondrial biogenesis [215,216]. In addition to deacetylation, PGC-1 α requires AMPK-mediated phosphorylation for its complete activation. CycloZ increases AMPK phosphorylation in the liver and epididymal adipose tissue. The cycloZ effect was also evaluated in a severe hyperglycemia model, yielding results consistent with those previously reported [214].

4. Future Perspective

This review demonstrates the advantages and potential of cyclic dipeptides as small-molecule protein kinase inhibitors. Cyclic peptides predominantly originate from natural sources; however, the advancements in peptide chemical synthesis have brought many advantages. These include the capacity to incorporate molecular modifications into analogs and derivatives with improved pharmacokinetic and pharmacodynamic properties. Moreover, they possess the benefit of being able to traverse the cell membrane. Recent advancements have made it possible to design cell-permeable, biologically active CPs rationally. These advances have consequently made cyclic peptides a promising therapeutic modality, perhaps offering a definitive solution to the “undruggable” intracellular PPI targets.

Nevertheless, considerable exploration remains concerning this class of compounds as emerging issues, such as gatekeeper mutations, promoting the intense research of numerous compounds, including CPs, which have been growing and gaining their space as therapeutic agents performing diverse activities. The most significant potential of CPs lies in their capacity to target intracellular proteins currently undruggable by small molecules or monoclonal antibodies. A second area of opportunity is multi-kinase inhibitor CPs, which would have a significant advantage over the current biologics regarding only one target. Many predominantly hydrophobic CPs have already exhibited favorable oral bioavailability. Due to their structural and metabolic stability properties, high binding affinity to targets, and low toxicity, CDPs show promising potential as therapeutic effects on complex diseases involving protein kinases, such as diabetes, cancer, obesity, aging, neurodegenerative diseases, and fibrosis. Although some protein targets of CDPs have already been found, such as the CLIC1 protein target of cHP, everything indicates that it has more molecular targets, so the panorama is opened to look for the mechanisms by which CDPs exert their effects at the molecular level.

ePK	eucaryotic catalytic domain
ErbB	erb-b tyrosine kinase receptor
ERK1/2	extracellular signal-regulated kinase
FAS	tumor necrosis factor receptor superfamily member 6 (TNFRSF6)
FDA	food and drug administration
FGF	Fibroblast Growth Factor
FGFR	fibroblast growth factor receptor
FKBP12	FK506-binding protein 12
Flt3	fms related tyrosine kinase 3
Fpa	1-4-fluorophenylalanine
G12D	K-Ras gene
Gab1	GRB2 associated binding protein 1
GRTH	gonadotropin-regulated testicular RNA helicase
GSH	glutathione
HCC	hepatocellular carcinoma
HCD	Higher-energy collisional dissociation
hEGF-Hegfr	human EGF receptor bound ligand
HER2	epidermal factor receptor 2
HGF	hepatocyte growth factor
HIF-1 α	hypoxia-inducible factor 1-alpha
HiP-8	HGF-inhibitory peptide-8
HIP2K	homeodomain-interacting protein kinase 2
HIV	human immunodeficiency virus
H-Ras	Harvey rat sarcoma viral oncogene homolog
IC ₅₀	half-maximal inhibitory concentration
iNOS	inducible oxide nitric synthase
IRF-1	interferon regulatory factor-1
JAKs	Janus kinases
KD	dissociation constant
KIR	kinase inhibitory region
Kit	KIT proto-oncogene
KLIFS	kinase-ligand interaction fingerprint and structure
KLU	lytic peptide
K-Ras	Kirsten Ras oncogene homolog
LD ₅₀	lethal dose 50
LKB1	liver kinase B1
LMP	lysosomal membrane permeabilization
McoTI	<i>Momordica cochinchinensis</i> trypsin inhibitor
MEK	mitogen-activated protein kinase kinase 7
MET	mesenchymal-epithelial transition factor
MF	intermediate-risk myelofibrosis
mRNAs	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic fatty liver disease and steatohepatitis
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NMDARs	N-methyl-D-aspartate receptors
nNOS	neuronal oxide synthase
Nrf2	nuclear factor erythroid-related factor 2
NSCLC	non-small cell lung cancer
OK2	7-mer cyclic peptide
P1GF	placental growth factor
P53	Tumor Suppressor Protein p53
PARP	Poly(ADP-ribose) polymerase
PBD	polo-box domain
PDGFR	platelet derived growth factor receptor
PDZ	binding domain PDZ

PGC-1alpha	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKI	protein kinase inhibitors
Plk1	polo like kinase
PPI	protein-protein interactions
PSD-95	Postsynaptic density protein-95
PTEN	phosphatase and tensin homolog
QR	VEGFR-targeting peptide VEGF125–136
RA	rheumatoid arthritis
Rac1	Rac family small GTPase 1
RAF	RAF kinase
RAGE	receptor of advanced glycation end products
Ral-GDS	Ral guanine nucleotide dissociation stimulator
RBD	RAS binding domain
Ras-GDP	inactive GDP-bound state
Ras-GTP	active GTP-bound state
RGD	arginine-glycin-aspartic acid
ROCK	Rho kinase
ROS	reactive oxygen species
ROS1	ROS proto-oncogene 1 receptor tyrosine kinase
ROSN	reactive oxygen-nitrogen species
RTK	receptor tyrosine kinase
SAPK	Stress-activated MAP kinases
SARS-CoV2	severe acute respiratory syndrome coronavirus 2
scHGF	single chain HGF
SFTI-1	sunflower trypsin inhibitor
SH2	Src homology 2
SOCS	suppressors of cytokine signaling
Src	stored response chain kinase
STAT	signal transducer and activator of transcription
SYK	spleen tyrosine kinase
tcHGF	two chain HGF
TK	tyrosine kinase
TK-EGFR	tyrosine kinase domain of EGFR
TKI	tyrosine kinase inhibitor
TNBC	triple negative breast cancer
TRH	hypothalamic thyrotropin-releasing hormone
TrkA	Tropomyosin receptor kinase A
TRKs	tropomyosin receptor kinases
TyKs	tyrosin kinases
Val	valine
VEGFs	vascular endothelial growth factors
VEGFRs	vascular endothelial growth factor receptors
Wnt	Wingless-related integration site

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CAPITULO IV: ARTICULO DE DIVULGACIÓN CIENTÍFICA: METÁSTASIS, ¿CUÁNDO Y A DÓNDE MIGRAR?

ARTÍCULO

Metástasis: ¿Cuándo y a dónde migrar?

Mayra Xochitl Durán-Maldonado y Jesús Campos-García



https://www.freepik.com/premium-photo/oncology-cancer-cells-chemotherapy-treatment-malignant-human-body-caused-by-carcinogens-genetics-with-cancerous-cell-as-immunotherapy-symbol-medical-therapy_42092342.htm#query=met%C3%A1stasis&position=7&from_view=search&track=sph

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Tal vez, una de las cosas que más nos aterra en la actualidad, es enfermarnos de cáncer o que a algún ser querido se lo diagnostiquen. Recibir un pronóstico de este tipo es difícil de aceptar, pero lo es aún más cuando nos dicen que hay metástasis.

¿A qué se refiere esto?

La metástasis es la etapa crítica de la progresión del cáncer en la que las **células cancerosas se diseminan desde el sitio primario a diferentes partes del cuerpo**, lo que conlleva a incrementar el índice de mortalidad asociada con el tipo de cáncer

diagnosticado. Si bien, los tumores primarios pueden curarse mediante cirugía y terapia adyuvante, las células metastásicas **son muy resistentes a la terapia y pueden pasar desapercibidas durante largos períodos de tiempo**. Por lo tanto, un tratamiento efectivo depende en gran medida de la capacidad para tratar la metástasis, quedando así el pronóstico de los pacientes condicionado a la existencia o no de esta.

El cáncer se puede propagar a casi cualquier parte del cuerpo; sin embargo, ciertos tipos tienen más probabilidades de diseminarse a ciertas áreas. **Los sitios más comunes donde hace metástasis son los huesos, el hígado, el cerebro y los pulmones**. El concepto de que el patrón de propagación metastásica del cáncer no es aleatorio y que las células cancerosas exhiben preferencias al hacer metástasis en órganos, se remonta a 1889, cuando Steven Paget publicó su **hipótesis de «semilla y suelo»**. Propuso que la propagación de las células tumorales se rige por la interacción y la cooperación entre las células cancerosas (semilla) y el órgano huésped (suelo). Un sinnúmero de estudios

durante las últimas décadas ha proporcionado una mejor comprensión del proceso de propagación metastásica del cáncer.

¿Qué es la metástasis y cómo ocurre?

El cáncer ocurre cuando las células anormales del cuerpo humano se multiplican. A veces, estas células forman una masa llamada tumor. Cuando esas células **se propagan desde su lugar de origen y migran a otras partes del cuerpo**, se denomina metástasis. En otras palabras, cuando el cáncer ha hecho metástasis, nos referimos a que **se ha diseminado**. Cuando un tumor ha crecido demasiado, las células que lo componen comienzan a tener cambios fisiológicos y moleculares que le permiten desprenderse de ese tumor, **viajar por la sangre o los ganglios linfáticos y llegar a cualquier órgano o tejido del cuerpo**. Las células cancerosas requieren de una gran cantidad de energía, ya que se dividen muy rápidamente, entonces cuando el tumor es muy grande y los nutrientes (glucosa, oxígeno, factores de crecimiento, ácidos grasos o aminoácidos) ya no son suficientes para abastecer a todas

Metástasis

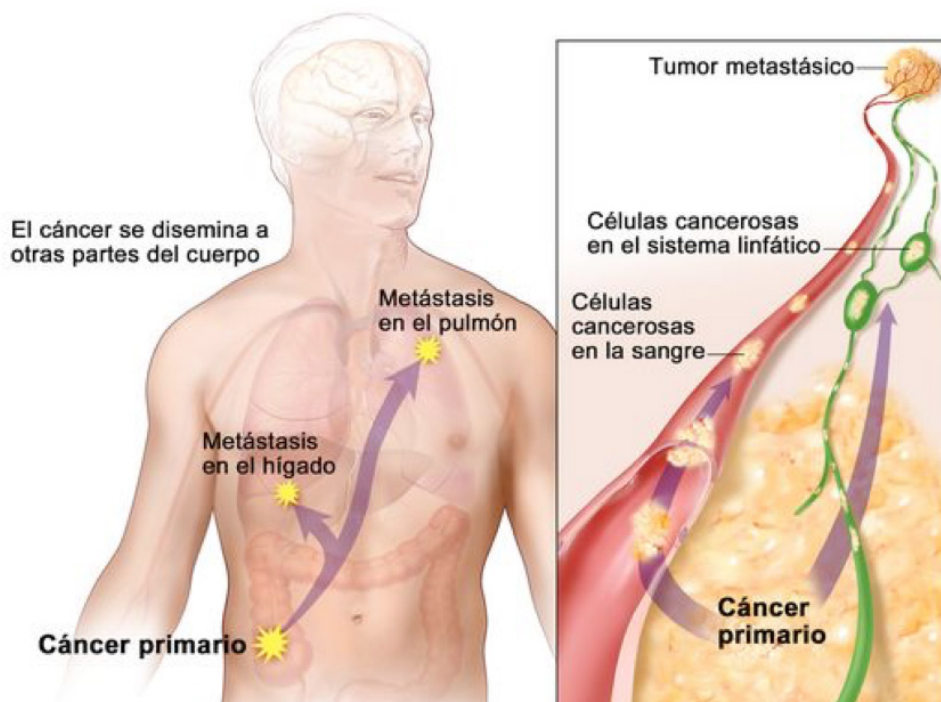
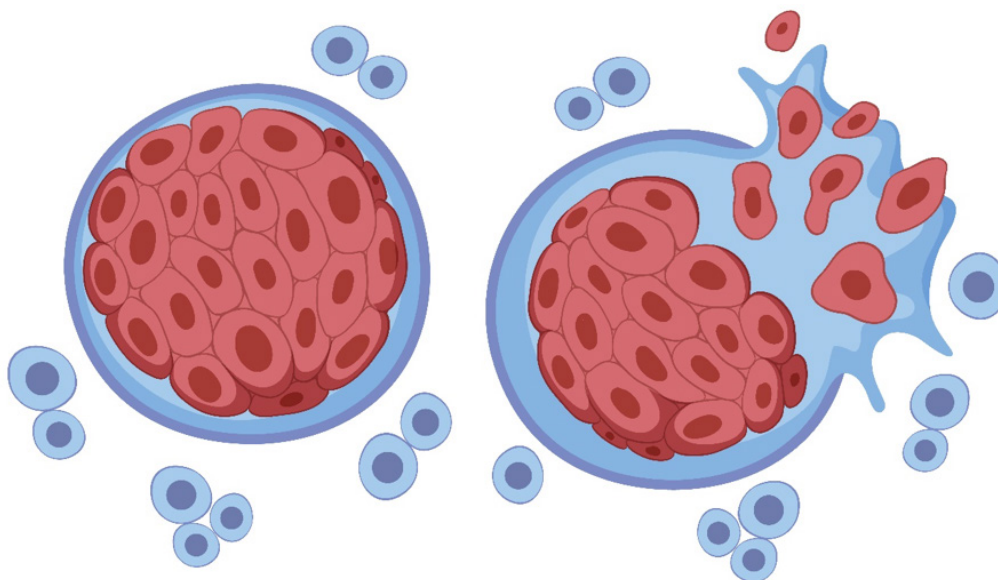


Imagen tomada de la página del Instituto Nacional del Cáncer (INC).



https://www.freepik.com/free-vector/tumor-cell-cancer-development_39024234.htm#query=met%C3%A1stasis&position=4&from_view=search&track=sph

las células tumorales, estas buscarán expandirse y llegar a otros órganos y tejidos que les brinden todo lo necesario para seguir reproduciéndose.

Imagina que un tumor que inició en el tejido mamario, crece tanto que algunas células de este se desprenden, atraviesan los vasos linfáticos o sanguíneos que estén cerca, viajando a través de ellos y concluyendo en la **dispersión por todas las partes del cuerpo**, por ejemplo, en el pulmón, donde **se instalan las células cancerosas** y comienzan a dividirse descontroladamente, creando un **tumor secundario**. Estas células no son cáncer de pulmón porque provienen y siguen siendo del tejido mamario, pero ahora tienen capacidades diferentes que les permitieron sobrevivir al proceso de invasión y colonizar el nuevo nicho —el pulmón—. Con los cambios adquiridos para sobrevivir a este proceso, **estas células probablemente sean un poco más difíciles de aniquilar** con el tratamiento inicial. Se considera entonces al tumor mamario como cáncer primario y el del pulmón, como el sitio donde ocurrió el proceso de la metástasis.

Desafortunadamente, **la metástasis resulta en la muerte de la mayoría de los pacientes con cáncer**. En el momento de diagnosticarlo, al menos la mitad de los pacientes ya presentan enfermedad metastásica clínicamente detectable. Un mayor número de pacientes también presentará **micro-metástasis** que no es fácil de detectar con las téc-

nicas convencionales. Por lo tanto, la **metástasis es el evento que más amenaza la vida en pacientes con cáncer**.

La transferencia de células tumorales de un tumor primario a otro órgano o tejido, es un fenómeno multiescalonado, activo, continuo y complejo que involucra la **respuesta combinada tanto de células tumorales como de células normales**. Los pasos que dan lugar a las metástasis se pueden resumir en eventos que son conocidos como «cascada metastásica».

Cascada metastásica

La pérdida de la **capacidad de adhesión de las células tumorales es el primer paso para dar origen a una metástasis**, ya que permite que las células tumorales malignas se disocien de la masa tumoral primaria otorgándoles la movilidad necesaria para invadir. Una vez que ocurre esto, comienza la migración celular al interior de los vasos sanguíneos y/o linfáticos y, con ello, la diseminación de las células tumorales por el torrente circulatorio. El viaje por el torrente sanguíneo es uno de los puntos de selección clave para las células diseminadas. En este punto, las células deben sortear dos amenazas: por un lado, **evadir las defensas del huésped**; y por el otro, el **daño mecánico propio de atravesar la circulación**. Estas células tienen la capacidad de formar grupos (también llamados *clusters*) de 20

a 50 células, asociación que promueve la supervivencia de las células tumorales a través de su paso por el sistema circulatorio.

Una vez que la célula tumoral ha llegado a un punto probable de nueva invasión, estas salen del torrente sanguíneo e **infiltran el órgano de localización secundaria**. La colonización es la **etapa final de la metástasis** y reside en la habilidad de las células tumorales en adaptarse al nuevo microambiente. Para que las células tumorales sean capaces de dividirse y formar colonias, es necesario recrear las **características del microambiente del tumor primario**. Otro punto crucial para el crecimiento del tumor secundario es la **generación de nuevos vasos sanguíneos** (angiogénesis), siendo un punto crucial de este proceso, ya que el vaso sanguíneo dentro de la vecindad del tumor no solo proporcionará oxígeno y nutrientes al nuevo tumor, sino que también puede proporcionar una ruta para que otras células ingresen al sistema circulatorio y hagan metástasis en otros sitios.

Los órganos de futuras metástasis **son modificados selectiva y activamente por el tumor primario antes de que ocurra la diseminación metastásica**. En la actualidad, se sabe que antes de la llegada de las células tumorales, los tumores preparan el terreno en el órgano que más tarde colonizarán. Diferentes investigaciones han revelado que los tumores **inducen la formación de un microambiente en órganos distantes** que promueven su supervivencia antes de la llegada de las células can-

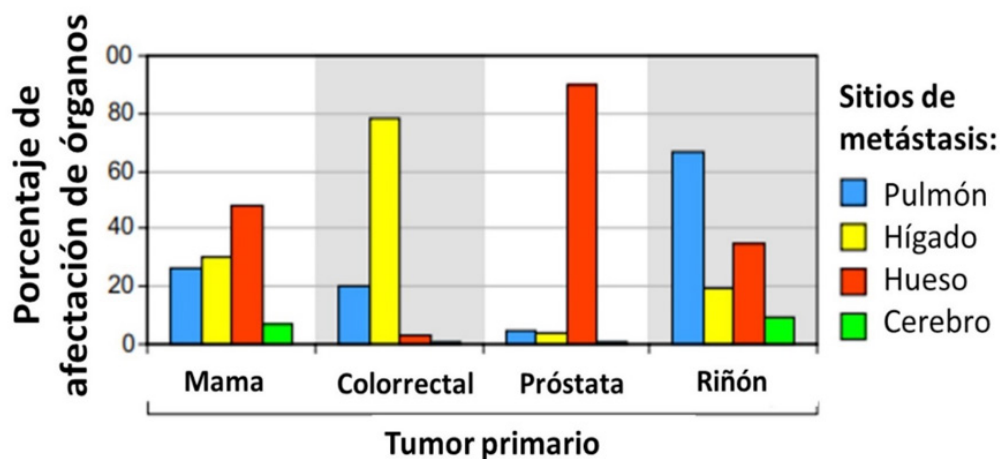
cerosas. Este microambiente predeterminado se denomina **«nicho premetastásico»**.

Con toda la información recabada de los muchos pacientes con este tipo de enfermedades, los médicos e investigadores han logrado encontrar tendencias de los sitios de metástasis dependiendo de donde se originó el tumor primario, es decir, se sabe que el cáncer de mama y próstata, tienen una gran predisposición por hacer metástasis en huesos, pulmón y cerebro, mientras que el cáncer de ovario hará metástasis o invadirá preferentemente cavidad abdominal e hígado. Pero, entonces, ¿qué determina la preferencia de las células cancerosas por ciertos órganos?, ¿cómo determinan las células cancerosas dónde hacer metástasis y dónde no?...

¿Cuándo y a dónde migrar?

Teoría de la «semilla y suelo»

El origen celular, las propiedades intrínsecas del tumor, las afinidades tisulares y los patrones de circulación, determinan no solo los sitios de diseminación del tumor, sino también el curso temporal y la gravedad de la metástasis en órganos vitales. Investigaciones actuales sobre este tema, describen el tipo de características asociadas con las células tumorales metastásicas para preferir ciertos tejidos. Por ejemplo, se sabe que los factores más relevantes serán la presencia de ciertas moléculas o factores de crecimiento, las características metabólicas, la presencia de ciertos componentes del sistema inmunológico, o incluso las características



Patrones de distribución de metástasis asociado con diferentes tipos de cáncer (Tomada de Xin Lu et al. 2017).

genéticas que tienen las células tumorales invasoras. **A la afinidad por un tejido u órgano para desarrollar metástasis se le conoce como «organotropismo metastásico».**

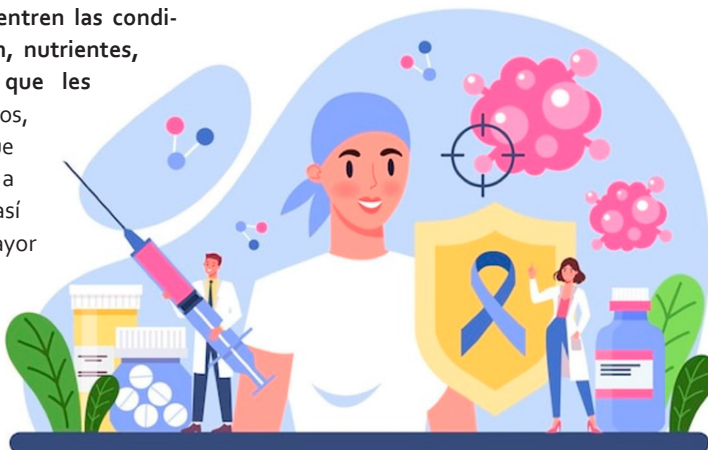
El organotropismo metastásico se puede explicar a través de la **teoría de la «semilla y suelo»**. En sus estudios, Paget argumentó que la distribución de las metástasis no se debía al azar, y sugirió que la propagación de las células metastásicas era específica del órgano e involucraba la **interacción entre las células cancerosas y el órgano huésped**. Concluyó que las metástasis se desarrollaban únicamente cuando la semilla y el suelo eran compatibles.

Ahora analicemos el ejemplo más claro. Supongamos que tomamos semillas de una manzana y las vamos regando por todos los lugares por los que pasamos y a lo largo de muchos municipios de la región; aunque pongamos muchas semillas en todos esos lugares, estas únicamente germinarán en aquellos lugares donde el suelo tenga las condiciones óptimas de nutrientes, temperatura, humedad, etc. En el caso del cáncer, supongamos que, de un tumor de mama, se desprenden cierto número de células cancerosas y, a través de la sangre o la linfa, se dispersan por todo el cuerpo; a pesar de que serán capaces de llegar a todos los órganos y tejidos, **solo van a establecerse y a desarrollarse en aquellos sitios donde encuentren las condiciones óptimas de oxigenación, nutrientes, componentes inmunológicos que les benefician, etc.** También sabemos, por ejemplo, que hay frutas que son más exigentes en cuanto a sus condiciones de crecimiento, así como hay tipos de cáncer con mayor

tendencia a progresar a metástasis. También es importante mencionar que a medida que avanza la enfermedad, las células comienzan a ser cada vez más heterogéneas, y así como muchas serán sensibles a los fármacos y morirán, un buen número no lo hará (quimio resistencia).

Entre más conozcamos la naturaleza de la enfermedad, tendremos mayor éxito para poder tratarla. Conforme se tiene mayor información sobre la metástasis, es posible pensar en nuevas opciones de tratamientos, cada vez más específicos y eficientes.

Prevenir la metástasis en pacientes de alto riesgo sería mucho mejor que tener que tratarla más tarde. Existen tratamientos para controlar la mayoría de los tipos de cáncer metastásico. A menudo, el **objetivo de los procedimientos de cáncer metastásico es controlarlo, deteniendo o ralentizando la progresión de la patología**. Si el cáncer metastásico es controlado, los pacientes podrán vivir por más años. Actualmente, los investigadores continúan buscando nuevas formas de inhibir o detener el crecimiento de las células cancerosas, además, existe un sinnúmero de estudios clínicos en fase tres (pruebas clínicas en pacientes voluntarios) de esquemas de tratamiento para cáncer en etapa avanzada o con metástasis.



sabermás

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DISCUSIÓN GENERAL

El cáncer de mama representa un importante problema de salud pública, especialmente los subtipos con fenotipo agresivo, que suelen presentar un alto riesgo de metástasis, como el cáncer de mama triple negativo, que carece de los receptores hormonales RH y HER2, lo que reduce las posibilidades de tratamiento (Xie Y *et al.*, 2022; Witt B.L. *et al.*, 2023). Las estadísticas muestran que hasta un 30 % de las mujeres con cáncer de mama presentarán metástasis en los primeros tres años tras el diagnóstico (Sung *et al.*, 2021; Won *et al.*, 2020). Esto convierte el tratamiento exitoso del cáncer de mama en un verdadero reto, a pesar de que actualmente existen muchas y muy diversas opciones (Witt *et al.*, 2023).

El uso de nuevos compuestos de origen biológico, como las terapias con dianas específicas, ha aportado numerosos beneficios en el tratamiento de diversos tipos de cáncer. Gracias a su selectividad frente a las células tumorales, estos suelen producir pocos efectos secundarios en las pacientes. Los ciclodipéptidos PAO1 de *Pseudomonas aeruginosa* se han estudiado ampliamente y se ha descrito su efecto antineoplásico en diferentes líneas celulares y en un modelo *in vivo* de melanoma murino no metastásico. Además, la vía de señalización PI3K/Akt/mTOR, que se encuentra inhibida, ha sido bien caracterizada en nuestro grupo de trabajo en células HeLa tratadas con PAO1-CDPs. Además, mediante análisis transcriptómicos, los PAO1-CDP han mostrado actividad al regular otras vías implicadas en la proliferación, la síntesis de lípidos, el metabolismo energético, la invasión y la metástasis, lo que indica que la interacción de los CDPs con las células cancerosas podría deberse a múltiples blancos, como proteínas clave para el mantenimiento de la neoplasia (Hernández-Padilla *et al.*, 2020). Cuando hay metástasis, el tratamiento puede convertirse en un reto, ya que las células tumorales han adquirido mecanismos de resistencia a las terapias que, en etapas tempranas, podrían resultar exitosas, por ejemplo, la sobreactivación de vías de señalización redundantes de supervivencia y de resistencia a la apoptosis (Vasan *et al.*, 2019). De este modo, que los PAO1-CDPs sean capaces de interactuar con distintos blancos moleculares podría explicar su efecto antineoplásico en distintos tipos de células cancerosas.

Una de las primeras estrategias utilizadas para resolver el problema de la resistencia a los tratamientos individuales, naturalmente, ha sido el uso de terapias combinadas, con las cuales se han observado buenos resultados en diversos tipos de tumores, como los linfomas, el cáncer de mama o el cáncer testicular. Esto ha dado lugar al uso de esquemas de tratamiento cada vez más complejos en ensayos clínicos; además, se ha recurrido al aumento de las dosis y al acortamiento de los intervalos de administración de los fármacos (DeVita *et al.*, 1980; Bonadonna *et al.*, 1976). Más recientemente, se han utilizado inmunoterapias combinadas que inicialmente muestran buena respuesta, pero con la eventual aparición de resistencia tumoral (Vasan *et al.*, 2019). Todo esto nos llevó a probar los CDPs en combinación con metotrexato, otro fármaco utilizado en el tratamiento de diferentes tipos de tumores, entre ellos los mamarios; incluso se ha investigado su uso en combinación con otros fármacos para el tratamiento del cáncer metastásico (Yamashita *et al.*, 2018; Choi *et al.*, 2013).

Por otro lado, el uso de modelos preclínicos para la investigación de moléculas con potencial antineoplásico constituye una herramienta valiosa para predecir el éxito de nuevos tratamientos en pacientes con cánceres de difícil tratamiento (Li Y. *et al.*, 2022; Dobrolecki *et al.*, 2016). En estudios previos, probamos ciclodipéptidos en un modelo murino de melanoma con resultados muy prometedores. Sin embargo, dado que los melanomas se generaron con una línea celular murina no metastásica, persistía la interrogante sobre cómo funcionarían estos compuestos en tumores inducidos con una línea celular cancerosa de origen humano y si estos tumores también serían tratados en un estado avanzado y con metástasis ya establecidas. Para ello, utilizamos un modelo

ortotópico de cáncer de mama metastásico que presenta varias ventajas, entre ellas, que este modelo utiliza una línea celular de origen humano, que la localización anatómica del tumor simula la localización anatómica real del tipo de cáncer utilizado, que con este modelo pudimos obtener metástasis en un corto período de tiempo y que, además, se trata de un tipo de cáncer con alta morbilidad, ya que es el principal en mujeres a nivel mundial.

Se ha debatido en varios trabajos el uso de modelos preclínicos con ratones inmunodeprimidos, así como sus ventajas y desventajas, ya que el sistema inmunitario desempeña un papel crucial en el microambiente tumoral y mantiene una interacción constante y compleja con las células tumorales. Sin embargo, dado que la progresión tumoral puede investigarse desde diferentes perspectivas, la elección del modelo de investigación se basa en los objetivos de la investigación; este modelo nos ayudó a responder a las preguntas del proyecto (Witt *et al.*, 2023; Szadvari *et al.*, 2016).

Los ratones Balb/c Foxn1nu carecen de un timo funcional y bien desarrollado y, por lo tanto, no poseen linfocitos T capaces de generar una respuesta inmunitaria específica contra patógenos o células malignas, aunque sí presentan mecanismos de respuesta inmunitaria innata (Suhail *et al.*, 2019). Esta característica nos permite disponer de un modelo biológico para inducir tumores de origen humano sin rechazo inmunológico y, de esta manera, estudiar diversos aspectos del cáncer con mayor similitud con lo que ocurre en los pacientes.

Además, en los ensayos *in vitro* utilizamos una línea de cáncer de mama luminal (MCF-7) como control de bajo potencial metastásico y una línea de macrófagos para evaluar la interacción con células cancerosas en el contexto de la invasión celular.

En los ensayos de viabilidad, obtuvimos porcentajes significativamente menores de células viables en los tratamientos con dosis crecientes de PAO1-CDP, con una LD50 de 63 µg/mL en células MCF-7 y de 23 µg/mL en MDA-MB-231, lo que representa una dosis letal similar a la reportada en líneas celulares de cáncer de cuello uterino, cáncer de colon, melanoma y leucemia (Hernández-Padilla *et al.*, 2024; Durán-Maldonado *et al.*, 2020). Además, se observó hasta un 80 % de células apoptóticas a una concentración de 0,05 mg/mL de PAO1-CDP, lo que indica que estos compuestos inducen la apoptosis. Los ensayos de herida para medir la capacidad migratoria de las células cancerosas tratadas con PAO1-CDP mostraron un área de herida dos veces mayor que la del grupo de control y 1,5 veces mayor que la del grupo tratado con MTX. Cabe destacar que las células MCF-7 mostraron una menor capacidad migratoria en todas las condiciones que las células MDA. Esto podría deberse a que las células MCF-7 tienen un fenotipo epitelial, lo que se traduce en una menor movilidad que la de las células MCF-7, mientras que las células MDA tienen un fenotipo mesenquimal y se ha reportado ampliamente que presentan mayor movilidad y, por lo tanto, mayor potencial metastásico.

Por otro lado, se realizaron ensayos en cámara Transwell con Matrigel en ambas líneas celulares, cultivadas en cocultivo con macrófagos, para simular la interacción de estos con las células cancerosas y evaluar cómo estos pueden favorecer la invasión. Los resultados mostraron, en primer lugar, que la presencia de macrófagos aumentó el porcentaje de células cancerosas que degradaron el Matrigel y atravesaron la membrana porosa, en contraste con el cultivo simple en ambas líneas celulares. En segundo lugar, el tratamiento con PAO1-CDPs disminuyó el número de células invasoras tanto en el cultivo simple como en el cocultivo de ambas líneas celulares, lo que podría indicar una característica favorable de los CDPs como tratamiento antimetástico. En este ensayo, también se comparó la eficacia de los CDPs con el tratamiento con MTX, un medicamento aprobado por la FDA para el tratamiento de diversos tipos de cáncer; incluso existen informes que han evaluado su uso en

combinación con otros fármacos para el tratamiento de la enfermedad metastásica, por ejemplo, en la metástasis ósea (Kazuki Nakamura *et al.*, 2003).

Como se mencionó anteriormente, el microambiente tumoral abarca diversas interacciones con las células tumorales que determinan la progresión; una de ellas es la que existe con las células del sistema inmunitario. Los macrófagos asociados a tumores (MAT), además de ser las células inmunitarias predominantes en el microambiente tumoral, secretan diversas quimiocinas y factores de crecimiento que facilitan la proliferación, la migración y la supervivencia (Hanahan y Weinberg *et al.*, 2011; Mantovani *et al.*, 2008). También se ha descrito que la interacción entre los MAT, los fibroblastos asociados a cáncer (FAC) y los neutrófilos asociados a tumor (NAT) y la célula tumoral afecta la eficacia de los fármacos anticancerígenos, por lo que estas células inmunitarias se han propuesto como dianas terapéuticas (Borros A, 2019). Los resultados de nuestros ensayos de invasión en cocultivo podrían indicar que, además de su capacidad proapoptótica, los CDP pueden inhibir la comunicación entre los MAT y las células cancerosas y, por lo tanto, obtener una ventaja como tratamiento antimetástico.

La innovación en los modelos de investigación del cáncer ha permitido desarrollar métodos para estudiar células en cultivo con diferentes grados de complejidad, lo que permite crear un entorno lo más similar posible a un tumor en un paciente. El modelo de esferoides multicelulares permite evaluar el comportamiento de las células cancerosas en un arreglo tridimensional que simula una agregación de células con diferentes estratos, en los que las características metabólicas y proliferativas varían en cada estrato, como ocurre en los tumores. Esta heterogeneidad celular, a su vez, provoca que exista una población más susceptible a los tratamientos y otra que presente resistencia, generando así células remanentes después de un tratamiento que causan recurrencia o recaída en los pacientes. Además, en estas agregaciones tridimensionales se puede encontrar una subpoblación de células con características de células madre cancerosas, capaces de autorrenovarse, generar metástasis y desarrollar resistencia a fármacos (Patel *et al.*, 2021). Por lo tanto, el uso de microesferoides para evaluar la efectividad de los tratamientos contra el cáncer es una excelente herramienta que nos acerca un poco más a lo que podríamos observar en los pacientes.

Nuestros resultados en micro esferoides de células MCF-7 y MDA mostraron, por un lado, que las diferencias fenotípicas entre ambas líneas causaron ciertas diferencias entre los esferoides obtenidos: los de MCF-7 eran más compactos y grandes, mientras que los de MDA eran más laxos y heterogéneos en tamaño, probablemente debido a diferencias en las proteínas de unión en su membrana, ya que MCF-7 suele expresar una mayor cantidad de E-cadherina y, por lo tanto, un fenotipo epitelial. Por otro lado, observamos que el tratamiento con PAO1-CDP disminuyó significativamente el tamaño y el número de microesferoides en ambas líneas celulares, tanto en comparación con el control sin tratamiento como con MTX. Previamente, se reportó un efecto potenciador de CDP en combinación con β -ciclodextrinas para mejorar la solubilidad y la permeabilidad celular (Duran-Maldonado *et al.*, 2020).

Respecto al modelo ortotópico de cáncer de mama metastásico, logramos un rendimiento del 100 % en los tumores inducidos en la almohadilla grasa mamaria con ayuda de Matrigel al momento de la inoculación, ya que se ha reportado que la inducción tumoral con suspensión celular en solución salina fisiológica no genera tumores en el 100 % de los ratones (Zhang *et al.*, 2019). Una vez establecido el modelo, administramos diversos esquemas de tratamiento. Se determinó el peso corporal a lo largo de los tratamientos y se observó que el patrón general de ganancia de peso fue similar en todos los grupos; sin embargo, los grupos tratados con PAO1-CDPs en estadios tempranos y avanzados presentaron una disminución temporal del peso después de cada ronda de tratamiento, que se recuperó durante el tiempo de descanso entre rondas. Nuestro grupo de trabajo ha reportado

que en células HeLa expuestas a PAO1-CDPs se observa una disminución en la expresión de genes relacionados con la síntesis de lípidos y con la vía del mevalonato (Lázaro-Mixteco *et al.*, 2022). Más recientemente, dentro de nuestro grupo de trabajo, Figueroa-Guzmán y colaboradores reportaron una disminución de la grasa visceral en ratones inducidos a la enfermedad de hígado graso no alcohólico tratados con los PAO1-CDPs (Patente No. 426355). Esto podría sugerir que la pérdida de peso tras cada ronda de tratamiento se deba a este efecto de los CDP, además de que los ratones no mostraron ningún efecto negativo aparente y mantuvieron un consumo normal de alimento y agua. Esta característica también podría favorecer el efecto antimetástico de nuestros compuestos, ya que existe evidencia de que los adipocitos podrían interactuar con las células cancerosas, produciendo adipocinas que promueven la invasión, la metástasis y la resistencia a la muerte por apoptosis o ferroptosis (Xie *et al.*, 2022).

Posteriormente, observamos que los grupos tratados con CDPs disminuyeron significativamente el volumen y el peso de los tumores primarios y el número de focos metastásicos en hígado, pulmón y hueso, en contraste con los ratones sin tratamiento, e incluso observamos un efecto sinérgico con el fármaco metotrexato, que se utiliza como parte del tratamiento de varios tipos de cáncer con metástasis en combinación con otros fármacos (Kazuki Nakamura *et al.*, 2003; Bazan *et al.*, 2019). Por otro lado, el análisis hematológico mostró una restauración de los valores de hemoglobina y hematocrito, casi como en el control, en los grupos tratados con CDPs, lo cual habíamos observado previamente en melanoma murino, mientras que con el tratamiento con MTX se obtuvieron niveles bajos de estos parámetros. Esta disminución es consistente con los efectos adversos reportados para MTX (Misra *et al.*, 2020). En cuanto al recuento de células sanguíneas, observamos un perfil hematológico similar al reportado en otras investigaciones en los grupos tratados con PAO1-CDP, mientras que en el grupo sin tratamiento y con MTX se observó un aumento significativo en el recuento de neutrófilos y monocitos, lo cual, según la literatura, se relaciona con una interacción entre estos y las células cancerosas que promueve la resistencia a la quimioterapia, la invasión y la metástasis (Worm MR *et al.*, 2023). Por otro lado, la determinación de las enzimas de daño tisular AST, ALT y LDH mostró un aumento drástico en los grupos con tumor sin tratamiento, así como en el grupo tratado con MTX, mientras que en los grupos tratados con CDPs los niveles séricos de estas enzimas se restablecieron de manera muy similar a los del control sano. De esta manera, nuestros compuestos muestran un perfil potencial como fármacos anticancerígenos en estas dos líneas de cáncer de mama: luminal y triple negativo y, de manera importante, un efecto antimetástico en el modelo *in vivo*. Cabe destacar que en este ensayo se utilizó una dosis mayor que en el modelo de melanoma y no se observaron efectos adversos graves, salvo la pérdida de peso transitoria, lo cual podría representar una ventaja frente a otras terapias utilizadas para el tratamiento del cáncer metastásico.

Aproximadamente el 30 % de los pacientes diagnosticados en etapas tempranas de cáncer de mama presentarán metástasis distantes, siendo el cerebro, el hígado, el pulmón y el hueso los órganos más afectados en este tipo de cáncer (Lu y Kang, 2007). La histología sigue siendo, a la fecha, una importante herramienta en el diagnóstico, pronóstico y seguimiento del tratamiento en pacientes con cáncer.

Realizamos un análisis de cortes de tumor primario, hígado y pulmón teñidos con hematoxilina y eosina, en el que pudimos observar las características propias del tumor primario desarrollado en ratones inmunosuprimidos inoculados con células de cáncer de mama metastásico MDA-MB-231. Estos tumores mostraron estar formados por células pleomórficas con un alto índice proliferativo y una arquitectura desordenada del tejido, así como una alta irrigación sanguínea, evidenciada por la

presencia de pequeños vasos, y presentaron núcleos con hiperchromasia, una característica propia de lesiones cancerosas, relacionada con un mayor contenido de ADN.

Los grupos tratados con los CDPs solos y en combinación con MTX mostraron una reducción de la hiperchromasia nuclear, además de cambios relacionados al tratamiento como lo son áreas focales de necrosis y células inflamatorias

Varios grupos de trabajo han investigado la importancia del microambiente tumoral y la interacción de las células cancerosas con él; incluso actualmente se considera que el TME es un factor importante en la progresión de la neoplasia, la invasión y la metástasis (Arneth, 2019; Rubenich *et al.*, 2023). Por ejemplo, se ha reportado que los tumores con un alto contenido de matiz extracelular podrían afectar la deposición de agentes quimioterapéuticos y, así, presentar resistencia a los tratamientos contra el cáncer (Borros, 2019). Además, se ha estudiado la importancia de la interacción de las células tumorales con otras presentes en el microambiente tumoral, como células madre cancerosas, fibroblastos, adipocitos, macrófagos y células polimorfonucleares, en las que dichas interacciones suelen favorecer la invasión tumoral y la metástasis. Por otra parte, los adipocitos protegen a las células de cáncer de mama, lo que favorece la aportación de ácidos grasos monoinsaturados (Xie *et al.*, 2022). Observamos una gran cantidad de gotas lipídicas a lo largo del tejido tumoral en ratones sin tratamiento, mientras que los tratados con CDPs mostraron una disminución significativa de dichas gotas. Esto concuerda con lo reportado previamente en nuestro grupo de trabajo: en un análisis transcriptómico de células HeLa tratadas con CDPs, se observó que nuestros compuestos regulan a la baja genes relacionados con el metabolismo de lípidos y con la vía de mevalonato (Lázaro-Mixteco *et al.*, 2022). Además, se ha reportado el efecto favorable de los CDPs en ratas obesas con enfermedad esteatótica hepática no alcohólica (Figueroa-Guzmán: artículo en revisión y patente No. 426355). Esto podría apoyar la idea de que los ciclodipeptidos, además de inducir apoptosis en las células tumorales, también disminuyen el metabolismo de lípidos y la cantidad de adipocitos en el microambiente tumoral y, de este modo, tienen un mejor efecto antimetastático que el fármaco MTX.

Se sabe que la obesidad representa un factor predisponente para desarrollar cáncer de mama y que además suele ser una condición que afecta el éxito del tratamiento, esto sumado a que la interacción de los adipocitos con las células tumorales favorece la malignidad y metástasis (Wang *et al.*, 2017) posiciona a los CDPs como buenos candidatos como tratamiento en cáncer de mama metastático debido la característica de atacar diferentes blancos moleculares involucrados en vías de señalización relacionadas con cáncer, pero también en vías relacionadas con el metabolismo de lípidos.

El organotropismo metastático es una característica en la que las células tumorales tienen preferencia por metastatizar a ciertos órganos, de acuerdo con las características metabólicas y genéticas de la célula tumoral y con las condiciones del microambiente del tejido blanco. Observamos que los órganos más afectados por focos metastáticos fueron el pulmón, el hígado y el hueso, lo cual concuerda con lo descrito en diversas revisiones sobre el organotropismo metastático (Lu *et al.*, 2007; Chen *et al.*, 2017).

Respecto a la histología pulmonar, este fue el órgano donde se encontró el mayor número de focos metastáticos, los cuales se redujeron en los grupos tratados con los CDPs, solos o en combinación con MTX, tanto en etapas tempranas como avanzadas del tumor primario. Los focos más evidentes y de mayor tamaño se presentaron en zonas adyacentes a los vasos sanguíneos, pero también se

encontraron células cancerosas diseminadas más allá de estos y a lo largo de todo el tejido pulmonar.

Se ha encontrado que genes como EGFR, COX2, MMP-1 y MMP-2 están asociados a la metástasis pulmonar al facilitar la angiogénesis en el tumor mediante la liberación de células en la circulación y su irrupción en los capilares pulmonares. De manera consistente, la inhibición de EGFR y COX2 minimiza la metástasis pulmonar (Chen *et al.*, 2018). En nuestro grupo de trabajo (Hernández-Padilla, 2024), se ha descrito que los CDPs disminuyen la fosforilación de EGFR en células MDA-MB-231, MCF-7 y HeLa, lo cual se correlaciona con la marcada disminución de la metástasis pulmonar observada en ratones tratados con CDPs.

Por otra parte, las células de cáncer de mama en hígado exhiben un programa metabólico único, equiparable al de las metástasis en hueso y pulmón, caracterizado por una conversión incrementada del piruvato derivado de la glucosa a lactato y por una disminución del metabolismo mitocondrial. La reprogramación metabólica dependiente de PDK1 es clave en la metástasis al hígado del cáncer de mama. Además, genes regulados por HIF-1, como LOX, OPN, VEGF y TWIST, coordinan la formación de metástasis hepáticas en el cáncer de mama. También se ha reportado que la desregulación de moléculas de adhesión, como E-cadherina y N-cadherina, y la alta expresión de proteínas de proliferación, como Ki-67, contribuyen al desarrollo de metástasis hepáticas (Chen *et al.*, 2018). En 2020, reportamos que ratones con melanoma tratados con los CDPs presentaron una disminución significativa de HIF-1 α , Ki-67 y N-cadherina (Durán Maldonado *et al.*, 2020), lo cual apoya lo observado en la histología del hígado de este trabajo, donde no se observaron focos metastásicos en los ratones tratados con CDPs en etapas tempranas y avanzadas del tumor primario de mama.

El MTX es un fármaco análogo del ácido fólico ampliamente utilizado como quimioterapéutico; sin embargo, este puede ocasionar efectos adversos graves en los pacientes, como toxicidad en distintos tipos celulares, como las células sanguíneas y los hepatocitos (Misra *et al.*, 2020; Koźmiński *et al.*, 2020). En este trabajo comparamos el resultado del tratamiento con MTX con el efecto de nuestros compuestos y nuestros resultados en conjunto muestran que los CDPs tienen un efecto superior al de MTX, además de la ventaja de no haber mostrado efectos adversos aparentes en los diferentes órganos analizados y en las concentraciones probadas *in vivo* hasta el momento. Sin embargo, el tratamiento combinado mostró un efecto sinérgico entre los CDPs y el MTX, lo que podría constituir un esquema de tratamiento prometedor para las metástasis.

En 2021 se describió que FoxO1 (Forkhead box transcription factor O1) actúa como regulador transcripcional de Gab1, que la capacidad de “homing” de células de leucemia linfocítica crónica (LLC) depende del eje FoxO1/Gab1 y que, además, la actividad de este eje promueve la activación sostenida de AKT, lo que mantiene la supervivencia celular, y proponiendo a GAB1 como un blanco terapéutico muy prometedor (Seda *et al.*, 2021).

Las proteínas de unión asociadas a GRB-2 incluida Gab1 funcionan como proteínas de anclaje en interacción proteína-proteína de diversas vías de señalización, por ejemplo, PI3K/AKT y el complejo PAR y, por ende, la sobreexpresión de Gab1 se ha asociado con proliferación y metástasis en tumores mamarios de fenotipo metastásico, hepatocarcinoma y en carcinoma tiroideo anaplásico, por mencionar algunos (Wang *et al.*, 2019; Song *et al.*, 2022; Bongartz *et al.*, 2024).

Por otra parte, existe evidencia de que diferentes estímulos, como el estrés oxidativo o los altos niveles de glucosa, pueden inducir la regulación de FOXO1 vía PI3K/AKT, JNK, ligasas E3 de ubiquitina, entre otros. Y que la función de FOXO1 depende de la modulación de blancos río abajo, tales como genes asociados a apoptosis, autofagia, ciclo celular, metabolismo y respuesta inmune (Xing *et al.*, 2018; Ebrahimnezhad *et al.*, 2023). Sin embargo, se sabe que la degradación de FOXO1 evita que este promueva la transcripción de genes relacionados con la pluripotencialidad (stemness), como SOX2, OCT4, NANOG, KLF4 y c-Myc, mediada por el eje TRIB3-AKT1-FOXO1-SOX2 en células con sobreexpresión de TRIB3 (Yu *et al.*, 2019).

Ya se ha descrito previamente en nuestro grupo de trabajo la inhibición de la vía de proliferación PI3K/AKT/MTOR y el efecto inhibitorio de los CDPs sobre la fosforilación y la activación de esta vía, a través de diferentes receptores de membrana, como Met, HGF, Cmyc, etc. Recientemente se reportó una disminución de la actividad de GAB1 en células Hela, MDA y MCF7 tratadas con nuestros compuestos (Hernández-Padilla *et al.*, 2024), por lo que quisimos investigar el rol de GAB1/FoxO1 en la capacidad metastásica de los tumores.

En este trabajo medimos la expresión de Gab1 y FOXO1 en el lisado de tumores metastásicos de cáncer de mama tratados con los CDPs, con MTX y con la combinación de ambos. Se observa una disminución significativa de estas proteínas, tanto en el estado desfosforilado como en el fosforilado, lo que indica una disminución tanto de su expresión como de su actividad. La disminución de p-AKT y Gab1 en los diferentes tratamientos concuerda con lo reportado previamente en distintas líneas celulares cancerosas, entre ellas MDA y MCF-7, así como con la disminución de la proliferación, migración e invasión celulares *in vitro*, y con una disminución significativa del tamaño de los tumores mamarios metastásicos y del número de focos metastásicos en hígado y pulmones en un modelo *in vivo*. Además, en diversas publicaciones anteriores de nuestro grupo de trabajo, hemos discutido la interacción de los CDPs con diferentes RTKs y quinasas citoplasmáticas implicadas en el fenotipo canceroso, lo cual podría respaldar la idea de los CDPs como una terapia multiblanco prometedora.

CONCLUSIÓN

Los ciclodipéptidos de *P. aeruginosa* PAO1 ejercen un efecto antimetástico en un modelo de cáncer de mama *in vivo* e *in vitro* mediante la inhibición de la fosforilación de la vía Akt/mTOR/S6K, la disminución de marcadores de metástasis como vimentina, Gab1 y FOXO1, y la modulación de la expresión de genes como SNAIL, GADD45A, PTEN, CXCL12 y CDKN1A, vinculados con *agresividad*, regulación inmune y respuesta al estrés en el cáncer.

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