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Citotoxicidad de las Defensinas PaDef (*Persea americana* var. *drymifolia*) y γ-tionina (*Capsicum chinense*) Contra la Línea Celular de Cáncer de Mama MCF-7

TESIS

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1. RESUMEN

El cáncer es un problema de salud importante a nivel mundial. En México es la tercera causa de muerte siendo el cáncer de mama uno de los de mayor incidencia. Las terapias convencionales tienen índices terapéuticos bajos y efectos secundarios severos por lo que es necesaria la búsqueda de nuevas terapias alternativas. Los péptidos antimicrobianos (PAs) son moléculas que han sido considerados una prometedora alternativa para tratar el cáncer debido a su habilidad para inactivar un amplio rango de células cancerosas. La actividad anticancerosa de los PAs incluye la lisis de la membrana citoplasmática y la inducción de apoptosis. En este trabajo se evaluó la citotoxicidad de las defensinas de plantas PaDef (*Persea americana* var. *drymifolia*) y γ-tionina (*Capsicum chinense*) contra la línea celular de cáncer de mama MCF-7 y se analizó su mecanismo de acción. La defensina PaDef disminuyó la viabilidad de las células MCF-7 de manera dependiente de la concentración, con una concentración inhibitoria 50 (IC_{50}) de 141.62 µg/ml; las células PBMC no se vieron afectadas con la defensina, pero la viabilidad de las CEMB disminuyó por el tratamiento con la defensina PaDef (datos no mostrados). La defensina γ-tionina tuvo un efecto mayor sobre la viabilidad de las células MCF-7 respecto a PaDef, con una IC_{50} de 117.29 µg/ml; sin embargo, la viabilidad de las CEMB y PBMC no se vio afectada, sugiriendo que la γ-tionina es selectiva hacia las células cancerosas. Para determinar el mecanismo citotóxico de las defensinas PaDef y γ-tionina (IC_{50}) se evaluó el daño a la membrana citoplasmática, el calcio extracelular y el potencial eléctrico de la membrana citoplasmática. Ninguna de las defensinas modificó estos parámetros, lo que sugiere que el mecanismo no está relacionado con la formación de poros. Por otro lado, se determinó que ambas defensinas incrementaron la tasa de apoptosis de manera dependiente del tiempo, alcanzando un máximo de 44 y 76% para PaDef y γ-tionina, respectivamente. Para elucidar la ruta de señalización de apoptosis activada por las defensinas se mostró que ambas defensinas favorecen la pérdida del potencial eléctrico de la membrana mitocondial (ΔΨm) lo que sugiere que las defensinas activan la ruta de

apoptosis intrínseca. Adicionalmente se evaluó la expresión de genes típicos de la apoptosis extrínseca (*FasL*, *Fas* y *caspasa-8*) e intrínseca (*citocromo c*, *Apaf-1* y *caspasa 9*) así como la *caspasa 7*. En el caso de la defensina PaDef, la expresión de genes de la ruta intrínseca se indujo de manera dependiente del tiempo, mientras que la de los genes de la ruta extrínseca no se modificó. La defensina γ-tionina generó un patrón de expresión diferente, ya que los genes de la ruta intrínseca se inducen desde las 6 h de tratamiento; sin embargo, esta inducción ya no se observó a las 24 h; a este tiempo se incrementó significativamente la expresión de los genes de la ruta extrínseca (*FasL, Fas* y *caspasa-8*). Adicionalmente, las células MCF-7 tratadas con la defensina PaDef mostraron un incremento en la fosforilación de la cinasa p38. Asimismo, se observó que la defensina PaDef favorece la activación de la caspasa 9 (ruta intrínseca) pero no la de la caspasa 8 (ruta extrínseca). Por otro lado la defensina γ-tionina indujo la activación de ambas caspasas, lo que es congruente con la expresión de los genes. En conclusión, este es el primer reporte de defensinas de plantas con efecto citotóxico sobre las células MCF-7 a través de mecanismos diferenciados de inducción de apoptosis, lo que sugiere que estos PAs de plantas son moléculas con potencial en el tratamiento del cáncer.

Palabras clave: cáncer de mama; aguacate; chile habanero; péptidos antimicrobianos; apoptosis.

2. ABSTRACT

Cancer is a major health problem worldwide. In Mexico is the third cause of death, being the breast cancer one of the most prevalent. Conventional therapies have a low therapeutic index and severe side effects therefore alternative therapies are necessary. Antimicrobial peptides (APs) have been considered as promising anticancer agents because their ability to inactivate a wide range of cancer cells. The anticancer activities of APs include the lysis of cytoplasmic membrane and apoptosis induction. In this work, we assessed the cytotoxicity of plant APs PaDef (*Persea americana* var. *drymifolia*) and γ-thionin (*Capsicum chinense*) defensins against cancer cell line MCF-7 and we analyzed their mechanism of action. The PaDef defensin decreased the viability of MCF-7 cells in a concentration-dependent manner showing an IC_{50} of 141.62 μg/ml. In addition, PBMC viability was unaffected but BMEC viability decreased. On the other hand, γ-thionin significantly inhibited MCF-7 viability with an IC_{50}= 117.29 μg/ml. Interestingly, BMEC and PBMC viability was unaffected; suggesting that γ-thionin is selective to cancer cells. To determine the cytotoxicity mechanism of PaDef and γ-thionin on MCF-7 cells, we evaluated calcium efflux and membrane electrical potential. Both plant defensins did not modify these parameters, which indicate that the mechanism of these peptides on MCF-7 cells is not related to pore formation. On the other hand, both defensins increased the rate of apoptosis in a time-dependent manner reaching a maximum of 44 and 76%, respectively. To elucidate the apoptosis pathway induced by both plant defensins in MCF-7 cells, we evaluated the mitochondrial membrane potential (ΔΨm). Cells treated with PaDef and γ-thionin IC_{50} showed the loss of ΔΨm suggesting that intrinsic apoptosis has been activated. Additionally, we evaluated whether defensins regulate the expression of genes from extrinsic apoptosis (*FasL, Fas* and *caspase-8*) and intrinsic apoptosis (*cytochrome c, Apaf-1* and *caspase 9*) as well as *caspase-7*. PaDef induced the expression of genes of the intrinsic pathway in a time-dependent manner, whereas the expression of genes from extrinsic apoptosis was not affected. Otherwise, γ-

thionin induced a different expression pattern, increased the expression of genes of the intrinsic pathway since 6 h but this induction was down regulated at 24 h. Interestingly, at this time (24 h) γ-thionin induced significantly the expression of genes of the extrinsic pathway. Additionally, MCF-7 cells treated with PaDef IC_{50} defensin showed an increase in the phosphorylation of p38. Furthermore, PaDef favors the activation of caspase 9 (intrinsic pathway) but not caspase 8 (extrinsic pathway). However, γ-thionin induced activation of both caspases, which is consistent with the gene expression. In conclusion, this is the first report of plant defensins cytotoxic to MCF-7 cells through differential mechanisms of apoptosis induction, which suggests that these plant AMPs could be potential molecules in the treatment of cancer.

Keywords: Breast cancer; avocado; habanero pepper; antimicrobial peptides; apoptosis.

3. INTRODUCCIÓN GENERAL

3.1. Generalidades del cáncer

"El cáncer" es un término genérico que se refiere a más de 100 enfermedades diferentes que afectan a muchos tejidos y tipos celulares [1]. Las teorías actuales definen el cáncer como una proliferación celular descontrolada causada por la acumulación progresiva de mutaciones aleatorias en los genes críticos que controlan el crecimiento celular y la diferenciación [2]. Las mutaciones responsables de la formación de tumores cancerosos se agrupan en tres tipos de genes: 1) los oncogenes, 2) los genes supresores de tumores y 3) los genes de estabilidad. A diferencia de otras enfermedades de origen genético, como la distrofia muscular que se presenta por la mutación de un gen único, para el desarrollo del cáncer es necesaria la mutación en varios genes [3], de ahí la importancia de conocer cuáles son los mecanismos celulares que se ven afectados y como se relacionan entre sí (Figura 1).

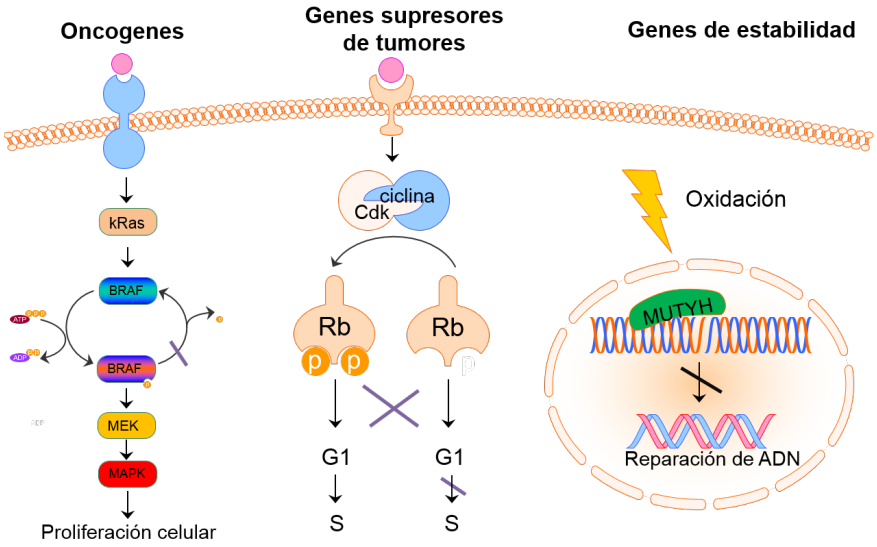

Figura 1. Genes relacionados con el desarrollo del cáncer. Los oncogenes son genes cuyas proteínas están implicadas en la inducción de la proliferación celular (por ejemplo, kRas). Los genes supresores de tumores se encargan de inhibir la proliferación celular (Rb), y los genes de estabilidad (Mutyh) reparan el daño producido al ADN por agentes externos como la radiación UV [3].

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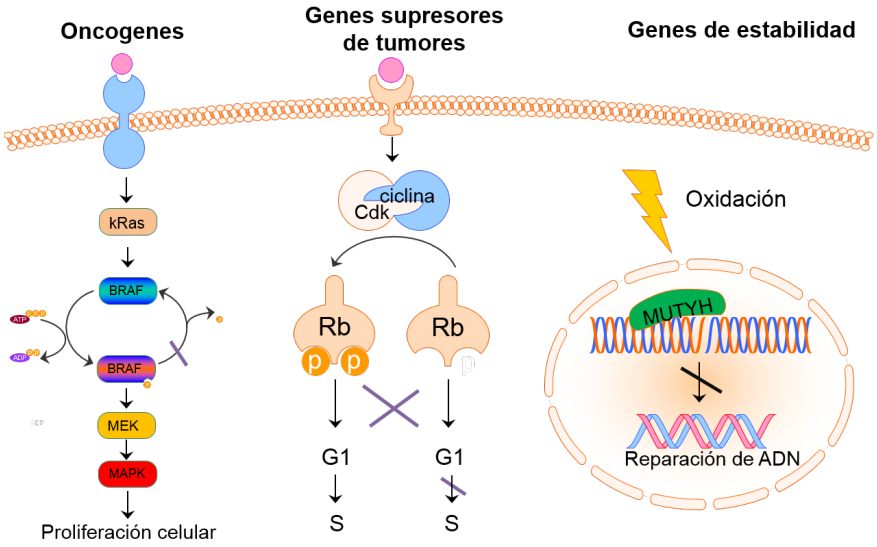

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A pesar de los avances recientes en la diversidad de tratamientos, el cáncer sigue siendo una fuente importante de morbilidad y mortalidad en todo el mundo. La Organización Mundial de la Salud (OMS) estimó en 2008 que la principal causa de muerte en el mundo es el cáncer (7.6 millones de casos), siendo los más importantes el de pulmón, estómago, hígado, colon y mama [4]. Particularmente el cáncer de mama es el cáncer más común en las mujeres en todo el mundo, en el 2012 se estimó la aparición de 1.7 millones de casos nuevos. En general la incidencia de este tipo de cáncer muestra que en países desarrollados como Estados Unidos e Inglaterra, tienen mayores incidencia en comparación con países menos desarrollados como Nepal y Ruanda [5].

En México, el cáncer es la tercera causa de muerte, y según estimaciones de la Unión Internacional contra el Cáncer, cada año se suman más de 128,000 casos. Entre los tipos de cáncer con mayor incidencia en el país se encuentran el cáncer de mama y el cervicouterino. El cáncer de mama es la principal neoplasia que presenta la población mexicana mayor de 20 años, principalmente en las mujeres (31%) [6]. En el 2012, de cada 100 fallecimientos por tumores malignos en mujeres mayores de 20 años, 15 correspondían a cáncer de mama. La entidad con la tasa más alta de mortalidad por cáncer de mama en 2012 fue Coahuila (28.58 de cada 100 mil mujeres mayores de 20 años) [6]. Con esta información es clara la necesidad de mejorar los tratamientos contra esta enfermedad, así como concientizar a la sociedad de una cultura de prevención, que en conjunto, podría ayudar a disminuir este problema de Salud Pública.

Las terapias citotóxicas convencionales, como la radiación y la quimioterapia, son los métodos de elección actuales para el tratamiento del cáncer. La terapia de radiación es relativamente precisa y es utilizada para lograr el control local, mientras que la quimioterapia ejerce un efecto sistémico y se utiliza como tratamiento de diversos tipos de cáncer. Aunque estos tratamientos se han utilizado con éxito, presentan graves efectos secundarios y las limitaciones en

la dosificación son frecuentes. Esto se debe a que no son específicos y se dirigen a cualquier célula que se divida rápidamente, sin discriminar entre las células sanas y malignas [7]. Por lo anterior, el desarrollo de una nueva clase de fármacos que carezcan de la toxicidad de los agentes terapéuticos convencionales sería un avance importante en el tratamiento del cáncer.

Otro aspecto importante a considerar en la aplicación de nuevas moléculas anticancerosas, es el estudio del tipo de muerte celular que desencadenan, principalmente la necrosis o la apoptosis. Esto se debe a que existen efectos secundarios graves que pueden ser consecuencia del mecanismo de muerte celular, como el síndrome de lisis del tumor [8, 9]; debido a ésto, a continuación se detallan los mecanismos moleculares implicados en la muerte celular por necrosis y apoptosis.

3.2 Muerte celular por necrosis y apoptosis

El término necrosis (del griego nekrós “muerte”) reúne los procesos violentos y catastróficos, donde la degeneración celular es pasiva sin un requerimiento de energía en forma de ATP. Aparece frecuentemente como consecuencia de un daño traumático o por la exposición a toxinas. En ella tiene lugar la pérdida aguda de la regulación y de la función celular que conlleva un proceso osmótico desmesurado y finaliza con la lisis de la membrana celular, liberando el contenido intracelular. Este fenómeno conduce a las células vecinas también hacia la muerte, atrayendo al mismo tiempo a las células inflamatorias, lo que hace que en las áreas donde se observan células necróticas sea frecuente encontrar nuevas células que desarrollan este tipo de muerte celular, además de originar una reacción de inflamación y una cicatriz fibrosa que deforma el tejido y el órgano afectado [10, 11].

La apoptosis es un tipo de muerte celular que se realiza de una forma ordenada y silenciosa, este mecanismo es empleado por los organismos multicelulares durante el desarrollo, la morfogénesis y en el mantenimiento de la homeostasis tisular en el organismo adulto, así como para controlar el número de células y eliminar células infectadas, mutadas o dañadas [12, 13, 14]. La muerte

por apoptosis es más limpia que la necrosis; se detectan cambios morfológicos particulares y la membrana celular, que no se destruye, engloba a los cuerpos apoptóticos o material celular. No se produce inflamación ya que las células fagocitarias reconocen, captan y eliminan los cuerpos apoptóticos. La apoptosis se puede definir como “el conjunto de reacciones bioquímicas que tienen lugar en la célula y que determinan su muerte de una forma regulada en respuesta a una serie de acontecimientos fisiológicos o patológicos” [15,16]. El programa de autodestrucción es complejo y se requiere una precisa coordinación entre la activación y la ejecución de varias rutas de la maquinaria de muerte. Las características generales de la muerte celular por apoptosis y necrosis se resumen en la Tabla 1.

Tabla 1. Características generales de la muerte por apoptosis y necrosis

APOPTOSIS	NECROSIS
Muerte fisiológica. Proceso regulado y controlado.	Muerte no fisiológica; muerte accidental traumática. Proceso no regulado
Se produce durante el desarrollo, mantiene la homeostasis tisular y elimina células dañadas	No se produce durante el desarrollo
Inducida por estímulos intra o extracelulares	Producida por un daño celular o tisular
Proceso energéticamente activo	Proceso energéticamente pasivo
Sigue un orden específico de eventos. Condensación de la cromatina y fragmentación del ADN. Mantenimiento estructural de los organelos.	La célula se hincha, se lisan los organelos y se desintegra de forma desordenada
Mantenimiento de la integridad de la membrana plasmática. El contenido se queda encapsulado en los cuerpos apoptóticos. No se produce la liberación del contenido celular. No se produce inflamación.	La ruptura de la membrana conduce a la liberación del contenido celular al espacio extracelular; asociada con la inflamación.
Participación activa de componentes celulares. Degradación mediada por caspasas.	Proceso pasivo.
Fagocitosis de cuerpos apoptóticos	Lisis celular y daño a las células vecinas

El proceso de apoptosis se desarrolla en varias etapas. En la primera, etapa efectora o de determinación, la célula reacciona ante un estímulo determinado o ante su ausencia (señales de desarrollo, estrés celular, alteración del ciclo celular, etc.) decidiendo iniciar el proceso de apoptosis. En la segunda, etapa de ejecución o degradativa, la célula sufre un conjunto de alteraciones moleculares que desencadenan la muerte celular. A éstas hay que añadir la fase de limpieza o eliminación de los cuerpos apoptóticos [17, 18].

3.2.1. Inducción de apoptosis por vía extrínseca o de receptores de muerte

Una primera ruta de señalización del proceso de apoptosis tiene su origen en la membrana celular a través de lo que se conoce como vía extrínseca o de receptores de muerte. Estos receptores pertenecen a la superfamilia del receptor de TNF (TNFR). La señal se inicia tras la unión de los correspondientes ligandos (FasL, TNF, etc.) a sus respectivos receptores de muerte (Fas, TNFR1, etc.) [19, 20]. La unión del ligando provoca la homotrimerización del receptor y, de este modo, el receptor de muerte es capaz de reclutar proteínas adaptadoras hacia la membrana celular. Este proceso implica la interacción homofílica entre los dominios de muerte DD (Death Domain) de los receptores con los de las moléculas adaptadoras (proteínas puente entre el receptor y la caspasa) como la proteína FADD (Fas Associated Death Domain). Las moléculas adaptadoras poseen los dominios efectores de muerte DEDs capaces de interaccionar homofílicamente con algunos miembros de la familia de las caspasas provocando su activación. Así, se forma un complejo de señalización de muerte (DISC; Death-Inducing Signaling Complex) que contiene a la proteína FADD y las caspasas 8 o 10. La activación de la procaspasa 8 requiere su asociación con la molécula adaptadora FADD a través de los dominios DED, situándose la caspasa 8 en la vía apoptótica mediada por receptores de muerte [21, 22]. La caspasa 8 se activa, dirigiendo de esta forma la ejecución de la apoptosis ya que, a su vez, activa a las caspasas ejecutoras (Figura 2).

3.2.2. Inducción de apoptosis por vía intrínseca o mitocondrial

Una gran parte de estímulos apoptóticos (estrés celular, drogas, radiaciones, agentes oxidantes, etc.) inducen una segunda ruta de señalización que se conoce como la vía mitocondrial o intrínseca, que está regulada por proteínas de la familia Bcl-2 [23]. Los cambios en la permeabilidad de la membrana mitocondrial causan la liberación al citoplasma de más de 40 moléculas implicadas en la apoptosis. Entre ellas se liberan al citoplasma diversas proteínas proapoptóticas como el citocromo c, el factor inductor de apoptosis AIF, la endonucleasa G, entre otras. Además, se genera un flujo de calcio y se liberan ROS. La liberación del citocromo c es un evento crítico ya que éste interacciona con la proteína citosólica Apaf-1 (Apoptotic protease activating factor-1). Esta última actúa como molécula adaptadora en esta vía, con dATP y, posteriormente con la procaspasa 9 se forma el megacomplejo heptamérico conocido como apoptosoma que ejecuta el programa apoptótico [24, 25, 26]. La formación del complejo conduce a un cambio conformacional y activación de la procaspasa 9 que, a su vez, rompe el prodominio de caspasas efectoras, como las caspasas 3, 6 y 7, activándolas para desencadenar el proceso de apoptosis (Figura 2).

Las diferencias entre apoptosis y necrosis son críticas en la observación del desarrollo de los tratamientos clínicos. La naturaleza controlada de la vía apoptótica permite la intervención o interrupción en la progresión de las patologías degenerativas. Sin embargo, en los casos de necrosis, el daño celular es irreversible e imparable. El conocimiento del mecanismo de señalización intracelular desde la activación de receptores hasta la fase ejecutora, permite establecer nuevas dianas farmacológicas para compuestos innovadores en el tratamiento de enfermedades como el cáncer. En este sentido, existe una gran variedad de productos naturales y terapias alternativas que se encuentran en estudio del tratamiento del cáncer [7, 27]. Dentro de estas alternativas destacan los péptidos antimicrobianos debido a su habilidad para inactivar un amplio rango de células cancerosas [28].

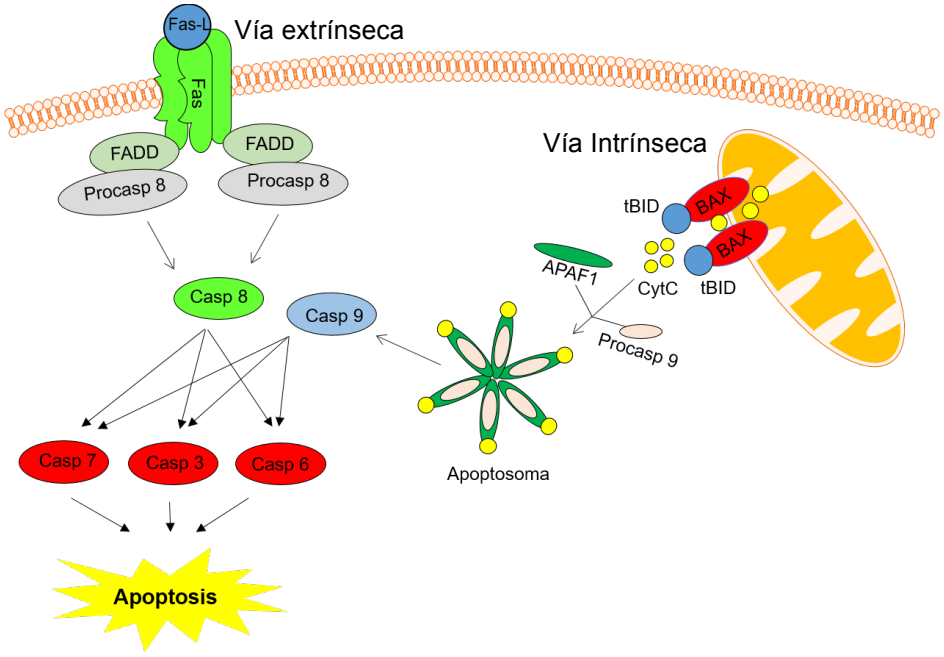


Figura 2. Rutas de activación de apoptosis. Se muestra la ruta de activación extrínseca, en la cual participan los denominados receptores de muerte, dicha activación eventualmente conduce a la apoptosis; así como la ruta de activación intrínseca, en la cual la liberación de moléculas como el citocromo c de la mitocondria conduce a la formación del apoptosoma para activar la caspasa 9 y posteriormente a las caspasas efectoras de la apoptosis [19-26].

3.3 Péptidos antimicrobianos (PAs)

Los PAs son pequeños péptidos anfipáticos y catiónicos en su mayoría, que protegen al hospedero contra una amplia gama de microorganismos. Estos péptidos son producidos por diversas especies incluyendo bacterias, insectos, plantas y animales [29]. Actualmente se han descrito más de 2700 PAs diferentes [30], los cuales están clasificados en 4 grupos de acuerdo a sus características estructurales: 1) Los que poseen en su estructura láminas-β, que son péptidos estabilizados por 2 a 4 enlaces disulfuro (por ejemplo, las α y β-defensinas humanas); 2) los que poseen hélices-α (por ejemplo la catelicidina LL-37, cecropinas o magaininas); 3) los de estructura extendida (ricos en glicina, prolina, triptófano, arginina y/o histidina) como la indolicidina; y 4) los péptidos “loop”, que cuentan con un solo enlace disulfuro (por ejemplo tanatina) (Figura 3). Estos PA

derivan de un péptido precursor o prepéptido, el cual después de uno o varios procesamientos proteolíticos libera el péptido activo [31].

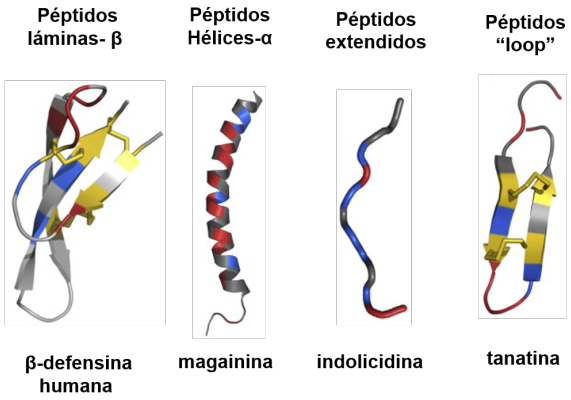


Figura 3. Clasificación estructural de los PAs. Esta clasificación se basa en las características estructurales de los péptidos, como la presencia de láminas-β, hélices-α, así como la configuración de éstas [31].

Los PAs son reconocidos principalmente por sus propiedades antimicrobianas, ya que protegen contra una gran variedad de agentes infecciosos, en mamíferos protegen contra bacterias, hongos, virus y algunos parásitos; sin embargo, poseen también una gran diversidad de funciones, entre ellas la inmunomodulación, la reparación de heridas, la neutralización de endotoxinas, la inducción de angiogénesis y la citotoxicidad [32].

3.4. Actividad citotóxica de los PAs

Una característica de los PAs que en los últimos años ha tomado relevancia es su citotoxicidad; en la tabla 1 se muestran algunos ejemplos de PAs con actividad citotóxica, las células que afectan así como su mecanismo de acción, en el cual se profundizará en el siguiente apartado.

Un ejemplo específico de esta actividad es la toxicidad de las catelicidinas de bovino BAMP-27 y BAMP-28 (1-6 μM) contra células de leucemia humana; este efecto se ha asociado con la liberación de calcio intracelular y la posterior fragmentación de ADN, que conduce a la apoptosis [33]; sin embargo, la

concentración efectiva de estas defensinas también daña algunos tipos celulares de tejido sano, por lo que dificulta su aplicación en procedimientos *in vivo* [33, 34]. Por otro lado, las cecropinas A y B obtenidas del gusano de seda (*Hyalophora cecropia*) presentan efectos citotóxicos contra células de leucemia sin afectar a las células sanas, incluso se han realizado estudios en ratones con cáncer de colon donde se encontraron disminuciones significativas en los tumores cancerosos [35]. Interesantemente, la cecropina B mostró un efecto sinergístico con agentes quimioterapéuticos convencionales como el 5-fluorouracilo y la citarabina contra líneas celulares de leucemia humana como la CCRF-SB [36]. Otros PAs con actividad citotóxica son las defensinas, de las cuales las de humanos son las más estudiadas. Las α-defensinas HNP-1, 2 y 3 son citotóxicas sobre varias líneas celulares cancerosas de linfoma, de riñon y de piel, además de que a concentraciones mayores de 25 μg/ml suprimen la síntesis de ADN en estas células [37]. Adicionalmente a su actividad citotóxica, las defensinas HNP-1, 2 y 3 interfieren con la neovascularización durante el desarrollo del tumor, debido a que inhiben la adhesión de las células endoteliales a la fibronectina [38].

Los PAs tienen varias ventajas sobre los fármacos utilizados actualmente en la terapéutica, particularmente para el tratamiento del cáncer algunos PAs presentan citotoxicidad selectiva hacia las células cancerosas, además de que no producen mecanismos de resistencia a múltiples fármacos, y que presentan efectos aditivos en la terapia de combinación [39].

3.5. Modo de acción de los PAs citotóxicos

Los PAs representan una nueva familia de agentes anticancerosos que pueden evitar algunas deficiencias de la quimioterapia convencional debido a que algunos de ellos exhiben una citotoxicidad selectiva contra un amplio espectro de células malignas humanas, incluyendo las células cancerosas multi-resistentes a los fármacos [40]. Los mecanismos citotóxicos de los PAs incluyen: 1) La activación de la necrosis por efecto de la lisis de la membrana celular, 2) la inducción del proceso de apoptosis, y 3) mecanismos de acción no relacionados con la ruptura de membranas (Tabla 2) [41].

Tabla 2. PAs con citotoxicidad contra células cancerosas

PA	Origen	Citotoxicidad	Mecanismo	Referencia
Lactoferricina B	*Bos taurus*	Neuroblastoma, leucemia y carcinoma	Membranolítico, inductor de apoptosis	[42]
Magainina 2	*Xenopus laevis*	Melanoma y retinoblasatoma	Membranolítico, inductor de apoptosis	[43]
Buforina IIb	Sintético	Hela	Inductor de apoptosis	[44]
LL-37	*Homo sapiens*	Jurkat T (Leucemia)	Membranolítico, inductor de apoptosis	[45]
Cecropina B	Sintético	Cáncer de vejiga y fibroblastos de humano	Membranolítico, inductor de apoptosis	[46, 47]
Melitina	*Apis mellifera*	Monocitos de leucemia	Membranolítico activador de fosfolipasas	[48, 49]
β-Defensina 2	*Homo sapiens*	Cáncer pulmonar y de carcinoma epidérmico	Controlador del ciclo celular	[41]
Penaeidina-2	*Penaeus vannamei*	Cáncer de riñon y de pulmón	Inductor de apoptosis	[50]
Pseudimenochirina-1Pb (Ps-1Pb) y pseudimenochirina-2Pa	*Pseudhymenochirus merlini*	Cáncer de pulmón de mama y de colon	Membranolítico	[51]
FK-16	Derivado de LL-37	Cáncer de colon	Inductor de autofagia	[52]

Existen varios modelos que explican la interacción de los Pas para producir la lisis de las membranas de las células cancerosas, los cuales son muy similares a lo que ocurre en células bacterianas. Uno de ellos es el denominado modelo de “carpeta”, donde inicialmente los péptidos cargados positivamente se asocian con la membrana celular a través de interacciones electrostáticas, la cual se cubre de una manera similar a una alfombra. Posteriormente, después de que se ha alcanzado una concentración umbral, los péptidos se insertan en la membrana formando poros. El mecanismo llamado “poro toroidal” presenta una etapa antes del colapso de la membrana e incluye la formación de poros, este se da cuando el

péptido es lo suficientemente largo para atravesar la membrana. Alternativamente, los Pas se pueden unir a través de interacciones hidrofóbicas con la membrana para formar canales transmembranales a través del mecanismo de "palo-barril" (Figura 4) [39]. Estos mecanismos eventualmente producen la lisis de la membrana y por lo tanto la muerte de la célula por necrosis.

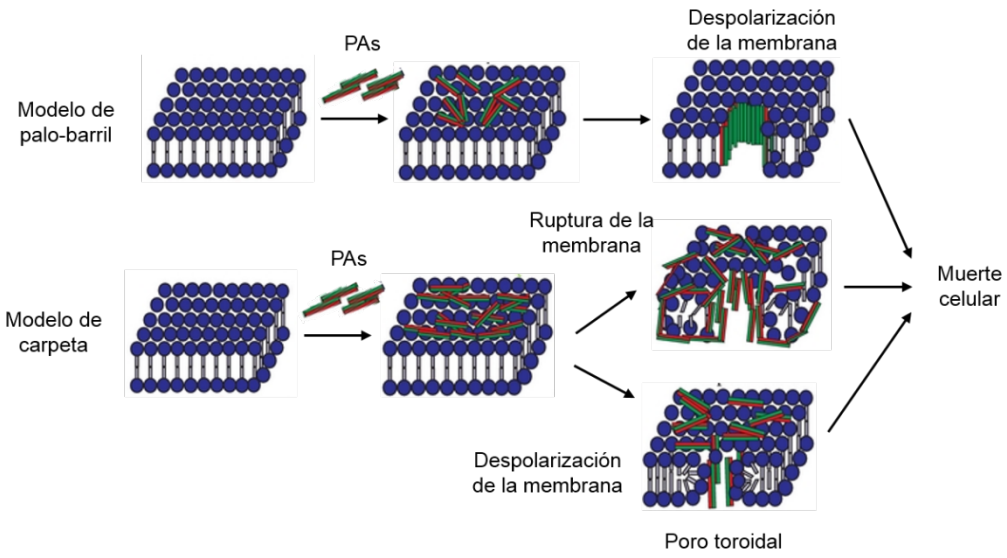


Figura 4. Mecanismos de acción membranolítico de los Pas sobre células animales. En el modelo de "palo-barril" los péptidos se unen a través de interacciones hidrofóbicas con la membrana para formar canales transmembranales. En el modelo de "carpeta" los péptidos cargados positivamente se asocian con la membrana celular a través de interacciones electrostáticas. El mecanismo llamado "poro toroidal" presenta una etapa antes del colapso de la membrana e incluye la formación de poros [39].

Adicionalmente, se ha demostrado que la primera interacción del péptido con la membrana citoplasmática puede ser solo para su internalización, ya que el PA puede tener un blanco intracelular. Uno de éstos, es la interacción directa con la membrana mitocondrial, lo que resulta en la liberación de citocromo c y la inducción de la apoptosis por la vía conocida como intrínseca (Figura 5) [53]. Un PA con esta actividad es la buforina Iib, la cual atraviesa las membranas celulares de las células cancerosas e induce la apoptosis vía mitocondrial, ésto se confirmó con la activación de la caspasa 9 y la liberación del citocromo c al citoplasma [54]. Por otro lado, se han encontrado PA capaces de activar la apoptosis por la vía extrínseca, es decir, mediante receptores (Figura 5). Ejemplo de ello es la

cecropina, que es capaz de inducir apoptosis en las células cancerosas a través de la activación de elementos característicos de esta ruta, como son las proteínas Fas, FasL y las caspasas 8 y 3 [46].

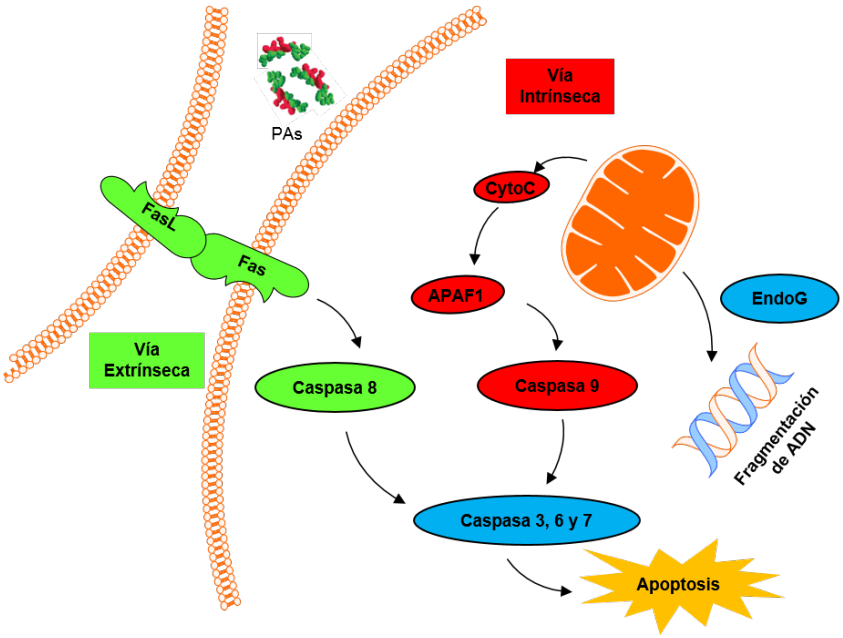


Figura 5. Proceso de inducción de apoptosis producida por los PAs. Diversos péptidos antimicrobianos pueden activar las dos vías clásicas de inducción de apoptosis, la intrínseca y extrínseca [46, 53 y 54].

Además de la interacción de los PAs con la membrana plasmática o mitocondrial en las células cancerosas, existen mecanismos de acción alternativos. Por ejemplo, la defensina humana hBD-2 mostró efecto sobre las células tumorales inhibiendo la proliferación celular, ya que detuvo el paso del ciclo celular de la fase G1 a S debido a una disminución de la fosforilación de la proteína Rb, así como a una disminución en la expresión de la ciclina D1 [41]. Estos resultados sugieren que los PA tienen propiedades reguladoras del ciclo celular que pueden ser usadas en un futuro en el desarrollo de nuevos enfoques quimioterapéuticos anticancerosos. Actualmente se realizan investigaciones para aprovechar las propiedades de los PA en el tratamiento del cáncer, siendo los más estudiados los provenientes principalmente de animales y plantas. Otro mecanismo alternativo, es la inducción de autofagia y de apoptosis independiente de caspasas en células de cáncer de colon, el cual fue descrito para el péptido FK-

16, un derivado de la catelicidina humana LL-37; la apoptosis se relacionó con la activación de la ruta p53-Bcl-2/Bax y la autofagia por la sobreexpresión de proteínas de ensamblaje del autofagosoma, además de que se demostró una regulación recíproca entre estas dos rutas de muerte celular [52].

Como se ha establecido anteriormente, la citotoxicidad de los PAs de animales es la más estudiada; sin embargo, las plantas son una fuente muy importante de moléculas bioactivas incluyendo PAs, el potencial citotóxico de estos péptidos se abordará en secciones posteriores.

3.6. Relación estructura-actividad

De acuerdo con los mecanismos de acción propuestos, los PAs interactúan con las membranas de la célula blanco y alteran físicamente la integridad de la misma, en consecuencia, la hidrofobicidad, la cationicidad y auto-asociación se consideran características esenciales que determinan las interacciones péptido-membrana. Para algunos péptidos, características como la hidrofobicidad y cationicidad determinan su capacidad para conformar un dominio transmembranal, mientras que para otros, estas mismas propiedades influyen en su actividad antimicrobiana [55].

Los estudios sobre la hidrofobicidad sugieren que en los PAs con mayor hidrofobicidad se favorece el incremento de su actividad hemolítica pero disminuye su actividad antimicrobiana; esto puede ser explicado por la fuerte auto-asociación que presentan las moléculas del péptido, lo que impide que pueda pasar a través de la pared celular [56]. Por otro lado, una distribución separada a lo largo de la secuencia de los aminoácidos cargados positivamente reduce la hemolisis de los péptidos con el mismo núcleo de hidrofobicidad. En este sentido, ya se ha demostrado que péptidos diseñados con una cationicidad incrementada muestran mayor potencia y espectro de actividad antimicrobiana, además de que se produce el mismo efecto en células de cáncer humano (MCF-7 y H460) [57]. Con base en la información anterior, es clara la necesidad de un balance específico entre la hidrofobicidad, cationicidad y auto-asociación para obtener un péptido con una actividad óptima, además de qué de estas características también puede

depender la selectividad de los péptidos, dadas las propiedades específicas de la membrana de la célula. En este sentido dentro de las secuencias nativas de PAs de animales, son relativamente pocos los que poseen toxicidad selectiva hacia células cancerosas, a diferencia de los PAs de plantas que hasta el momento muestran una selectividad importante, debido a ello los PA de plantas representan una alternativa que vale la pena estudiar más detalladamente.

3.7. Péptidos antimicrobianos de plantas (PAPs)

Las plantas han acumulado evolutivamente una serie de sistemas de defensa contra el ataque de insectos y patógenos, basados en la generación de una respuesta metabólica activa o a través de barreras químicas y estructurales. Por lo tanto, la resistencia de las plantas frecuentemente se divide en defensa constitutiva, expresada como una característica normal del desarrollo de la planta; o de defensa inducible, la cual se activa al contacto con un organismo invasor. Este último requiere de un sistema de vigilancia, el cual permite el reconocimiento de la amenaza generando un sistema de transducción de señales y una ruta de respuesta, usualmente regulada a nivel transcripcional por medio de la expresión de genes relacionados con la defensa [58, 59].

Las plantas producen pequeños PAs ricos en cisteínas como una defensa natural contra los patógenos. Estos pueden ser producidos en todos los órganos, siendo generalmente más abundantes en las capas externas, lo que es coherente con el papel de los PAs en la defensa constitutiva del hospedero contra los invasores microbianos que atacan desde el exterior. Los PAs de plantas se expresan de manera constitutiva o son inducidos en respuesta al ataque de patógenos, e incluso pueden ser liberados inmediatamente después de iniciada la infección debido a que son producidos por un solo gen, lo que hace que se reduzca el gasto de energía en su expresión [60, 61].

La mayoría de los PAs de plantas reportados hasta ahora tienen un peso molecular entre 2 y 10 kDa, son básicos y contienen 4, 6, 8 ó 12 cisteínas que forman enlaces disulfuro que les confieren estabilidad estructural y termodinámica [62, 63]. Con base a la homología en la estructura primaria de los PAs es que se

han clasificado en diferentes grupos, se reconocen hasta el momento 12 familias [62]. Por su importancia destacan los PAs tioninas y defensinas ya que son los más diversos y estudiados.

3.8. Defensinas de plantas (DPs)

Las defensinas de plantas (DPs) son un grupo importante de PAs identificados en plantas que están estructuralmente relacionados con las defensinas de insectos y las de mamíferos, por lo que se les ha nombrado "defensinas vegetales" [63]. Las DPs tienen una alta actividad antifúngica, que refleja la importancia relativa de los hongos frente a los patógenos bacterianos en el mundo vegetal [64].

Las DPs tienen una importante similitud entre sus estructuras, las cuales están generalmente compuestas por hélices-α y láminas-β. A diferencia de las tioninas, las DPs tienen similitud tanto a nivel de estructura secundaria como terciaria. Esta homología entre las defensinas sugiere una alta conservación durante la evolución, lo que podría confirmar su importante papel en la defensa de las plantas contra patógenos [65].

Se han aislado DPs en diversos tejidos, principalmente en los que se encuentran expuestos al contacto con los patógenos como las semillas, hojas, vainas, tubérculos, frutos, raíces y cortezas [66]. Debido a esto, se ha estudiado la función específica de las DPs en los diferentes órganos; por ejemplo, la defensina de rábano (*Raphanus sativus*) Rs-AFPs se encuentra expresándose en la semilla cuando ésta se encuentra perforada, ya sea por la geminación o artificialmente, la cantidad de DP que se expresa en esa área es suficiente para inhibir el crecimiento de hongos protegiendo la semilla y asegurando así la supervivencia de las plántulas [67].

3.9. Estructura de las DPs

Todas las DPs identificadas hasta el momento tienen ocho cisteínas que forman cuatro puentes disulfuro que estabilizan su estructura. Estudios de la estructura tridimensional de una serie de defensinas vegetales han demostrado

que estos péptidos se componen de una hélice-α y tres láminas-β antiparalelas, ordenadas en una configuración βαββ (Figura 6) [59]. Un ejemplo representativo de las DPs es la Rs-AFP1 de rábano, la cual está constituida por una configuración estructural conocida como βαββ, además de los 4 enlaces disulfuro característicos de estos PAs [59].

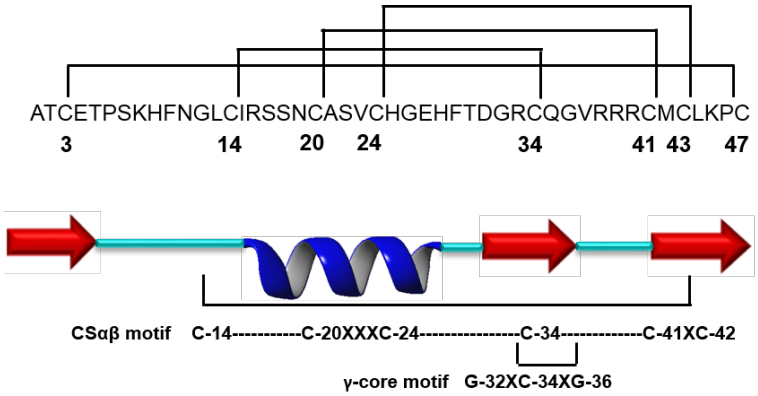


Figura 6. Estructura de las defensinas de plantas. Se presenta la DP Rs-AFP1 de rábano. La molécula se caracteriza por la presencia de 4 puentes disulfuro que estabilizan su estructura, mientras que la configuración de la estructura secundaria de las defensinas de plantas es conocida como βαββ [59].

Las DPs se pueden clasificar en dos tipos, dependiendo de la estructura de la proteína precursora de la que provienen: Tipo 1: En este grupo se encuentran la mayoría de las DPs; en ellas el prepéptido está constituido por un péptido señal unido a la secuencia de la defensina madura en el extremo N-terminal (por ejemplo, Rs-AFPs). Tipo 2: Se agrupa a las defensinas de plantas cuyo precursor está constituido por un péptido señal, el dominio activo de la defensina y un prodominio C-terminal. Un ejemplo de defensinas de este grupo es la NaD1 de *Nicotiana alata* [67].

La presencia de la secuencia conservada del péptido señal indica que estos PAs son secretados al exterior de la célula en su forma madura, como se demostró con la defensina de alfalfa alfAFP que regula la secreción del péptido hacia los espacios intercelulares del tallo en papas transgénicas [67].

A la fecha solo se ha demostrado la función específica del prodominio C-terminal en la defensina NaD1, en la cual esta secuencia actúa como una señal de localización subcelular, por lo que es importante en el tráfico subcelular y para el procesamiento postraduccional. Por otro lado, partiendo del hecho de que este prodominio está constituido en su mayoría por aminoácidos ácidos, se produce un equilibrio electrostático con las cargas positivas que presenta el dominio activo de la defensina, por lo cual el C-terminal tiene un efecto citoprotector evitando que la defensina interactúe con otras proteínas celulares o con lípidos de membrana durante la traslocación en la ruta de secreción [68].

3.10. Actividad citotóxica de las PDs

Las DPs son PAs identificados en plantas que tienen una alta actividad antifúngica, aunque también presentan efecto antibacteriano e insecticida [64], además de que influyen en el crecimiento y desarrollo de la planta [69].

Recientemente se ha encontrado que algunas de ellas presentan efecto citotóxico selectivo contra células cancerosas, un ejemplo de ello es una defensina extraída de semillas de una variedad de fríjol (*Phaseolus vulgaris*) la cual inhibe la proliferación de la línea celular de cáncer de mama (MCF-7) y de cáncer de colon (HT29), pero no la de hígado embrionario humano [70]. Por otro lado, la foratoxina C de *Phoradendron tomentosum* tiene efecto contra células de cáncer de mama [71]. En el mismo sentido, la γ-tionina de *Capsicum chinense* presentó efecto contra la línea celular de cáncer cervicouterino HeLa inhibiendo su crecimiento hasta en un 80%, además de que no mostró inhibición contra células de endotelio bovino sin transformar [72].

Para información más detallada respecto a la actividad citotóxica de los PAs de planta se refiere al lector al capítulo III de esta tesis.

4. ANTECEDENTES

Según la base de datos “The antimicrobial peptide database” [30] donde se registran 2712 PAs, 196 presentan actividad citotóxica y de ellos 70% son de animales y 30% de plantas. Por otro lado, del total de los PAs de animales reportados solo el 6% presentan citotoxicidad mientras que en los PAs de plantas el 14% posee esta propiedad. A pesar de que los PAs de animales son los más estudiados, solo un porcentaje relativamente bajo son selectivos hacia células cancerosas. Por lo anterior los PAs de plantas son un grupo prometedor en busca de alternativas de control contra el cáncer ya que existen reportes que soportan que los PAs, en particular las defensinas, pueden ejercer una acción citotóxica selectiva hacia las células cancerosas.

En este sentido, en nuestro grupo de trabajo se demostró el efecto citotóxico específico de la defensina γ-tionina de *Capsicum chinense* contra la línea celular de cáncer cervicouterino Hela, ya que al ser expresada en un sistema heterólogo inhibió el crecimiento de estas células hasta en un 80%, además de que no mostró inhibición contra células de endotelio bovino sin transformar [72].

A pesar de que el estudio de los PAs de plantas como herramienta alternativa para el tratamiento del cáncer va en aumento, aún existen muchos PAs de plantas sin evaluar su actividad citotóxica. Ejemplo de ello es la defensina PaDef de aguacate (*Persea americana* var. *drymifolia*), que fue recientemente descrita tanto a nivel estructural y funcional, ésta presentó actividad antibacteriana contra *Escherichia coli* y *Staphylococcus aureus* con porcentajes de inhibición de >90% y 85%, respectivamente [73]; sin embargo, a la fecha no se ha analizado su actividad citotóxica.

Es importante mencionar que las defensinas γ-tionina y PaDef fueron evaluadas utilizando un sistema de expresión heteróloga. Estas defensinas fueron expresadas en una línea celular inmortalizada de endotelio de cordón umbilical de bovino (BVE-E6E7), por lo que para realizar los ensayos de actividad se utilizó una

mezcla de proteínas producidas por la célula que incluye a la defensina recombinante; para confirmar que el efecto observado está dado por la defensina se incluyeron los controles necesarios. Sin embargo, para establecer un modelo de investigación con menos variables, además de tener la posibilidad de ampliar los rangos de concentración utilizados en los ensayos, en este proyecto se recurrió a la síntesis química de las defensinas γ-tionina y PaDef (> 95% de pureza).

Por todo lo anterior, en el presente proyecto, se determinó la citotoxicidad de las defensinas PaDef (*P. americana* var. *drymifolia*) y γ-tionina (*C. chinense*) sobre la línea de cáncer de mama MC-7, además de elucidar su mecanismo de acción.

5. JUSTIFICACIÓN

Debido a que el cáncer es un problema de salud mundial que va en aumento, y a que el cáncer de mama es uno de los de mayor prevalencia [6], constantemente se realizan investigaciones para encontrar un tratamiento más eficaz para tratar esta enfermedad [74]. Los procedimientos empleados actualmente presentan limitaciones ya que tienen bajos índices terapéuticos y son altamente tóxicos y con un amplio espectro de efectos secundarios [7]. En este sentido, la inducción de apoptosis es un blanco molecular importante debido a que se evitan los efectos secundarios producidos por la lisis de las células como es la inflamación [8, 9]. De ahí la necesidad del desarrollo de una nueva clase de fármacos que carezcan de la toxicidad de los tratamientos convencionales. Para tal efecto, las líneas celulares cancerosas, como la MCF-7 de cáncer de mama, son el modelo de elección para realizar la primera aproximación del efecto de los fármacos en el tratamiento del cáncer.

En los últimos años se ha evaluado como una prometedora herramienta a los péptidos antimicrobianos [28]. Estos péptidos tienen ventajas sobre los fármacos utilizados actualmente en la terapéutica, ya que algunos de ellos presentan citotoxicidad selectiva hacia las células cancerosas, además de que no producen mecanismos de resistencia y que presentan efectos aditivos en la terapia de combinación [40]. Los PAs son producidos por bacterias, insectos, plantas y animales; hasta el momento existen aproximadamente 2700 registrados, de los cuales 196 presentan citotoxicidad [30]. Los PAs de animales son los más estudiados, ejemplo de ello son las cecropinas, ya que presentan efectos citotóxicos contra células de leucemia sin afectar a las células sanas, por lo que ya se han realizado estudios en ratones con cáncer de colon donde se encontraron disminuciones significativas en los tumores cancerosos [36]. Recientemente ha crecido el interés por analizar el potencial citotóxico de los PAs de plantas, hasta el momento existen más de 40 con actividad citotóxica y ya se han reportado defensinas de plantas con citotoxicidad selectiva hacia células cancerosas, entre

ellas una defensina de fríjol que presenta inhibición de la proliferación de las líneas celulares HepG2, MCF-7, HT29 y SiLa, pero no afecta la de células de hígado embrionario humano [71]. Otra defensina selectiva hacia células cancerosas es la γ-tionina de *C. chinense*, que inhibe el crecimiento de las células de cáncer cerivcouterino Hela al 80% pero no afecta a células endoteliales bovinas sin transformar [72].

Como se mencionó anteriormente, todas estas investigaciones van encaminadas a la implementación de mejores alternativas para el tratamiento del cáncer; sin embargo, para que esto suceda es importante contar con información básica del mecanismo por el cual estos PAs ejercen su efecto. En este sentido existe información preliminar que indica que el mecanismo de acción de los PAs puede estar encaminado a la inducción de necrosis por la lisis de la membrana celular, como es el caso de la magainina 2 [43] y las cecropinas [47]. Sin embargo, es interesante observar que en estudios más recientes, se demostró que estos mismos PAs inducen apoptosis en células cancerosas [46]. Por lo que respecta a los PAs de plantas, es poco lo que se sabe de su mecanismo de acción.

Consecuentemente con lo aquí planteado, en este trabajo se analizó la citotoxicidad de las defensinas de plantas PaDef y γ-tionina sobre la línea celular de cáncer de mama MC-7, además de que se elucidó el mecanismo de acción de las dos defensinas sobre esta línea.

6. HIPÓTESIS

Las defensinas PaDef (*Persea americana* var. *drymifolia*) y γ-tionina (*Capsicum chinense*) son citotóxicas contra la línea celular de cáncer de mama (MCF-7) mediante la inducción de la apoptosis.

7. OBJETIVO GENERAL

Evaluar la citotoxicidad de las defensinas PaDef (*P. americana* var. *drymifolia*) y γ-tionina (*C. chinense*) contra la línea celular de cáncer de mama MCF-7 y elucidar su mecanismo de acción.

7.1. Objetivos específicos

1) Determinar la citotoxicidad de las defensinas PaDef y γ-tionina contra las células MCF-7, y los cultivos primarios (CEMB y PBMC).

2) Evaluar el daño a la membrana citoplasmática en las células MCF-7 tratadas con las defensinas.

3) Analizar la inducción de apoptosis por las defensinas PaDef y γ-tionina en las células MCF-7.

4) Analizar las rutas de señalización activadas por las defensinas PaDef y γ-tionina relacionadas con la muerte celular.

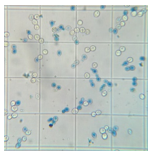
5) Realizar un análisis estructura-actividad de las defensinas PaDef y γ-tionina.

8. ESTRATEGIA EXPERIMENTAL

Objetivo 1:

Citotoxicidad de PaDef y γ-tionina

Viabilidad

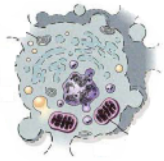


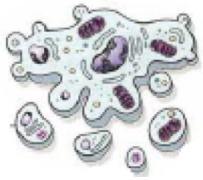
Objetivo 5:

Análisis comparativo

- Análisis estructural

MCF-7, PBMC y CEMB





Objetivo 2:

Daño a la membrana citoplasmática

- Potencial de membrana citoplasmático
- Liberación de calcio extracelular

Objetivos 3 y 4:

Apoptosis

- Externalización de fosfatidilserina
- Potencial de membrana mitocondrial
- Análisis de genes *cas7, cas8, fas, fasL, cas9, apaf1* y *cytC*
- Fosforilación de p38
- Activación de caspasas 8 y 9

9. RESULTADOS

Los resultados generados con la realización del presente trabajo se presentan en 3 capítulos.

1) El capítulo I corresponde al artículo de investigación:

Guzmán-Rodríguez J., R. López-Gómez, R. Salgado-Garciglia, A. Ochoa-Zarzosa and J. E. López-Meza. 2016. The defensin from avocado (*Persea americana var. drymifolia*) PaDef induces apoptosis in the human breast cancer cell line MCF-7. **Biomedicine and Pharmacotherapy**. 82(C): 620-627. ISSN: 0753-3322. doi:10.1016/j.biopha.2016.05.048. **Factor de impacto 2.326** (2015 JCR Science Edition).

2) En el capítulo II se presentan los resultados correspondientes a los efectos citotóxicos de la γ-tionina.

The plant defensin γ-thionin (*Capsicum chinense*) is cytotoxic against breast cancer cell line MCF-7 by apoptosis induction

3) El capítulo III comprende el artículo de revisión:

Guzmán-Rodríguez, J.J., A. Ochoa-Zarzosa, R. López-Gómez, and J.E. López-Meza. 2015. Plant antimicrobial peptides as potential anticancer agents. **BioMed Research International.** Volume 2015, Article ID 735087, 11 pages. http://dx.doi.org/10.1155/2014/735087. ISSN: 1110-7243 (Print). **Factor de impacto 2.134** (2015 JCR Science Edition).

9.1. CAPÍTULO I: The defensin from avocado (*Persea americana* var. *drymifolia*) PaDef induces apoptosis in the human breast cancer cell line MCF-7

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Original article

The defensin from avocado (*Persea americana* var. *drymifolia*) PaDef induces apoptosis in the human breast cancer cell line MCF-7

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ABSTRACT

Antimicrobial peptides (AMPs) are cytotoxic to cancer cells; however, mainly the effects of AMPs from animals have been evaluated. In this work, we assessed the cytotoxicity of PaDef defensin from avocado (*Persea americana* var. drymifolia) on the MCF-7 cancer cell line (a breast cancer cell line) and evaluated its mechanism of action. PaDef inhibited the viability of MCF-7 cells in a concentration-dependent manner, with an IC_{50} = 141.62 μg/ml. The viability of normal peripheral blood mononuclear cells was unaffected by this AMP. Additionally, PaDef induced apoptosis in MCF-7 cells in a time-dependent manner, but did not affect the membrane potential or calcium flow. In addition, PaDef IC_{50} induced the expression of *cytochrome c*, *Apaf-1*, and the *caspase 7* and *9* genes. Likewise, this defensin induced the loss of mitochondrial $\Delta\psi m$ and increased the phosphorylation of MAPK p38, which may lead to MCF-7 apoptosis by the intrinsic pathway. This is the first report of an avocado defensin inducing intrinsic apoptosis in cancer cells, which suggests that it could be a potential therapeutic molecule in the treatment of cancer.

1. Introduction

Cancer is a major public health concern worldwide. In 2012, cancer caused 8.2 million deaths, with breast cancer being one of the most prevalent cancers [1]. Cancer treatment requires conventional approaches, such as surgery, radiotherapy or chemotherapy; however, these approaches have a low therapeutic index and severe side effects [2]. These limitations have led to the search for new anticancer therapies. An attractive alternative is the use of antimicrobial peptides, or AMPs, which represent a novel family of anticancer agents that avoid the limitations of conventional treatments [3].

The AMPs are biologically active molecules, which are mainly cationic and amphipathic, and are produced by a wide variety of organisms as essential components of their innate immune response [4]. The primary role of AMPs is to defend the host against pathogenic microorganisms [4]; however, these peptides exhibit a wide range of properties, including modulation of the innate immune response and anticancer activity [5]. Currently, over 2500 AMPs are reported in The Antimicrobial Peptide Database (URL http://aps.unmc.edu/AP/main.php) [6], and 7% of them have shown cytotoxicity to cells from different types of cancer. The cytotoxic mechanisms of AMPs include necrosis (e.g., magainin 2) [7], apoptosis (e.g., lactoferricin and cecropin) [8,9], and alternative mechanisms, such as cell cycle arrest (e.g., beta-defensin-2) [10] and autophagy induction (e.g., FK-16) [11]. For the most part, these effects have been evaluated using AMPs from animals, and very little is known about AMPs from plants.

Plants produce small cysteine-rich AMPs as a mechanism of natural defense, and these AMPs are abundantly expressed in the majority of species [12]. Plant AMPs have a molecular weight between 2 and 10 kDa, are basic, and contain 4, 6, 8, or 12 cysteines that form disulfide bonds conferring structural and thermodynamic stability [13]. Numerous sequences of AMPs have been reported (≈320) [6], and twelve families have been described based on the identities of their amino acid sequences and the

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Table 1
Sequence of primers used in this study.

Gene	Sequence	Tm (°C)	Product size (bp)	Reference
Fas	5′ GTGAGGGAAGCGGTTTACGAGTGA 3′ 5′ TGGGCATTAACACTTTTGGACGAT 3′	66	256	This study
Fas L	5′ TGTGATCAATGAAACTGGGCTGTA 3′ 5′ ATCATCTTCCCCTCCATCATCACC 3′	57	233	This study
Caspase 8	5′ AGATCTGGCCTCCCTCAAGTTCCT 3′ 5′ AAATTTGAGCCCTGCCTGGTGTCT 3′	66	244	This study
Caspase 7	5′ AACCCAAACTCTTCTTCATTCAGG 3′ 5′ TAATAGCCTGGAACCGTGGAATAG 3′	57	145	This study
Cyt c	5′ TCAGCACCATGGCGGAAGACA 3′ 5′ TCCTTTAGCGGTCATTGCCTTCTG 3′	66	151	This study
Apaf-1	5′ AAATGGACACCTTCTTGGACGACA 3′ 5′ CAGAAAAGCAGGCATGGTAAACAG 3′	58	223	This study
Caspase 9	5′ AGGACATGCTGGCTTCGTTTCTG 3′ 5′ CCAAATCCTCCAGAACCAATGTCC 3′	66	257	This study
β-actin	5′ AAAACCTAACTTGCGCAGAAAACA 3′ 5′ TGTCACCTTCACCGTTCCACTTT 3′	57	317	This study

number and position of cysteines forming disulfide bonds. Recent reports have demonstrated the anticancer properties of three families: thionins, cyclotides and defensins [14]. Several studies have demonstrated that plant defensins inhibit the proliferation of cancer cells, including breast, colon and cervical cancer cells, without exerting side effects on normal cells [15–17]. However, the mechanism of action of plant defensins against cancer cells, as well as their selectivity, is poorly understood. In a previous study, we isolated the cDNA of PaDef defensin from avocado fruit (*Persea americana* var. drymifolia) and expressed it as a fusion protein in the endothelial cell line BVE-E6E7. We showed that the conditioned media from these cells have antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, important pathogens affecting animals and humans [18]. However, its cytotoxic effect has not been explored. In this study, we demonstrated that PaDef defensin from avocado induces the intrinsic apoptosis pathway in the breast cancer cell line MCF-7, which is a novel property for a plant defensin.

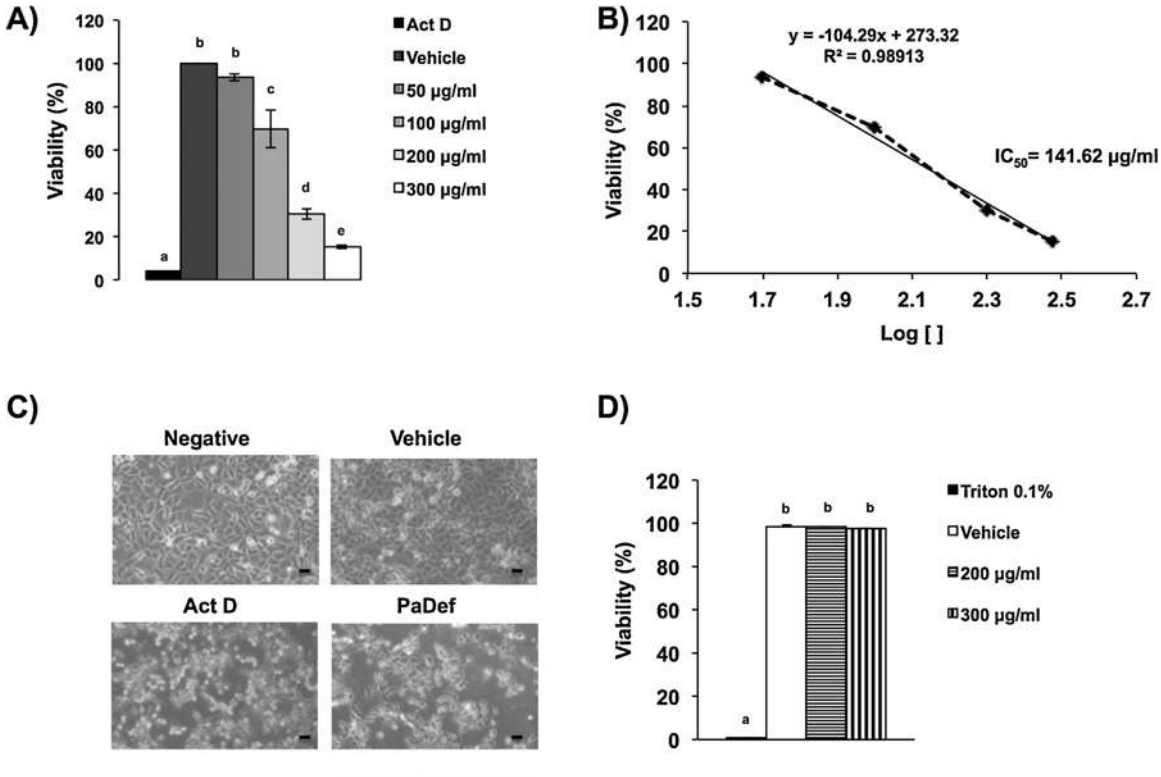

Fig. 1. PaDef defensin from avocado is cytotoxic to breast cancer MCF-7 cells. (A) Effect of PaDef on the viability of MCF-7 cells. Cells were treated with defensin (50, 100, 200 and 300 μg/ml), and viability was evaluated by trypan blue assays at 48 h. Cell viability is shown as the percentage of viable cells with respect to cells treated with vehicle (DMSO 0.4%). Data represent the mean of three independent experiments performed in triplicate. (B) Linear regression analysis of the concentration-response to calculate the half maximal inhibitory concentration (IC_{50}) of PaDef on MCF-7 cells; IC_{50} = 141.62 μg/ml; R^2 = 0.9891. (C) MCF-7 cell morphology after different treatments. Photographs are representative of at least two independent experiments and were taken with bright field microscopy. Scale bars: 10 μm. Act D (Actinomycin D), PaDef IC_{50} = 141.62 μg/ml. (D) Effect of PaDef on the viability of human peripheral blood mononuclear cells. Cells were treated with defensin (200 and 300 μg/ml), and viability was evaluated by trypan blue assays at 48 h. Cell viability is shown as the percentage of viable cells with respect to cells treated with vehicle (DMSO 0.4%). Data represent the mean of three independent experiments performed in triplicate. Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Peptide

PaDef used in this work corresponds to the mature region (ATCETPSKHFNGLCIRSSNCASVCHGEHFTDGRCQGVRRRCMCLKPC, 47 aa) (Genbank KC007441) [18], which was chemically synthesized and obtained from BIOMATIK. Prior to assays, PaDef (5 mg/ml) disulfide bond formation was accomplished by air oxidation in dimethyl sulfoxide (20%) for 5 days at room temperature. PaDef bond formation was confirmed by TOF-mass spectrometry [19]. For all of the experiments, the final concentration of vehicle was 0.4% DMSO, which was also used as a control. PaDef was used over a concentration range of concentrations of 50–300 μg/ml, in agreement with reports describing these concentrations as cytotoxic for mammalian defensins [8]. Additionally, PaDef biological activity was confirmed by evaluating its antifungal effect against *Candida albicans* ATCC 10231 [20].

2.2. MCF-7 cell culture

The human breast tumor cell line MCF-7 was obtained from American Type Culture Collection. Cells were routinely cultured in DMEM medium/nutrient mixture F-12 Ham (Sigma) supplemented with 10% (v/v) fetal bovine serum (Corning) and 100 U/ml penicillin and streptomycin (Gibco) and grown in an atmosphere of 5% CO_2 at 37 °C.

2.3. Trypan blue dye exclusion viability assay

MCF-7 cells (2×10^4 cells/well) were cultured in 96-well plates overnight to allow cell attachment. Fresh medium without serum was added, and cells were incubated for 12 h. Then, the medium was replaced with 100 μl of fresh medium containing PaDef (50–300 μg/ml), and cells were incubated for 48 h. Then, cells were harvested by trypsinization and 10 μl of the cell suspension was mixed with 10 μl of trypan blue (0.4%). Dead and viable cells were counted using a hematocytometer in an inverted microscope (Primo Vert, Zeiss). Cell viability was registered as the percentage of viable cells with respect to vehicle (DMSO 0.4%). The half maximal inhibitory concentration (IC_{50}) was calculated using Excel (Microsoft).

2.4. Calcium efflux testing

Calcium efflux was measured by flow cytometry in a BD Accuri™ C6 flow cytometer (BD Biosciences) using a Calcium Assay Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, MCF-7 cells (1×10^5/ml) were incubated with the indicator dye for 1 h. The baseline fluorescence was established (3 min), and

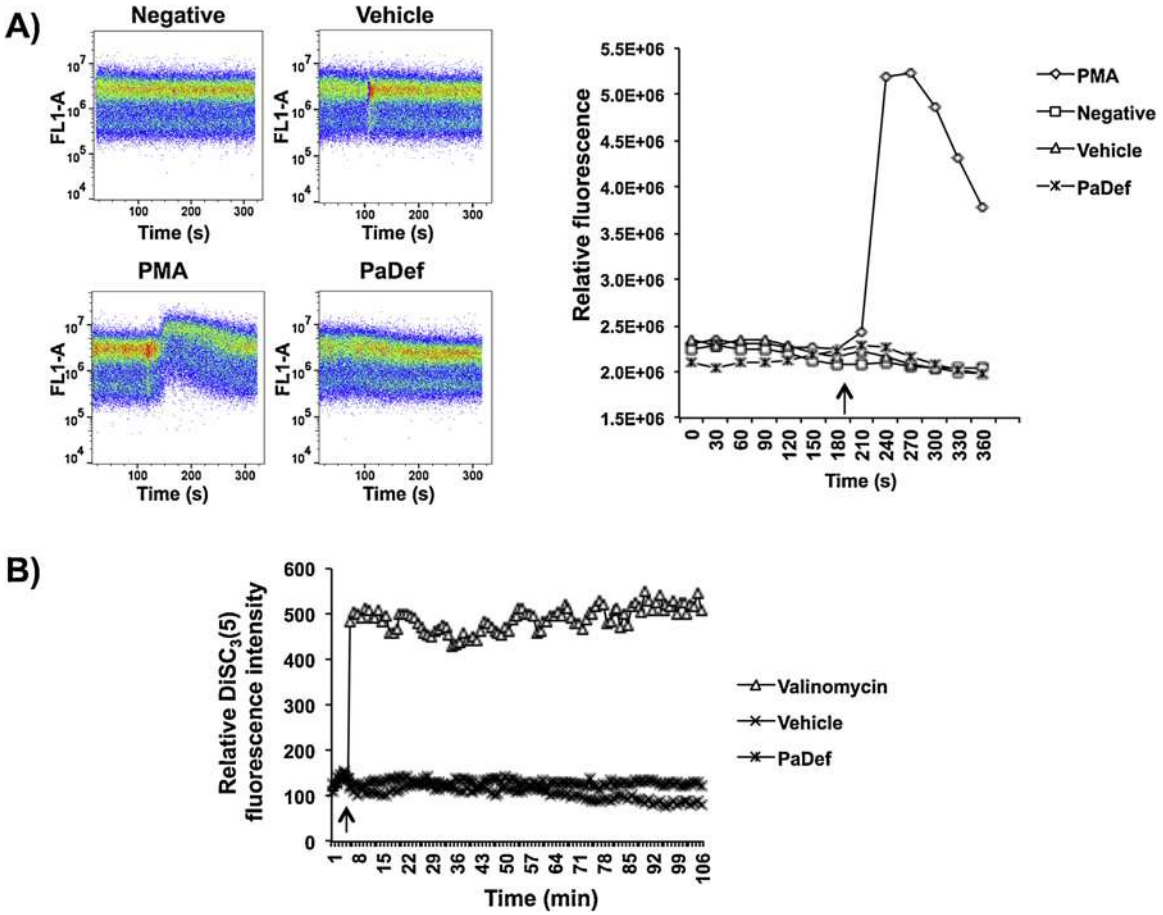

Fig. 2. PaDef defensin does not affect the membrane integrity of MCF-7 cells. (A) The efflux of cytosolic calcium was analyzed by flow cytometry. Measurements were performed for 6 min. The left panel shows representative plots of the different conditions. The right panel indicates the relative fluorescence intensities for extracellular calcium release. PMA (3 μM) was used as a positive control. (B) Changes in the membrane potential of MCF-7 cells were measured using a membrane potential-sensitive dye. Cells were incubated with 200 μM $DiSC_3(5)$ for 30 min at 37 °C and were then treated with PaDef IC_{50}. Valinomycin (0.2 mM) was used as a positive control. Arrows indicate the time at which the treatments were added.

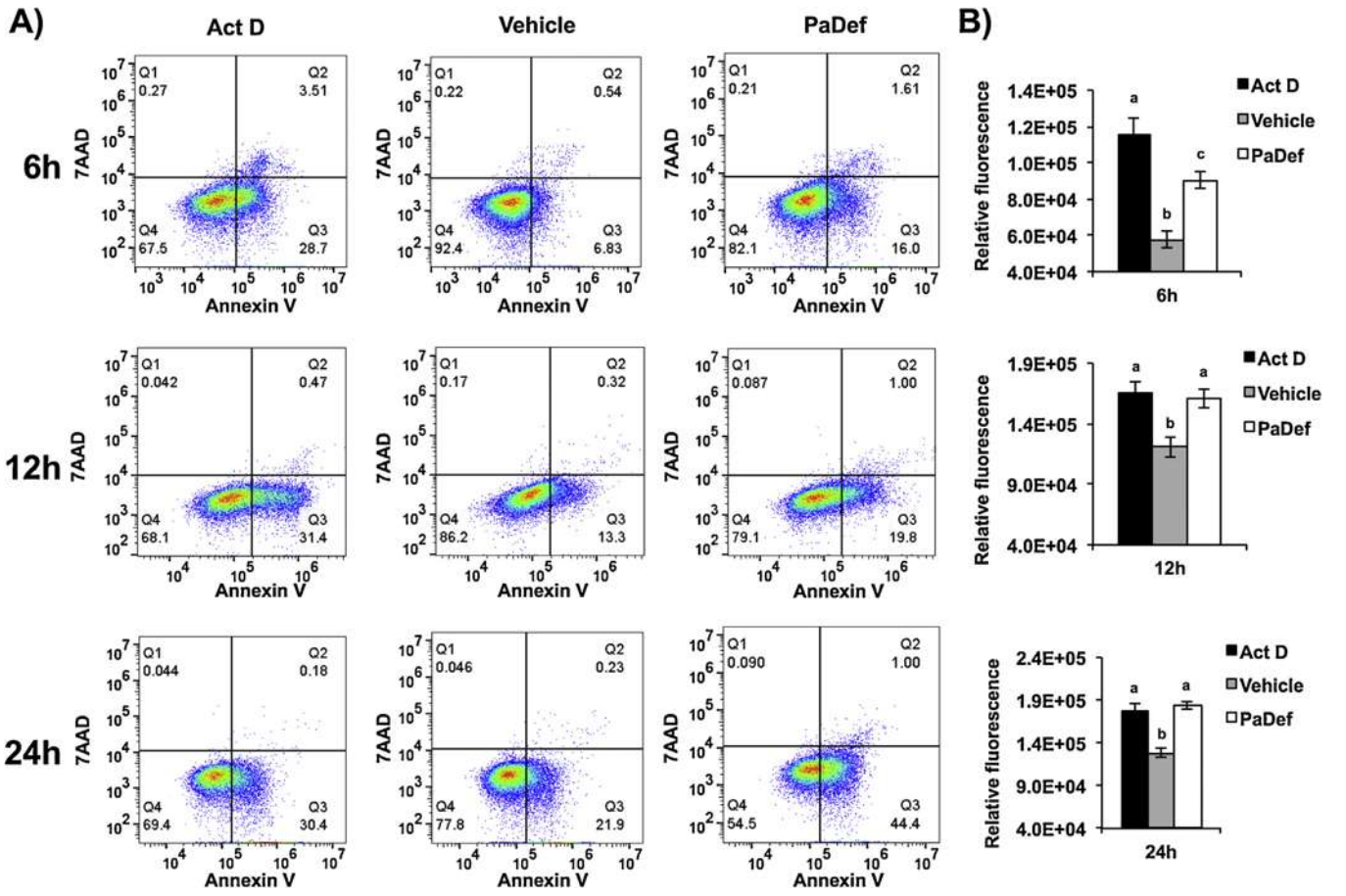

Fig. 3. PaDef defensin induces apoptosis in MCF-7 cells. Cells were treated with PaDef IC_{50} for 6, 12 and 24 h. (A) Apoptosis was analyzed by flow cytometry using Annexin V/7AAD staining. Viable cells did not bind to annexin V or 7AAD (lower left quadrant). The lower right quadrant of each histogram represents early apoptosis, the upper right quadrant represents late apoptosis, and the upper left quadrant contains necrotic cells. (B) The graphics show the fluorescence of each time of treatment (relative units). Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (One-way ANOVA and Tukey's pairwise comparison, $P < 0.05$).

then, treatments were added (PaDef IC_{50} or vehicle). The measurements were carried out for 3 min without interrupting the data collection. The changes of the fluorescence intensity in cell populations in response to the peptide were monitored using flow cytometry. Phorbol myristate acetate (3 μM; PMA, Sigma) was used as a positive control.

2.5. Measurement of the transmembrane potential

The cell transmembrane potential depolarization was measured using the membrane potential sensitive dye 3,3′-dipropylthiadicarbocyanine iodide, $DiSC_3(5)$ (Sigma). For this, MCF-7 cells (2×10^4 cells/well) were seeded in 96-well black-wall plates and cultured for 16 h. Then, cells were washed two times with Hanks'-HEPES buffer and were incubated with 100 μl of Hanks' HEPES buffer containing $DiSC_3(5)$ (0.2 mM) for 30 min in a CO_2 incubator. PaDef IC_{50} was added to each well, and the subsequent changes in the fluorescence intensity were monitored for 2 h in a Varioskan spectrophotometer (Thermo Scientific). Valinomycin (Sigma, 0.2 mM) was used as a positive control.

2.6. Apoptosis rate determined by flow cytometry

MCF-7 cells (2×10^4 cells/well) were cultured in 96-well plates and incubated with PaDef IC_{50} for 6, 12, and 24 h. After treatment, the cells were collected by trypsinization and washed with PBS and subsequently stained with Annexin V and 7AAD according to the manufacturer's instructions (Annexin V, Alexa Fluor® 488 conjugate, Invitrogen). The cell-surface phosphatidylserine (PS) exposure and plasma membrane integrity were analyzed using a BD Accuri™ C6 flow cytometer (BD Biosciences) using the FlowJo software (TreeStar, Inc.). Actinomycin D (Sigma, 80 μg/ml) was used as a positive control for apoptosis. A total of 10,000 events were analyzed.

2.7. Assessment of mitochondrial membrane potential ($\Delta\Psi m$)

MCF-7 cells (2×10^4 cells/well) were cultured in 96-well plates and treated with PaDef IC_{50} or vehicle and stained with JC-1 dye (BD Biosciences) for 15 min at 37 °C in the dark, according to the manufacturer's instructions. The cells were subsequently washed twice with assay buffer, and their fluorescence was measured in a BD Accuri™ C6 flow cytometer (BD Biosciences) using the FlowJo software (TreeStar, Inc.). JC-1 allows for differentiating healthy cells (red fluorescence) from those with mitochondrial damage (green fluorescence).

2.8. RNA isolation and gene expression analysis

MCF-7 cells (1×10^5 cells/well) were cultured in 24-well plates and incubated with PaDef IC_{50} for 6, 12, and 24 h. Total RNA was extracted from all of the conditions with Trizol reagent (Invitrogen) according to the manufacturer's instructions and then used to synthesize cDNA. Genomic DNA contamination was removed from

RNA samples with DNase I treatment (Invitrogen). cDNA synthesis was carried out as previously described [21].

The relative quantification of gene expression (qPCR) was performed using the comparative Ct method (ΔΔCt) in a StepOne Plus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The reactions were carried out with a SYBR Green PCR Master Mix (Applied Biosystems). The genes evaluated are shown in Table 1. β-actin was used as an internal control.

2.9. Phosphorylation of MAPK p38

To evaluate the MAPK p38 activation level by flow cytometry, MCF-7 cells (1×10^5 cells/well) were cultured in 24-well plates and treated with PaDef IC_{50} or vehicle. Samples of protein (30 μg) were prepared according to the manufacturer's protocol for adherent cells (BD Biosciences). Phospho-protein 38 (pp38, T180/Y182) was quantitatively determined using antibodies from a Flex Set Cytometric Bead Array (BD Biosciences) according to the manufacturer's protocol. Flow cytometric analysis was performed using the BD Accuri™ C6 and CBA analysis FCAP software (BD Biosciences). A total of 300 events were acquired following the supplied protocol. The minimum detection level for pp38 was 0.64 U/ml. A pharmacological inhibitor of p38 (2.5 μm SB203580) was used as a control.

2.10. Statistical analysis

The data were obtained from three independent experiments, each of which was performed in triplicate and compared by analysis of variance (ANOVA). The results are reported as the means ± the standard errors (SE) and the significance level was set at $P < 0.05$, except for RT-qPCR analysis where fold-change values greater than 2 or less than 0.5 were considered as significantly differentially expressed mRNAs according to Morey et al. [22].

3. Results

3.1. PaDef defensin is cytotoxic to MCF-7 cells

The cytotoxicity of PaDef (50, 100, 200 and 300 μg/ml) on MCF-7 cells was evaluated by trypan blue exclusion assays (at 24 and 48 h). PaDef did not affect the MCF-7 cells at 24 h (data not shown). However, PaDef defensin was cytotoxic to MCF-7 cells in a concentration-dependent manner after 48 h, reaching an inhibition of 85% at 300 μg/ml (Fig. 1A).The half maximal inhibitory concentration was calculated as IC_{50} = 141.62 μg/ml (Fig. 1B); this concentration was used in the rest of the experiments. Additionally, the morphology of MCF-7 cells showed alterations that were suggestive of apoptosis, such as the loss of adherence and cell rounding, similar to cells treated with actinomycin D (Fig. 1C). In addition, PaDef did not show cytotoxicity towards human peripheral blood mononuclear cells (Fig. 1D).

3.2. PaDef defensin does not affect the membrane integrity of MCF-7 cells

Antimicrobial peptides may induce membrane pore formation. To determine if PaDef cytotoxicity on MCF-7 cells is associated with membrane damage, we evaluated two different parameters: the calcium efflux and membrane electrical potential. PaDef IC_{50} did not affect the efflux of intracellular calcium as monitored by flow cytometry (Fig. 2A). The effect of PaDef IC_{50} on the plasma membrane electrical potential in MCF-7 cells was analyzed using the $DiSC_3(5)$ dye. As shown in Fig. 2B, PaDef defensin did not affect the membrane electrical potential of cells compared with the positive control

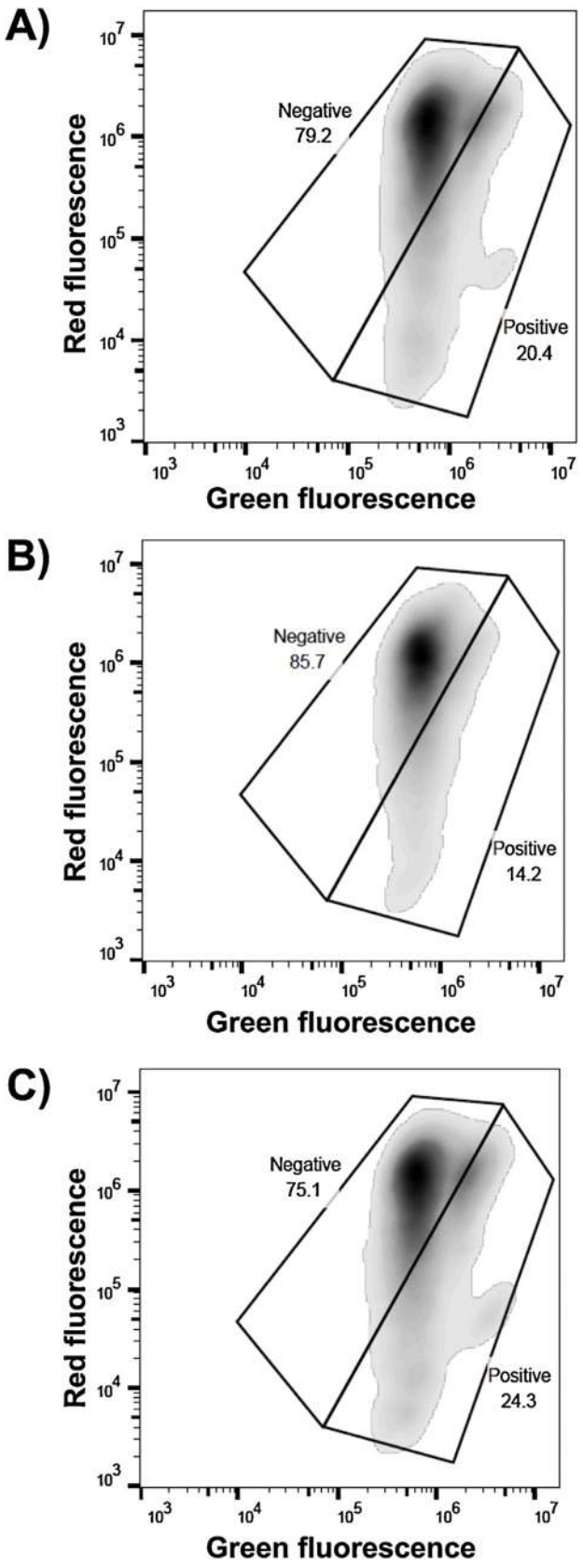

Fig. 4. PaDef defensin induces loss of mitochondrial membrane potential (ΔΨm) in MCF-7 cells. Cells were treated with ActD (A), Vehicle (DMSO 0.4%) (B), and PaDef IC_{50} **(C)** for 24 h, stained with JC-1 dye, and analyzed by flow cytometry.

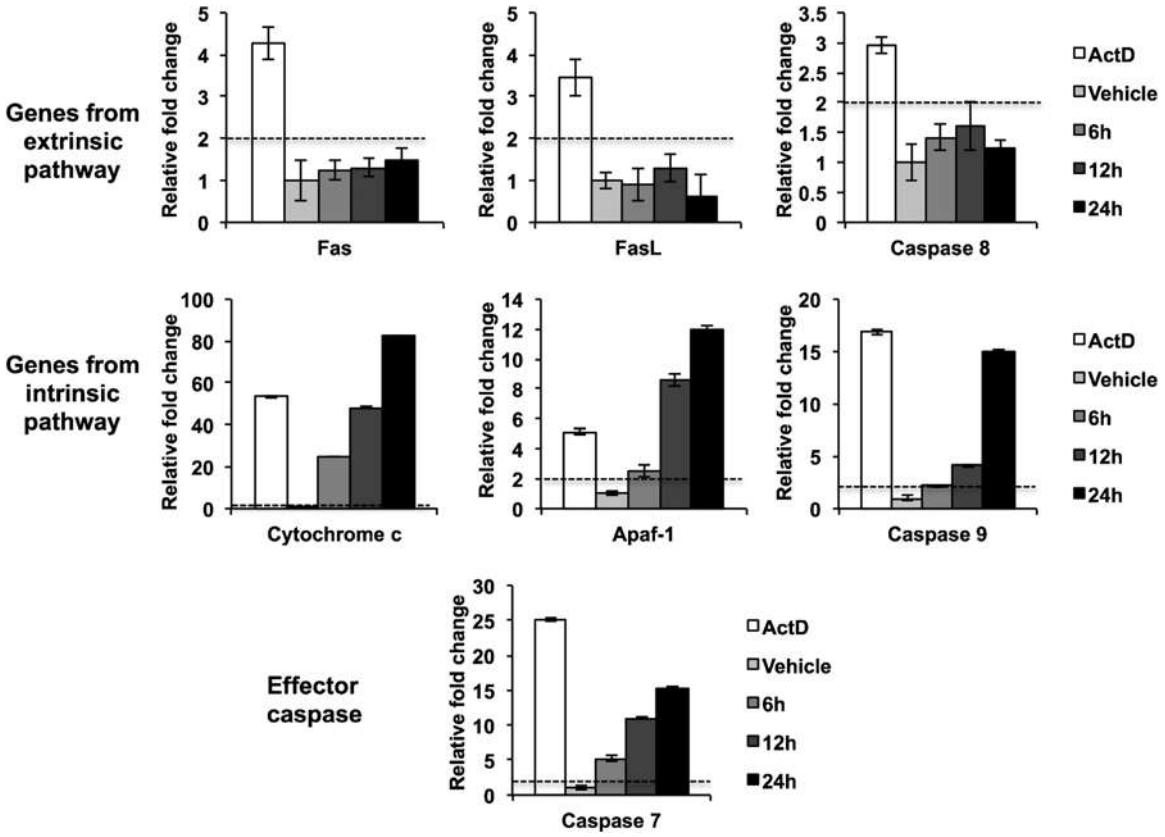

Fig. 5. PaDef defensin regulates the expression of genes related to intrinsic apoptosis in MCF-7 cells. Cells were treated with PaDef IC_{50} for 6, 12 and 24 h. mRNA expression was analyzed by RT-qPCR. mRNA expression of cells treated with actinomycin D and vehicle was analyzed at 24 h. Each bar shows the mean of triplicates ± SE of three independent experiments. Fold-change values greater than 2 or less than 0.5 were considered significant for differentially expressed mRNAs.

(valinomycin 0.2 mM). These results suggest that the mechanism of PaDef cytotoxicity on MCF-7 cells is not related to pore formation.

3.3. PaDef defensin induces apoptosis in MCF-7 cells

Apoptosis induction has been reported to be a cytotoxic mechanism for antimicrobial peptides. We evaluated whether PaDef IC_{50} induces apoptosis in MCF-7 cells by flow cytometry using Annexin V. Fig. 3 shows representative dot plots for 6, 12 and 24 h PaDef treatments. PaDef defensin induced apoptosis (>40%) in MCF-7 cells in a time-dependent manner, an effect similar to that exhibited by actinomycin D at 24 h. We also evaluated necrosis, and the results showed that it did not increased significantly.

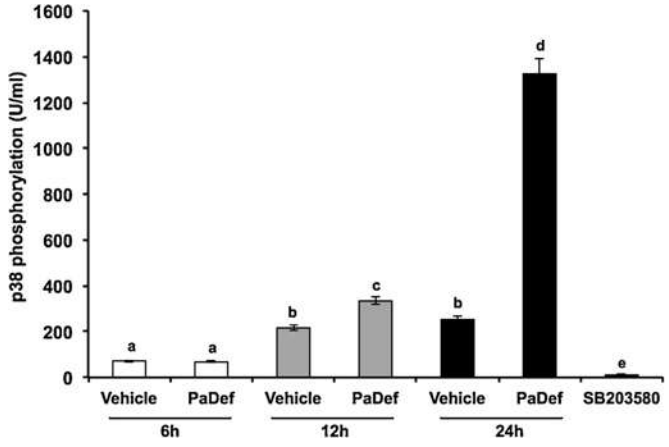

Fig. 6. PaDef defensin induces p38 phosphorylation in MCF-7 cells. Phosphorylation was measured in cells that were treated with vehicle (DMSO 0.4%) or PaDef IC_{50} for 6, 12 and 24 h by flow cytometry. The phosphorylated pp38 concentration (units/ml) is represented. Each bar represents the mean of 300 events. SB203580: p38 inhibitor. Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (One-way ANOVA and Tukey's pairwise comparison, $P < 0.05$).

3.4. PaDef defensin induces the loss of $\Delta\Psi m$ and regulates the expression of genes related to intrinsic apoptosis in MCF-7 cells

To elucidate the apoptosis pathway induced by PaDef in MCF-7 cells, we evaluated mitochondrial function by flow cytometry using the $\Delta\Psi$m-sensitive molecular probe dye JC-1. MCF-7 cells treated with PaDef IC_{50} for 24 h showed an increase in the number of fluorescent cells (Fig. 4), indicating the loss of $\Delta\Psi$m. These data suggest that intrinsic apoptosis has been activated.

Additionally, we evaluated whether PaDef IC_{50} regulates the expression of genes typical of extrinsic apoptosis (*FasL, Fas* and *caspase-8*) and intrinsic apoptosis (*cytochrome c, Apaf-1* and *caspase 9*). Additionally, we evaluated the effect or *caspase7*. qPCR analysis showed that PaDef significantly induced the expression of intrinsic pathway genes (*cytochrome c, Apaf-1* and *caspase 9*) beginning at 6 h of treatment, as well as *caspase 7* (Fig. 5). This expression increased as a function of time, reaching maximal expression at 24 h. Interestingly, the expression of the extrinsic pathway genes was not modified (Fig. 5). These results indicate that PaDef defensin induces apoptosis in MCF-7 cells by the intrinsic pathway.

3.5. PaDef defensin activates p38 MAPK in MCF-7 cells

The activation of the p38 MAPK pathway leads to the mitochondrial release of cytochrome c and, as a consequence, to

apoptosis [23]. To demonstrate the participation of p38 MAPK in the PaDef induction of apoptosis of MCF-7 cells, we determined the levels of active phospho-p38 by flow cytometry at different times. MCF-7 cells treated with PaDef IC_{50} defensin showed an increase in phosphorylation of p38 after 12 h of treatment, and phosphorylation reached its maximal level at 24 h (Fig. 6).

4. Discussion

The search for bioactive peptides from plants is an attractive field in the pursuit of alternatives to cancer therapy. To this end, several reports describe the anticancer activity of plant defensins; however, the anticancer activity mechanism of these AMPs is poorly understood [14]. In this work we demonstrated the anticancer activity of PaDef defensin from avocado on MCF-7 cells and showed evidence that this effect is due to induction of the intrinsic apoptosis pathway.

PaDef was cytotoxic to MCF-7 cells in a concentration-dependent manner, with an $IC_{50} = 141.62\ \mu g/ml$. This is in agreement with the reported IC_{50} values of the plant defensins NaD1, sesquin and limenin [24–26]. Additionally, PaDef defensin was not cytotoxic to peripheral blood mononuclear cells, suggesting that the peptide could be selective to cancer cells; however, further experiments are required to demonstrate it. It has been established that cancer cells are more sensitive to oxidative stress than normal cells and have an important role in cell death [27]. Accordingly, PaDef induced the loss of the inner mitochondrial transmembrane potential in MCF-7 cells, which has been associated with an increase in the generation of reactive oxygen species (ROS) and may explain the PaDef selectivity.

The cytotoxic mechanisms described for AMPs include membrane damage because AMPs may form pores that lead to cell lysis [28]. Few studies have been conducted to describe the cytotoxic mechanism of plant defensins. The mechanism best characterized is that of the NaD1 defensin from tobacco, which causes plasma membrane blebbing and permeabilization of human cells [29]. Interestingly, PaDef did not modify the calcium release and the membrane potential of MCF-7 cells (Fig. 3). From these results, we concluded that MCF-7 cytoplasmic membrane stability was not affected by treatment with PaDef, suggesting a different cytotoxic mechanism.

Apoptosis induction has been reported to be a relevant cytotoxic mechanism for diverse AMPs [8,9]. Notably, PaDef defensin induced apoptosis in MCF-7 cells after 6 h, but necrosis was not detected. Apoptosis induction has been reported only for the radish defensin RsAFP2 (which activates caspases or caspase-like proteases) and HsAFP1 from coral bells (which induces ROS accumulation) in *Candida albicans* [30,31]. To our knowledge, this is the first report of a plant defensin inducing apoptosis in cancer cells.

Apoptosis is a process of death cell that occurs by extrinsic and intrinsic pathways. The analysis of gene expression suggested that PaDef induces intrinsic apoptosis in MCF-7 cells because only genes from this pathway had modified expression patterns. PaDef also induced mitochondrial depolarization, which is consistent with gene expression analysis. Furthermore, the MAPK p38 signaling pathway has been related to mitochondrial apoptosis [23]. PaDef defensin significantly induced the phosphorylation of p38. Taken together, these results support the fact that PaDef defensin induces cell death in MCF-7 cells by the intrinsic apoptosis pathway.

5. Conclusions

This is the first report of an avocado defensin that is cytotoxic to cancer cells. PaDef defensin from avocado (*Persea americana)* fruit is cytotoxic to MCF-7 cells via the induction of mitochondrial apoptosis, which suggests that this defensin could be a potential molecule in the treatment of cancer.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Complementary results

To confirm whether PaDef actives the intrinsic apoptosis pathway, we measured the activation of caspase 8 (extrinsic pathway) and caspase 9 (intrinsic pathway) by a luminescent assay. PaDef IC_{50} significantly increased the activation of caspase 9 at 24 h in relation to control; however, caspase 8 was not altered (Fig 1S). These results are consistent with the gene expression analysis, and confirm that PaDef induces intrinsic apoptosis pathway in MCF-7 cells.

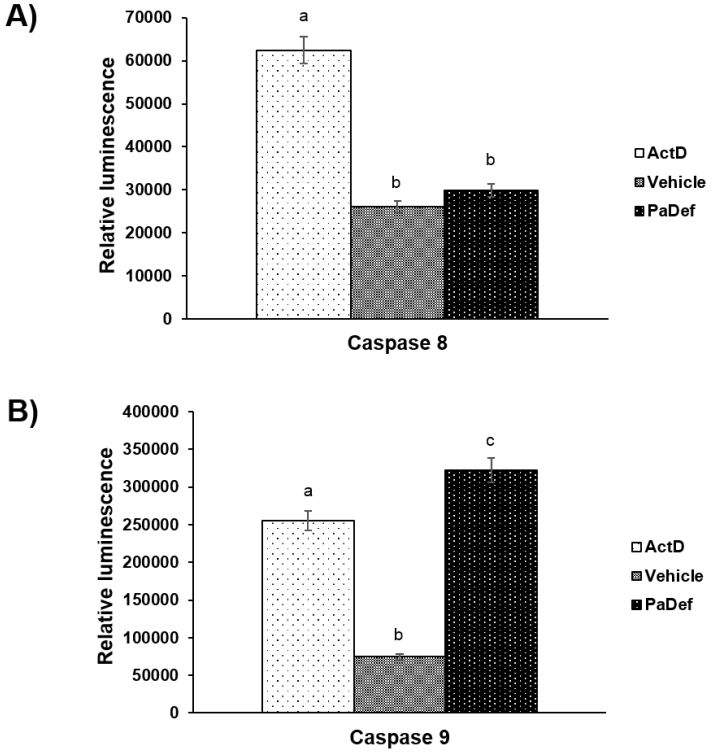

Figure 1S. PaDef induces the activation of caspase 9 in MCF-7 cells. A) Shows the measurement of caspase 8. B) Shows the measurement of caspase 9. Cells were treated for 24 h with PaDef IC_{50} and then were treated with Caspase-Glo reactive and luminescence was measured after 45 min. When caspase is activated, substrate for luciferase (aminoluciferin) is released and luminescence can be measured. Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$). Each bar represents the mean of three replicates

9.2. CAPÍTULO II:

The plant defensin γ-thionin (*Capsicum chinense*) is cytotoxic against breast cancer cell line MCF-7 by apoptosis induction

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Abstract

Plant antimicrobial peptides have cytotoxic activity against a variety of cancer cells. However, its mechanism of action has been poorly studied. In this study we showed that defensin γ-thionin (*Capsicum chinense*) was cytotoxic against breast cancer cell line (MCF-7) with an IC_{50}=117.29 µg/ml. According to calcium efflux and cytoplasmic membrane electric potential, the effect of γ-thionin is not related to the membrane damage. However, γ-thionin increased the apoptotic rate in a manner time-dependent manner; also, the mitochondrial electric potential (ΔΨm) was modified, which is associated with intrinsic apoptosis. In addition, γ-thionin induced the expression of intrinsic pathway genes (*cytochrome c*, *apaf-1* and *caspase 9*) reaching the maximum level at 6 h, which subsequently decreased and was inhibited at 24 h; however, the activation of caspase 9 remained. Interestingly, at 24 h of treatment the expression of extrinsic pathway genes was induced (*fasL, fas and caspase-8*) as well as the activation of caspase 8. To our knowledge, this is the first report of a plant defensin that induces both apoptosis pathways in MCF-7 cells, which suggest that γ-thionin could be a alternative for future therapeutic applications.

Keywords: Breast cancer, apoptosis, antimicrobial peptides, *Capsicum chinense*

1. Introduction

Nowadays natural products still represent an important tool for identifying new drugs for the treatment of various diseases such as cancer [1], this condition is a public health problem that requires the application of natural products as an alternative treatment due to deficiencies in conventional therapy, e.g. low therapeutic index and severe side effects [2]. In this respect, diverse molecules with anticancer potential produced in many plant species have been studied, for example the plant defensins [3]; these are members of a group known as antimicrobial peptides (AMPs), which are biologically active peptides, mainly cationic and amphipathic produced by a wide variety of organisms as essential components of their innate immune response [4]. However, these peptides exhibit a wide range of properties, including modulation of the innate immune response and anticancer activity [5].

Plant defensins are small cysteine-rich AMPs produced as a mechanism of natural defense, which are abundantly expressed in the majority of species [6]. Several studies have demonstrated that plant defensins inhibit the proliferation of cancer cells, including breast, colon and cervical cancer cells by cell lysis and apoptosis induction, without exerting side effects on normal cells [7-10]. In a previous study, we expressed the cDNA of *γ-thionin* defensin from *Capsicum chinense* as a fusion protein in the endothelial cell line BVE-E6E7. We showed that the conditioned media (CM) from these cells have antimicrobial activity against *Candida albicans* [11]. Also, CM inhibited the viability of HeLa cells (~80%) but did not affect

untransfected endothelial cells. However, the mechanism of action of γ-thionin has not been explored. In this work we showed that γ-thionin was cytotoxic to MCF-7 cells but does not affect bovine mammary epithelial cells (bMEC) or peripheral blood mononuclear cells (PBMC). Also, we demonstrated that the cytotoxic mechanism includes intrinsic apoptosis pathway in the early stages (6 and 12 h) and extrinsic apoptosis pathway at 24 h.

2. Materials and methods

2.1. Peptide

γ-thionin used in this work corresponds to the mature region (QNAICETTSKHFNGLCIASSNCASVCIGEDKFTDGHCSGVQRKCLCLKNC, 50 aa) (Genbank AF128239.1) [12], which was chemically synthesized and obtained from BIOMATIK. Prior to assays, γ-thionin (5 mg/ml) disulfide bond formation was accomplished by air oxidation in dimethyl sulfoxide (20%) for 5 days at room temperature. γ-thionin bond formation was confirmed by TOF-mass spectrometry [18]. For all of the experiments, the final concentration of vehicle was 0.4% DMSO, which was also used as a control. γ-thionin was used over a concentration range of concentrations of 50 to 300 μg/ml, in agreement with reports describing these concentrations as cytotoxic for mammalian defensins [13]. Additionally, γ-thionin biological activity was confirmed by evaluating its antifungal effect against *Candida albicans* ATCC 10231 [14].

2.2. MCF-7 cell culture

The human breast tumor cell line MCF-7 was obtained from American Type Culture Collection. Cells were routinely cultured in DMEM medium/nutrient mixture F-12 Ham (Sigma) supplemented with 10% (v/v) fetal bovine serum (Corning) and 100 U/ml penicillin and streptomycin (Gibco) and grown in an atmosphere of 5% CO_2 at 37°C.

2.3. Trypan blue dye exclusion viability assay

MCF-7 cells ($2x10^4$ cells/well) were cultured in 96-well plates overnight to allow cell attachment. Fresh medium without serum was added, and cells were incubated for 12 h. Then, the medium was replaced with 100 µl of fresh medium containing γ-thionin (50-300 µg/ml), and cells were incubated for 48 h. Then, cells were harvested by trypsinization and 10 µl of the cell suspension was mixed with 10 µl of trypan blue (0.4%). Dead and viable cells were counted using a hematocytometer in an inverted microscope (Primo Vert, Zeiss). Cell viability was registered as the percentage of viable cells with respect to vehicle (DMSO 0.4%). The half maximal inhibitory concentration (IC_{50}) was calculated using Excel (Microsoft).

2.4. Calcium efflux testing

Calcium efflux was measured by flow cytometry in a BD Accuri™ C6 flow cytometer (BD Biosciences) using a Calcium Assay Kit (BD Biosciences) according to the manufacturer´s instructions. Briefly, MCF-7 cells ($1X10^5$/ml) were incubated with the indicator dye for 1 h. The baseline fluorescence was established (1 min), and then, treatments were added (γ-thionin IC_{50} or vehicle). The measurements were carried out for 3 min without interrupting the data collection. The changes of the fluorescence intensity in cell populations in response to the peptide were

monitored using flow cytometry. Phorbol myristate acetate (3 μM; PMA, Sigma) was used as a positive control.

2.5 Measurement of the transmembrane potential

The cell transmembrane potential depolarization was measured using the membrane potential sensitive dye 3,3′-dipropylthiadicarbocyanine iodide, $DiSC_3(5)$ (Sigma). For this, MCF-7 cells ($2X10^4$ cells/well) were seeded in 96-well black-wall plates and cultured for 16 h. Then, cells were washed two times with Hanks'-HEPES buffer and were incubated with 100 μl of Hanks' HEPES buffer containing $DiSC_3(5)$ (0.2 mM) for 30 min in a CO_2 incubator. γ-thionin IC_{50} was added to each well, and the subsequent changes in the fluorescence intensity were monitored for 2 h in a Varioskan spectrophotometer (Thermo Scientific). DMSO 10% (Sigma) was used as a positive control.

2.6 Apoptosis rate determined by flow cytometry

MCF-7 cells ($2x10^4$ cells/well) were cultured in 96-well plates and incubated with γ-thionin IC_{50} for 6, 12, and 24 h. After treatment, the cells were collected by trypsinization and washed with PBS and subsequently stained with Annexin V and 7AAD according to the manufacturer's instructions (Annexin V, Alexa Fluor® 488 conjugate, Invitrogen). The cell-surface phosphatidylserine (PS) exposure and plasma membrane integrity were analyzed using a BD Accuri™ C6 flow cytometer (BD Biosciences) using the FlowJo software (TreeStar, Inc.). Actinomycin D (Sigma, 160 μg/ml) was used as a positive control for apoptosis. A total of 10,000 events were analyzed.

2.7 Assessment of mitochondrial membrane potential (ΔΨm)

MCF-7 cells ($2X10^4$ cells/well) were cultured in 96-well plates and treated with γ-thionin IC_{50} or vehicle by 6, 12 and 24 h; subsequently cells were stained with JC-1 dye (BD Biosciences) for 15 min at 37°C in the dark, according to the manufacturer's instructions. The cells were subsequently washed twice with assay buffer, and their fluorescence was measured in a BD Accuri™ C6 flow cytometer (BD Biosciences) using the FlowJo software (TreeStar, Inc.). JC-1 allows for differentiating healthy cells (red fluorescence) from those with mitochondrial damage (green fluorescence).

2.8 RNA isolation and gene expression analysis

MCF-7 cells ($1x10^5$ cells/well) were cultured in 24-well plates and incubated with γ-thionin IC_{50} for 6, 12, and 24 h. Total RNA was extracted from all of the conditions with Trizol reagent (Invitrogen) according to the manufacturer's instructions and then used to synthesize cDNA. Genomic DNA contamination was removed from RNA samples with DNase I treatment (Invitrogen). cDNA synthesis was carried out as previously described [15].

The relative quantification of gene expression (qPCR) was performed using the comparative Ct method (ΔΔCt) in a StepOne Plus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. The reactions were carried out with a SYBR Green PCR Master Mix (Applied Biosystems). The genes evaluated are shown in Table 1. β-actin was used as an internal control.

2.9 Analysis of caspase 8 and 9 activation

Activation of caspases 8 and 9 was measured with caspase-Glo 8 and 9 kit (Promega) according to manufacturer´s instructions. Briefly, cells were seeded in

white 96-well plates at a density of $2x10^5$/well and incubated for 24 h. Further, cells were incubated with serum-free medium for 12 h and γ-thionin IC_{50} was added and the luminescence was measured in a Varioskan spectrophotometer (Thermo Scientific). Actinomycin D was used as positive control.

2.9 Statistical analysis

The data were obtained from three independent experiments, each of which was performed in triplicate and compared by analysis of variance (ANOVA). The results are reported as the means ± the standard errors (SE) and the significance level was set at $P < 0.05$, except for RT-qPCR analysis where fold-change values greater than 2 or less than 0.5 were considered as significantly differentially expressed mRNAs according to Morey et al. [16].

3. Results

3.1 Cytotoxic activity of γ-thionin against MCF-7 cells

γ-thionin (50 to 300 µg/ml) toxicity on MCF-7 cells was evaluated by trypan blue exclusion assays at 24 and 48 h. γ-thionin did not show effect at 24 h of treatment (data not shown). However, this AMP showed an effect concentration-dependent from 50 µg/ml at 48 h (Fig. 1A), with a half maximal inhibitory concentration of IC_{50}= 117.29 µg/ml (Fig. 1B). This concentration was used in the rest of the experiments by 6, 12 and 24 h. MCF-7 cells shows loss of refractivity and cell rounding similarly to cells treated with actinomycin D (Fig. 1C). Interestingly, γ-thionin did not show cytotoxic effect on primary culture of bovine mammary epithelial cells (bMEC) neither on human mononuclear cells from peripheral blood (PBMC).

3.2 Effect of γ-thionin on membrane integrity of MCF-7 cells

To determine whether γ-thionin cytotoxicity on MCF-7 cells is associated with membrane damage, we evaluated calcium efflux and membrane electrical potential. γ-thionin IC_{50} did not affect the efflux of intracellular calcium as shows in figure 2A. On the other hand, γ-thionin IC_{50} did not affect the membrane electrical potential of MCF-7 cells compared to the positive control (valinomycin 0.2 mM). These results suggest that the mechanism of γ-thionin cytotoxicity on MCF-7 cells is not related to membrane damage.

3.3 Apoptosis induction by γ-thionin in MCF-7 cells

Apoptosis is a cytotoxic mechanism that has been reported for many AMPs. We analyzed the phosphatidylserine externalization of MCF-7 cells at 6, 12 and 24 h using Annexin V by flow cytometry. The results showed that γ-thionin induces apoptosis in MCF-7 cells in a time dependent manner, reaching >75% of apoptotic cells at 24 h (Fig. 3). In addition, γ-thionin does not produce a significant increase of necrotic cells.

3.4 Analysis of mitochondrial membrane potential modification (ΔΨm) produced by γ-thionin

We evaluated the loss of ΔΨm produced by γ-thionin in order to elucidate if defensin can activate intrinsic apoptosis pathway. To this, we used the ΔΨm-sensitive molecular probe dye JC-1 and measurements were made at 6, 12 and 24 h of treatment. The results showed that γ-thionin induces the loss of ΔΨm since 6 h of treatment and this was maintained until 24 h (Fig. 4). This result supports the fact that γ-thionin activates intrinsic apoptosis pathway in MCF-7 cells.

3.5 Evaluation of intrinsic and extrinsic apoptosis pathways in MCF-7 cells

We evaluate the effects of γ-thionin on the expression of genes characteristic of extrinsic (*fasL, fas* and *caspase-8*) and intrinsic apoptosis (*cytochrome c, apaf-1* and *caspase 9*), as well as the effector *caspase7*. qPCR analysis shows that γ-thionin significantly induced the expression of intrinsic pathway genes beginning at 6 h of treatment, as well as *caspase 7* (Fig. 5). This expression decreases as a function of time, reaching basal expression at 24 h. Interestingly, the expression of the extrinsic pathway genes was not modified at 6 and 12 h; however, at 24 h of treatment the expression of these genes was increased significantly (Fig. 5). In agreement, the activation of caspase 8 and 9 was demonstrated at 24 h of treatment (Fig. 6).

4. Discussion

In search of drugs to treat important diseases such as cancer, in this study we analyze the cytotoxic activity of plant defensin γ-thionin (*Capsicum chinense*) against breast cancer cell line MCF-7. γ-thionin inhibited the viability of MCF-7 cells through apoptosis by activation of both intrinsic and extrinsic pathways without affecting cytoplasmic membrane or necrosis activation. γ-thionin was cytotoxic to MCF-7 cells in a concentration- dependent manner, with an IC_{50} of 117.29 μg/ml. This is in agreement with the reported IC_{50} values of the plant defensins NaD1, sesquin and limenin [9-10, 17-18]. Additionally, γ-thionin defensin was not cytotoxic to bMEC and PBMC, suggesting that the peptide could be selective to cancer cells. It has been established that AMPs selectivity depends on the combination of

different factors. Cancer cells possess different elements like phosphatidylserine, sialic acid residues, and sulfated glycans that may contribute to increase the interaction of cells with the peptide. Also, residue composition, length sequence, net charge and hydrophobicity of each peptide are important factors. Altogether, all these factors may contribute to the selectivity anticancer action [19-20]. However, further experiments are required to demonstrate the contribution of each one in the γ-thionin cytotoxicity on MCF-7 cells.

The cytotoxic mechanisms described for AMPs include membrane damage because AMPs may form pores that lead to cell lysis [21]. Few studies have been conducted to describe the cytotoxic mechanism of plant defensins. One of the mechanisms best characterized is that of the NaD1 defensin from tobacco, which causes plasma membrane blebbing and permeabilization of human cells [10]. Interestingly, γ-thionin did not modify the calcium release and the membrane potential of MCF-7 cells. From these results, we concluded that MCF-7 cytoplasmic membrane stability was not affected by the treatment with γ-thionin, suggesting a different cytotoxic mechanism.

Apoptosis induction has been reported to be a relevant cytotoxic mechanism for diverse AMPs [22-23]. In agreement γ-thionin induced apoptosis in MCF-7 cells since 6 h of treatment, but necrosis was not detected. Apoptosis induction has been reported for the radish defensin RsAFP2 (which activates caspases or caspase-like proteases) and HsAFP1 (from coral) bells in *Candida albicans* [24-25]. However, the only report about plant defensins that induces apoptosis in cancer cells is PaDef from avocado fruit, which induces apoptosis in MCF-7 cells at the same concentration range [9].

To elucidate the apoptosis pathway activated by γ-thionin, we evaluated the loss of the inner mitochondrial transmembrane potential in MCF-7 cells and it shows the loss of ΔΨm since 6 h of treatment, which was maintained until 24 h. This effect has already been shown by other AMPs like BMAP-28 in U937 cells, which leads to the release of cytochrome c and eventually to the induction of mitochondrial apoptosis [26]. In this sense, the analysis of gene expression shows that γ-thionin induces apoptosis intrinsic pathway at early times; interestingly, at 24 h of treatment the expression of this genes fall although apoptosis remains. On the other hand, gene expression of extrinsic pathway was induced at this time; besides we demonstrated the activation of caspases 8 and 9, these proteases corresponds to extrinsic and intrinsic pathway respectively. Extrinsic pathway is related to activation of death receptors [27]; however, so far no conclusive evidence showing the direct interaction of AMPs with such receptors [21]; however, has been reported that γ-thionin, like others defensins (HNP-1, 2 and 3) induces the expression of TNF-α [28-29]. This factor may eventually activate the route of receptor cell death receptors in MCF-7 line; however more experiments are needed to demonstrate it.

5. Conclusion

The defensin γ-thionin defensin has cytotoxic effects against MCF-7 cells by induction of intrinsic and extrinsic apoptosis. This is the first report of a plant defensin which activates the two pathways signaling apoptosis in cancer cells without affecting bMEC and PBMCs, which suggest that γ-thionin may be a promising alternative in the search for new molecules with anticancer potential.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. γ-thionin defensin is cytotoxic to breast cancer MCF-7 cells. (A) Effect of γ-thionin on the viability of MCF-7 cells. Cells were treated with defensin (50, 100, 200 and 300 µg/ml), and viability was evaluated by trypan blue assays at 48 h. Cell viability is shown as the percentage of viable cells with respect to cells treated with vehicle (DMSO 0.4%). Data represent the media of three independent experiments performed in triplicate. **(B)** Linear regression analysis of the concentration-dependent response to calculate the half maximal inhibitory concentration (IC_{50}) of γ-thionin on MCF-7 cells; IC_{50}= 117.29 µg/ml; R^2= 0.9321. **(C)** Effect of γ-thionin on the viability of bovine mammary epithelial cells and **(D)** human peripheral blood mononuclear cells. Viability was evaluated by trypan blue assays at 48 h. Cell viability is shown as the percentage of viable cells with respect to cells treated with vehicle (DMSO 0.4%). **(E)** MCF-7 cell morphology after different treatments. Photographs are representative of at least two independent experiments and were taken with bright field microscopy. Scale bars: 10 µm. Act D (Actinomycin D), γ-thionin IC_{50}= 117.29 µg/ml. Data represent the media of three independent experiments performed in triplicate. Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$).

Figure 2. γ-thionin does not affect the membrane integrity of MCF-7 cells. (A) The efflux of cytosolic calcium was analyzed by flow cytometry. Measurements were performed for 4 min. The upper panel shows representative plots of the different conditions. The lower panel indicates the relative fluorescence intensities

for extracellular calcium release. PMA (3 µM) was used as a positive control. **(B)** Changes in the membrane potential of MCF-7 cells were measured using a membrane potential-sensitive dye. Cells were incubated with 200 µM $DiSC_3(5)$ for 30 min at 37°C and were then treated with PaDef IC_{50}. DMSO 10% was used as a positive control. Arrows indicate the time at which the treatments were added.

Figure 3. γ-thionin induces apoptosis in MCF-7 cells. Cells were treated with γ-thionin IC_{50} for 6, 12, and 24 h. **(A)** Apoptosis was analyzed by flow cytometry using Annexin V/7AAD staining. Viable cells did not bind to annexin V or 7AAD (lower left quadrant). The lower right quadrant of each histogram represents early apoptosis, the upper right quadrant represents late apoptosis, and the upper left quadrant contains necrotic cells. **(B)** The graphics show the fluorescence of each time of treatment (relative units). Different letters denote significant differences (same letter denotes no difference) for all of the values compared with each other (one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$).

Figure 4. γ-thionin induces loss of mitochondrial membrane potential (ΔΨm) in MCF-7 cells. Cells were treated with ActD, vehicle (DMSO 0.4%) and γ-thionin IC_{50} for **(A)** 6 h, **(B)** 12 h and **(C)** 24 h, stained with JC-1 dye, and analyzed by flow cytometry. FL-1 corresponds to green fluorescence and FL-2 to red fluorescence.

Figure 5. γ-thionin regulates the expression of genes related to apoptosis in MCF-7 cells. Cells were treated with γ-thionin IC_{50} for 6, 12, and 24 h. mRNA expression was analyzed by RT-qPCR. mRNA expression of cells treated with actinomycin D and vehicle was analyzed at 24 h. Each bar shows the mean of

triplicates ± SE of three independent experiments. Fold-change values greater than 2 or less than 0.5 were considered significant for differentially expressed mRNAs.

Figure 6. γ-thionin induces activation of caspase 8 and 9 in MCF-7 cells. Cells were treated for 24 h with γ-thionin IC_{50}, and then were treated with Caspase-Glo reactive and luminescence was measured after 45 min of incubation. When caspase is activated the substrate for luciferase (aminoluciferin) is released resulting in the luciferase reaction and the production of light. Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$).

Table 1. Sequence of primers used in this study.

Gene	Sequence	Tm (°C)	Product size (bp)	Reference
Fas	5´GTGAGGGAAGCGGTTTACGAGTGA 3´ 5´ TGGGCATTAACACTTTTGGACGAT 3´	66	256	[30]
Fas-L	5´ TGTGATCAATGAAACTGGGCTGTA 3´ 5´ ATCATCTTCCCCTCCATCATCACC 3´	57	233	[30]
Caspase-8	5´AGATCTGGCCTCCCTCAAGTTCCT 3´ 5´AAATTTGAGCCCTGCCTGGTGTCT 3´	66	244	[30]
Caspase-7	5´ AACCCAAACTCTTCTTCATTCAGG 3´ 5´ TAATAGCCTGGAACCGTGGAATAG 3´	57	145	[30]
Cyt c	5´TCAGCACCATGGCGGAAGACA 3´ 5´TCCTTTAGCGGTCATTGCCTTCTG 3´	66	151	[30]
Apaf-1	5´ AAATGGACACCTTCTTGGACGACA 3´ 5´ CAGAAAAGCAGGCATGGTAAACAG 3´	58	223	[30]
Caspase-9	5´AGGACATGCTGGCTTCGTTTCTG 3´ 5´CCAAATCCTCCAGAACCAATGTCC 3´	66	257	[30]
β-actin	5´ AAAACCTAACTTGCGCAGAAAACA 3´ 5´ TGTCACCTTCACCGTTCCACTTT 3´	57	317	[30]

Figure 1

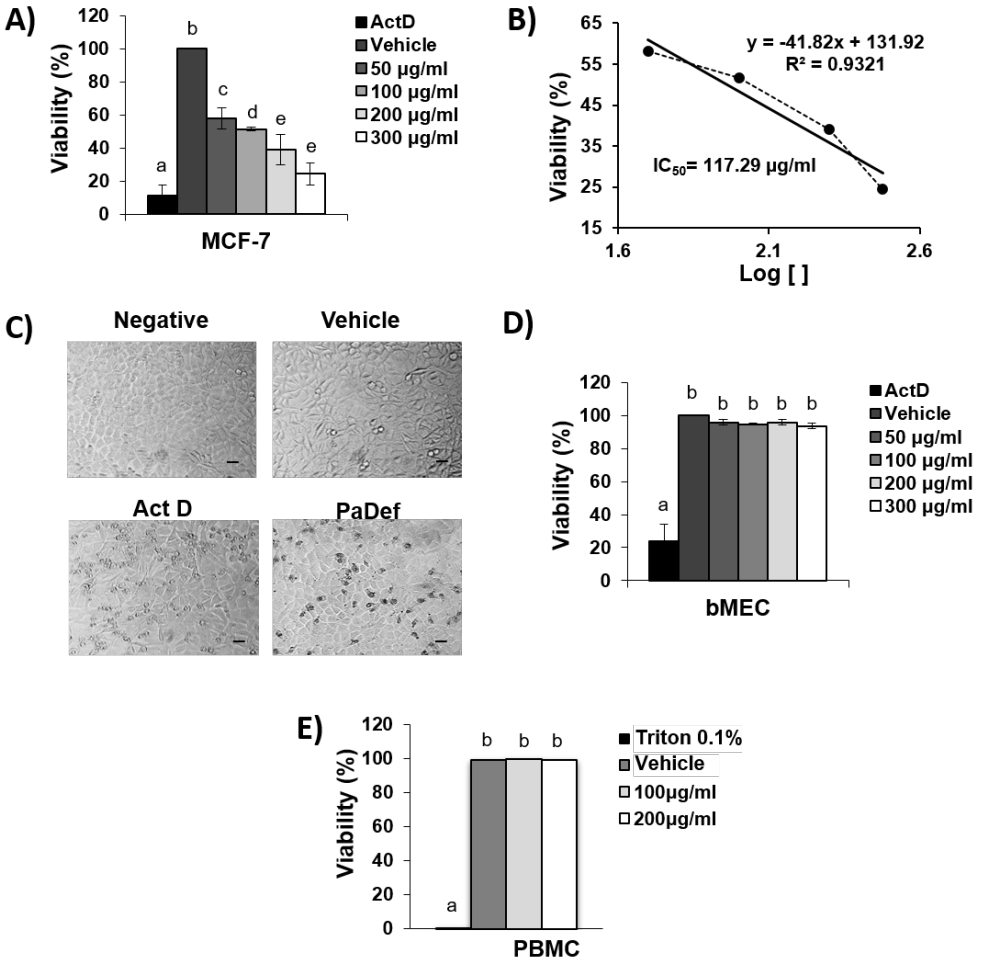
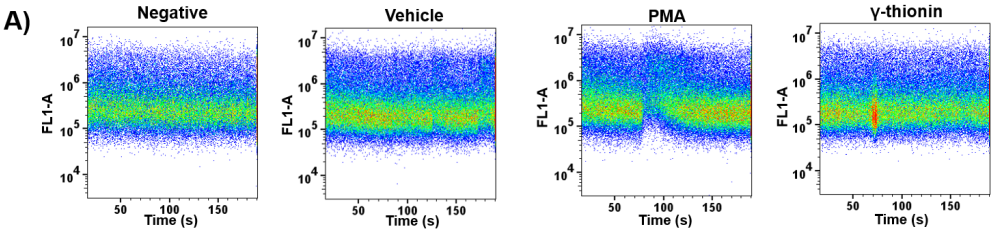

A)
Viability (%)
MCF-7
ActD
Vehicle
50 µg/ml
100 µg/ml
200 µg/ml
300 µg/ml
B)
Viability (%)
y = -41.82x + 131.92
R² = 0.9321
IC50= 117.29 µg/ml
Log [ ]
C)
Negative
Vehicle
Act D
PaDef
D)
Viability (%)
bMEC
E)
Viability (%)
PBMC
Triton 0.1%
Vehicle
100µg/ml
200µg/ml

Figure 2


A)
Negative
Vehicle
PMA
γ-thionin
FL1-A
Time (s)

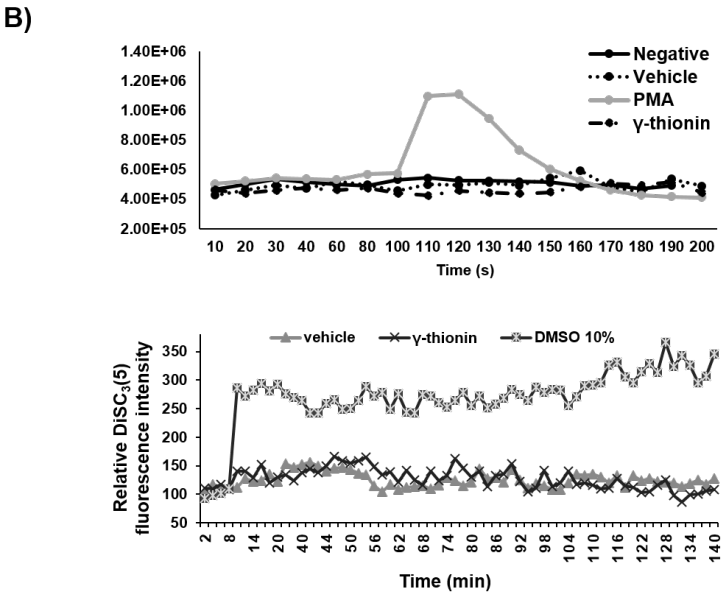

B)
Negative
Vehicle
PMA
γ-thionin
Time (s)
vehicle
γ-thionin
DMSO 10%
Relative DiSC3(5) fluorescence intensity
Time (min)

Figure 3

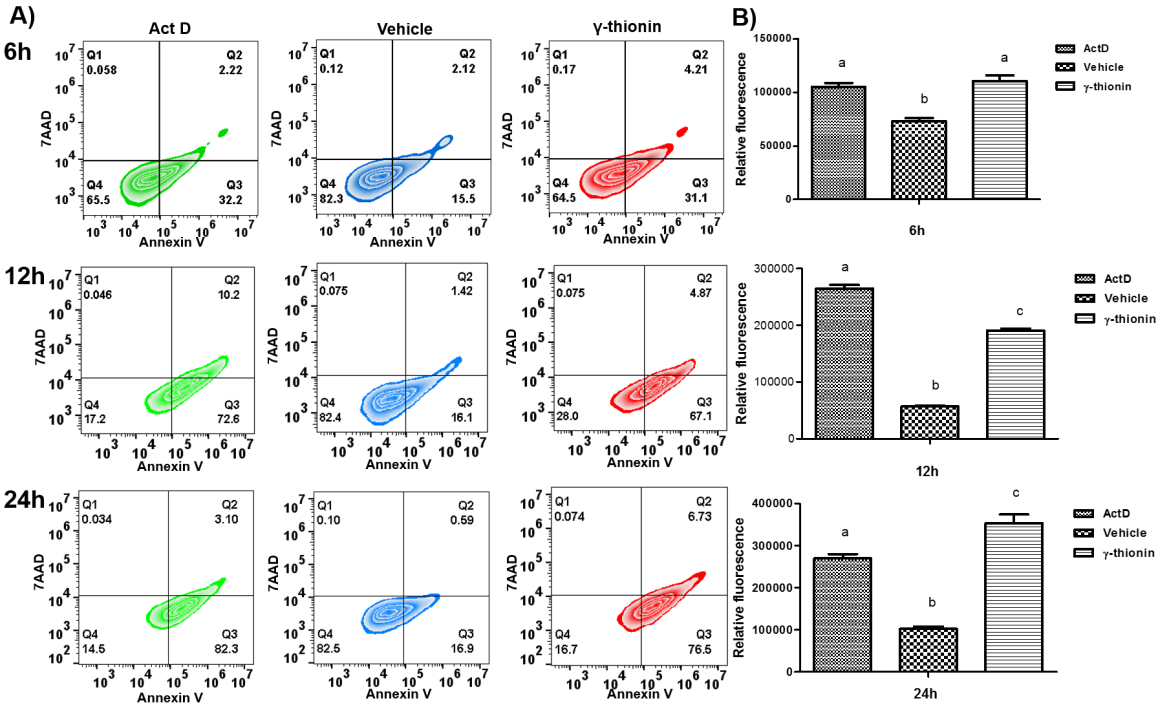

A)
Act D
Vehicle
γ-thionin
6h
12h
24h
7AAD
Annexin V
Q1
Q2
Q3
Q4
0.058
2.22
65.5
32.2
0.12
2.12
82.3
15.5
0.17
4.21
64.5
31.1
0.046
10.2
17.2
72.6
0.075
1.42
82.4
16.1
0.075
4.87
28.0
67.1
0.034
3.10
14.5
82.3
0.10
0.59
82.5
16.9
0.074
6.73
16.7
76.5
B)
Relative fluorescence
ActD
Vehicle
γ-thionin
a
b
c
6h
12h
24h

Figure 4

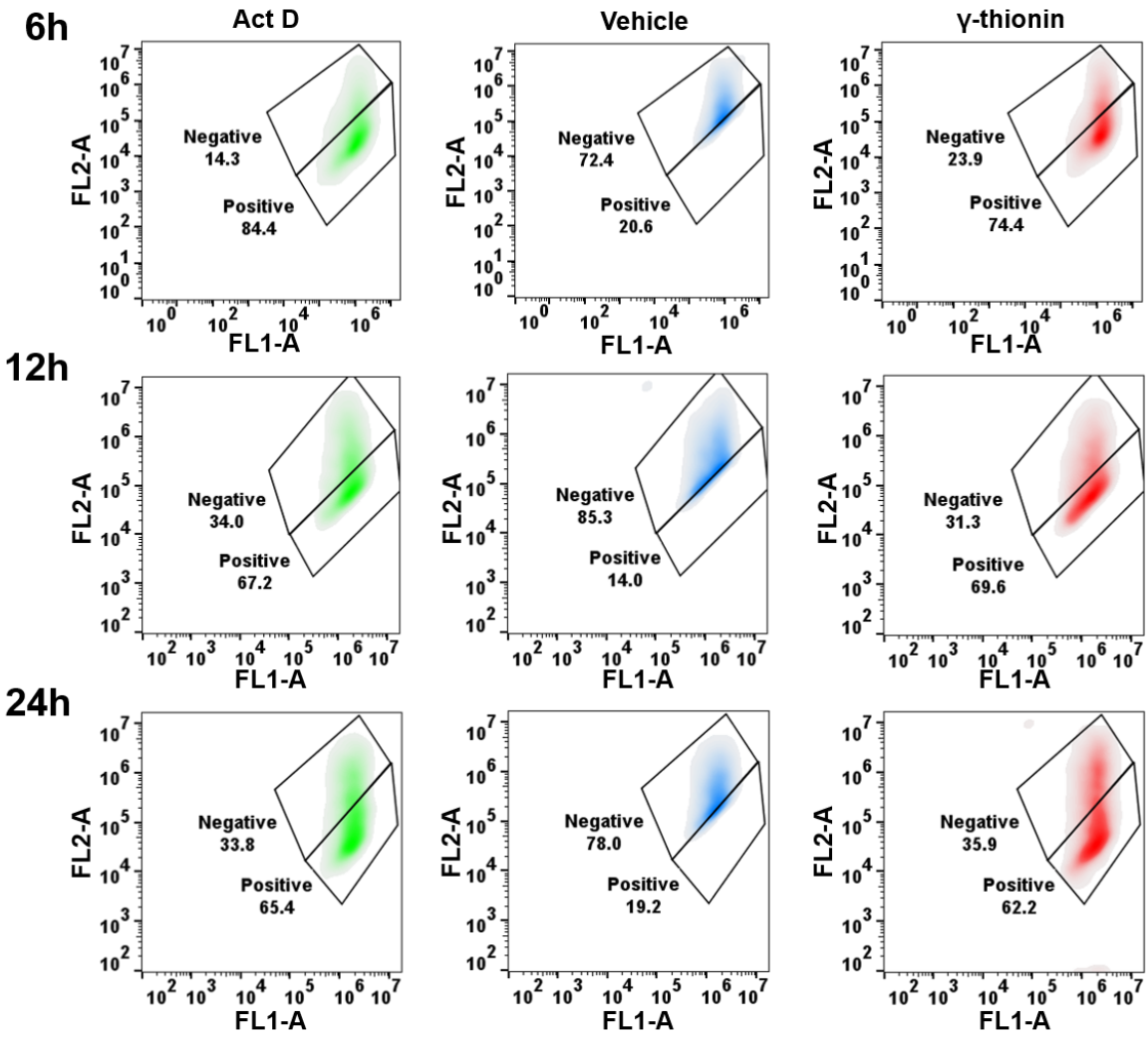
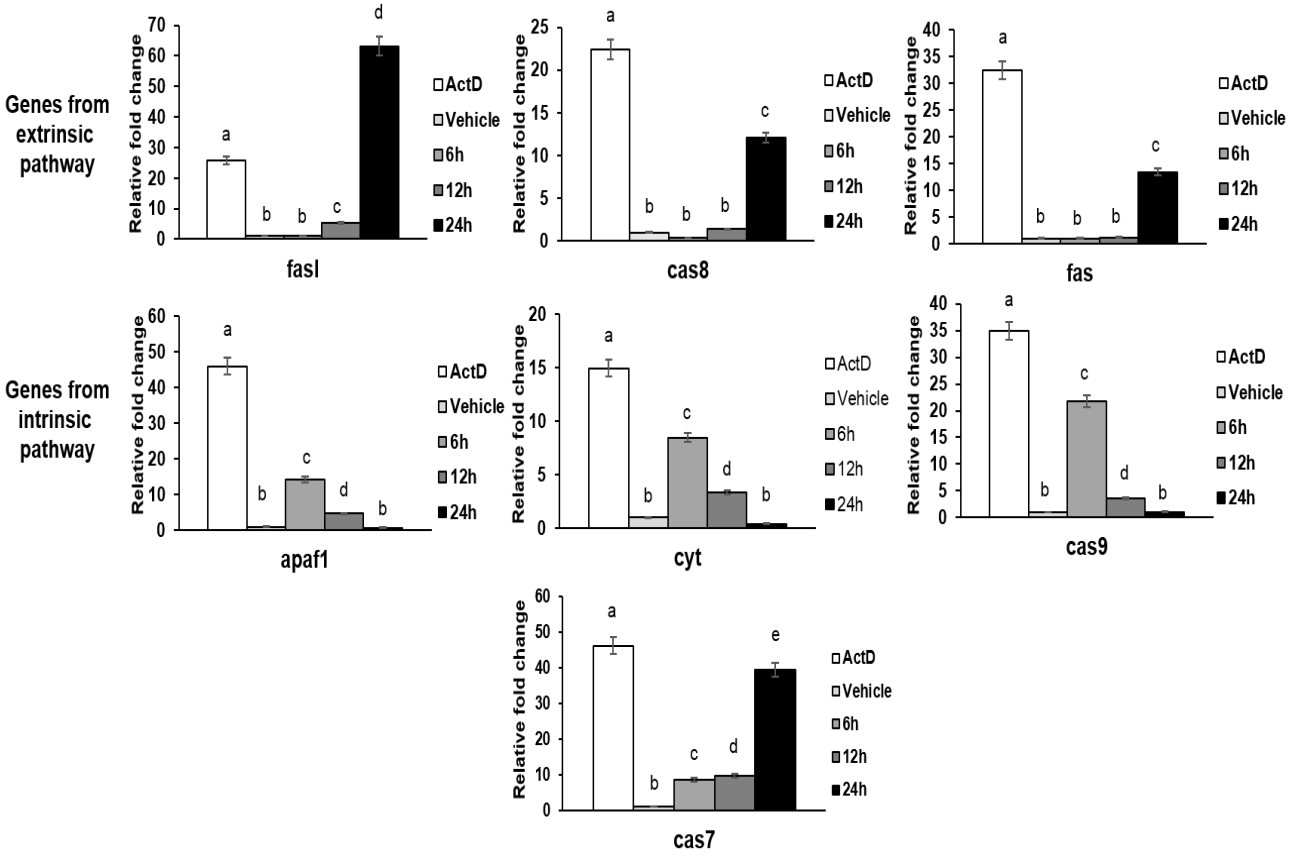

6h
12h
24h
Act D
Vehicle
γ-thionin
Negative
14.3
Positive
84.4
Negative
72.4
Positive
20.6
Negative
23.9
Positive
74.4
Negative
34.0
Positive
67.2
Negative
85.3
Positive
14.0
Negative
31.3
Positive
69.6
Negative
33.8
Positive
65.4
Negative
78.0
Positive
19.2
Negative
35.9
Positive
62.2
FL2-A
FL1-A

Figure 5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Genes from intrinsic pathway
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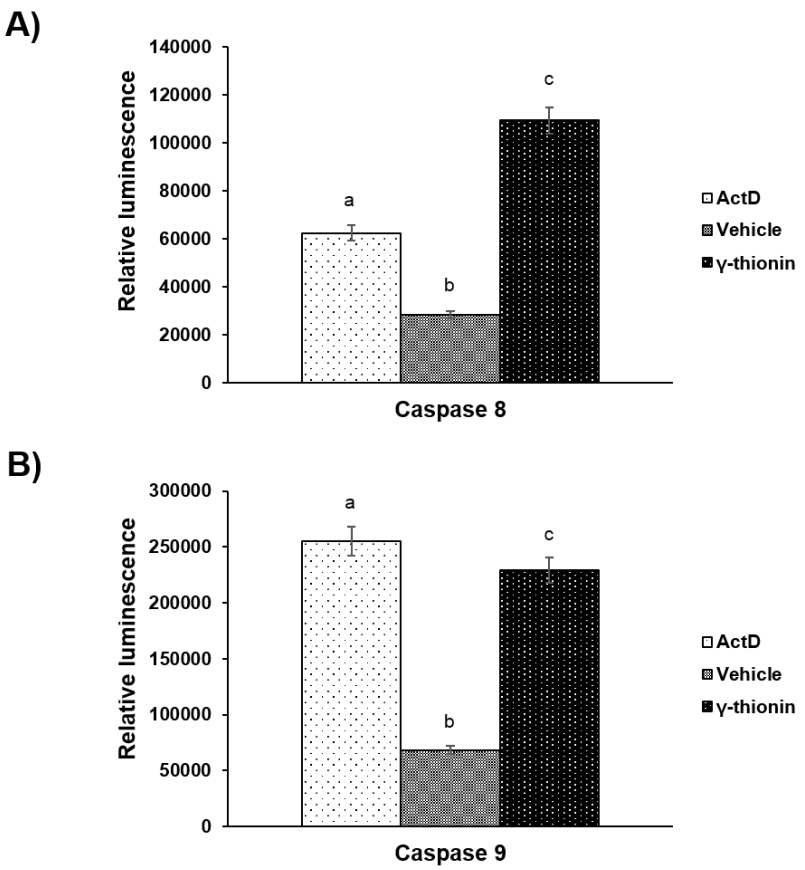
Figure 6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ActD
Vehicle
γ-thionin

9.3. CAPÍTULO III

Plant Antimicrobial Peptides as Potential Anticancer Agents

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Review Article

Plant Antimicrobial Peptides as Potential Anticancer Agents

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Antimicrobial peptides (AMPs) are part of the innate immune defense mechanism of many organisms and are promising candidates to treat infections caused by pathogenic bacteria to animals and humans. AMPs also display anticancer activities because of their ability to inactivate a wide range of cancer cells. Cancer remains a cause of high morbidity and mortality worldwide. Therefore, the development of methods for its control is desirable. Attractive alternatives include plant AMP thionins, defensins, and cyclotides, which have anticancer activities. Here, we provide an overview of plant AMPs anticancer activities, with an emphasis on their mode of action, their selectivity, and their efficacy.

1. Introduction

Cancer is a leading cause of death worldwide. In 2012, cancer caused 8.2 million deaths, and cancers of the lungs, liver, colon, stomach, and breast are main types [1]. A hallmark of cancer is the rapid growth of abnormal cells that extend beyond their usual limits and invade adjoining parts of the body or spread to other organs, a process known as metastasis. Cancer treatment requires careful selection of one or more therapeutic modalities, such as surgery, radiotherapy, or chemotherapy. Despite progress in anticancer therapies, the chemotherapeutic drugs used in cancer treatment have the serious drawback of nonspecific toxicity. Additionally, many neoplasms eventually become resistant to conventional chemotherapy because of selection for multidrug-resistant variants [2]. These limitations have led to the search for new anticancer therapies. An attractive alternative is the use of antimicrobial peptides or AMPs, which represent a novel family of anticancer agents that avoid the shortcomings of conventional chemotherapy [3].

AMPs are amphipathic molecules produced by a wide variety of organisms as part of their first line of defense (eukaryotes) or as a competition strategy for nutrients and space (prokaryotes) [4]. Currently, over 2400 AMPs are reported in The Antimicrobial Peptide Database (URL http://aps.unmc.edu/AP/main.php) [5]. The continuous discovery of new AMP groups in diverse organisms has made these natural antibiotics the basic elements of a new generation of potential biomedical treatments against infectious diseases in humans and animals. Moreover, the broad spectrum of biological activities and the low incidence of resistance to these molecules suggest a potential benefit in cancer treatment, which reinforces the importance of their study [6].

AMPs are usually short peptides (12–100 aa residues), which mainly have a positive charge (+2 to +9), although there are also neutral and negatively charged molecules [7]. AMPs are classified into the following four groups according to their structural characteristics: (1) cysteine-rich and β-sheet AMPs (α- and β-defensins); (2) AMPs possessing α-helices (LL-37 cathelicidin, cecropins, and magainins);

(3) AMPs with extended structure (rich in glycine, proline, tryptophan, arginine, and/or histidine); (4) peptide "loop," which have a single disulfide bond (bactenecin) [8]. In recent years, several reviews on the structures, mechanisms of action, and emergence of resistance to AMPs have been published, to which the reader is referred for additional information [9–11]. Furthermore, recent reviews of the anticancer activities and selectivity and efficacy of AMPs, particularly from animals, have been reported [12–15]. The mechanisms by which AMPs kill cancerous cells are poorly understood although evidences indicate that both membranolytic and nonmembranolytic mechanisms are involved. The membranolytic activity of AMPs depends on their own characteristics as well as of the target membrane [13]. Also, the selectivity of some AMPs against cancer cells has been related with the charge of membrane, which has a net negative charge [12]. Anionic molecules (phosphatidylserine, O-glycosylated mucins, sialylated gangliosides, and heparin sulfate) confer a net negative charge to cancer cells, which contrasts with the normal mammalian cell membrane (typically zwitterionic) [14, 15]. On the other hand, the nonmembranolytic activities of AMPs involve the inhibition of processes such as angiogenesis, which is essential for the formation of tumor-associated vasculature [14].

Despite the promising characteristics of anticancer agents such as AMPs, only a few of them have been tested using *in vivo* models. Cecropin B from *Hyalophora cecropia* increases the survival time of mice bearing ascitic murine colon adenocarcinoma cells [16]. In the same way, when magainin 2 was tested against murine sarcoma tumors, animals increase its life span (45%) [17]. However, there is little information related to the anticancer effects of plant AMPs. Here, we provide an overview of plant AMP anticancer activities with an emphasis on their mode of action, selectivity, and efficacy. We focus on the anticancer activity reported only for the defensins, thionins, and cyclotides because the cytotoxic effects of these families have been widely described.

2. Plant AMPs

Plants are a major source of diverse molecules with pharmacological potential. Over 300 AMP sequences have been described [5]. Plants produce small cysteine-rich AMPs as a mechanism of natural defense, which may be expressed constitutively or induced in response to a pathogen attack. Plant AMPs are abundantly expressed in the majority species, and small cysteine-rich AMPs may represent up to 3% of the repertoire of plant genes [18]. Plant AMPs are produced in all organs and are more abundant in the outer layer, which is consistent with their role as a constitutive host defense against microbial invaders attacking from the outside [19, 20]. Plant AMPs are released immediately after the infection is initiated. AMPs are expressed by a single gene and therefore require less biomass and energy consumption [19, 20]. The majorities of plant AMPs have a molecular weight between 2 and 10 kDa, are basic, and contain 4, 6, 8, or 12 cysteines that form disulfide bonds conferring structural and thermodynamic stability [21]. Plant AMPs are classified based on the identity of their amino acid sequence and the number and position of cysteines forming disulfide bonds. Twelve families have been described, which are listed in Table 1 [21–23].

Table 1: Classification of plant AMPs[1].

Family	Disulfide bonds	Activity
Thionins	3-4	Bacteria, fungi, and cytotoxic
Defensins	3-4	Bacteria, fungi, and cytotoxic
Cyclotides	3	Bacteria, virus, insects, and cytotoxic
Knottin-like	3	Gram (+) bacteria and fungi
Shepherdins	0 (linear)	Bacteria and fungi
MBP-1	2	Bacteria and fungi
Ib-AMPs	2	Gram (+) bacteria and fungi
LTP	3-4	Bacteria and fungi
Snakins	6	Bacteria and fungi
Hevein-like	4	Gram (+) bacteria and fungi
β-Barrelins	6	Fungi
2S albumins	2	Bacteria and fungi

[1]Modified from [21–23].

The primary biological activities of plant AMPs are antifungal, antibacterial, and against oomycetes and herbivorous insects [32, 34, 35]. Additionally, plant AMPs also exhibit enzyme inhibitory activities [36] and have roles in heavy metal tolerance [37], abiotic stress [38], and development [39]. In addition, some plant AMPs show cytotoxic activity against mammalian cells and/or anticancer activity against cancer cells from different origins [25, 28, 31, 40–56]. Of the 12 plant AMP families, 3 contain members with cytotoxic and anticancer properties, the defensins, thionins, and cyclotides. Here, the cytotoxic properties of these peptides are described and the possibility of their use in cancer treatment is discussed.

3. Thionins

Thionins were the first AMP isolated from plants [57]. These AMPs belong to a rapidly growing family of biologically active peptides in the plant kingdom and are small cysteine-rich peptides (~5 kDa) with toxic and antimicrobial properties [58]. Thionins are divided into at least four different types depending on the net charge, the number of amino acids, and the disulfide bonds present in the mature protein [59]. Type 1 thionins are highly basic and consist of 45 amino acids, eight of which are cysteines, forming four disulfide bonds. Type 2 thionins consist of 46 or 47 amino acid peptides, are slightly less basic than type 1 thionins, and also have four disulfide bonds. Type 3 thionins consist of 45 or 46 amino acid peptides with three or four disulfide bonds and are as basic as type 2 thionins. Finally, type 4 thionins consist of 46 amino acid peptides with three disulfide bonds and are neutral [58].

The primary role for thionins is plant protection against pathogens [57, 59]. However, they also participate in seed maturation, dormancy, or germination [58], as well as the packaging of storage proteins into protein bodies, or in their mobilization during germination [60]. In addition, thionins

Table 2: Thionins with anticancer and cytotoxic activity.

Name	Species	Activity against	Cytotoxic activity	Anticancer activity	Reference
Pyrularia	*Pyrularia pubera*	B16, HeLa, rat hepatocytes, and lymphocytes	Yes	Yes	[24]
Viscotoxin B2	*Viscum coloratum*	Rat sarcoma cells	Not tested	Yes	[25]
Viscotoxins 1-PS, A1, A2, A3, and B	*Viscum album*	Human lymphocytes	Yes	Not tested	[26]
Viscotoxin C1	*Coloratum ohwi*	Rat sarcoma cells	Not tested	Yes	[27]
Ligatoxin B	*Phoradendron liga*	U-937-GTB ACHN	Not tested	Yes	[28]
Ligatoxin A	*Phoradendron liga*	Animal cells	Yes	Not tested	[29]
Phoratoxins A and B	*Phoradendron tomentosum*	Mice	Yes	Not tested	[30]
Phoratoxins C, D, E, and F	*Phoradendron tomentosum*	10 cancer cell lines	Not tested	Yes	[31]
Thi2.1	*Arabidopsis thaliana*	HeLa, A549, MCF-7, and bovine mammary epithelial cells	Yes	Yes	[32]
β-Purothionin	*Tricum aestivum*	p388	Not tested	Yes	[33]

may play a role in altering the cell wall upon penetration of the epidermis by fungal hyphae or act as a secondary messenger in signal transduction [61].

3.1. Cytotoxic and Anticancer Activity of Thionins. In addition to the activities described, several plant thionins show cytotoxic and anticancer activities (Table 2). The pyrularia thionin from mistletoe (*Pyrularia pubera*) showed an anticancer activity against cervical cancer cells (HeLa) and mouse melanoma cells (B16) with an IC_{50} of 50 μg/mL (half maximal inhibitory concentration); however, the pyrularia thionin is cytotoxic because it causes hemolysis [24]. The anticancer effect is attributable to a cellular response that involves the stimulation of Ca^{2+} influx coupled to depolarization of the plasma membrane, which leads to the activation of an endogenous phospholipase A_2 and, as consequence, membrane alteration, and finally the cell death.

Another group of thionins with anticancer and cytotoxic activity are the viscotoxins from *Viscum* spp. Viscotoxin B2 showed anticancer activity against rat osteoblast-like sarcoma (IC_{50} 1.6 mg/L) [42]. On the other hand, viscotoxins A1, A2, A3, and 1-PS were cytotoxic to human lymphocytes, due the fact that they induce the production of reactive oxygen species (ROS) and cell membrane permeabilization [26]. Furthermore, a mixture of viscotoxins (50 μg/mL) induced apoptosis in human lymphocytes by activating caspase 3 [43]. Conversely, viscotoxins are far less hemolytic than other thionins. Under the same experimental conditions, pyrularia thionin (20 μg/mL) lysed 50% of human erythrocytes, whereas viscotoxin B (100 μg/mL) lysed only 10% [62]. An alignment of the amino acids sequences of both thionins shows that pyrularia has more hydrophobic amino acids compared to the viscotoxin B (Figure 1). These differences could explain the differential hemolytic activity of both thionins because greater hydrophobicity increases the hemolytic activity of AMPs [63].

Another thionin with anticancer activity is the ligatoxin B (*Phoradendron league*). This AMP (100 μg/mL) inhibited the growth of lymphoma cells (U937GTB) and human adenocarcinoma (ACHN). Ligatoxin B has a DNA binding domain, which may be related to the inhibition of nucleic acid and protein synthesis [28]. Unfortunately, the cytotoxic effects of ligatoxin B have not yet been tested on normal cells.

Several thionins (phoratoxins A–F) have been identified in *Phoradendron tomentosum*, all of which possess toxic activity. Phoratoxins A and B are toxic to rats at doses of 0.5–1 mg/kg, and their mechanism of action is related to changes in the electrical charge and the mechanical activity of the rat papillary muscle [30]. Furthermore, phoratoxins C–F showed differential anticancer activity against different types of solid tumor cells (NCI-H69, ACHN, and breast carcinoma) and hematological tumors (RPMI 8226-S and U-937 GTB). Phoratoxin C was the most toxic with an IC_{50} of 0.16 μM, whereas phoratoxin F had an IC_{50} value of 0.40 μM. Furthermore, phoratoxin C was tested on primary cultures of tumor cells from patients and showed selective activity to breast cancer cells from solid tumor samples. These cells were 18 times more sensitive to phoratoxin C than the hematological tumor cells [31]. These data suggest that these compounds are an alternative for developing a new class of anticancer agents with improved activity against solid tumor malignancies. Despite the marked differences in the activity of phoratoxins, they have a high percentage of identity (~90%) (Figure 1). The small changes in specific amino acids could be the key to the biological activity of these thionins; however, further studies are necessary.

Another thionin with anticancer activity against cancer cell lines is the Thi2.1 thionin from *Arabidopsis thaliana*, which was expressed in a heterologous system [32]. The conditioned media from cells that express Thi2.1 inhibited the viability of MCF-7 cells (94%), A549 (29%), and HeLa cells (38%); however, Thi2.1 also showed cytotoxicity against bovine mammary epithelial cells (89%) and bovine endothelium (93%). The mechanism of action of Thi2.1 has not yet been determined.

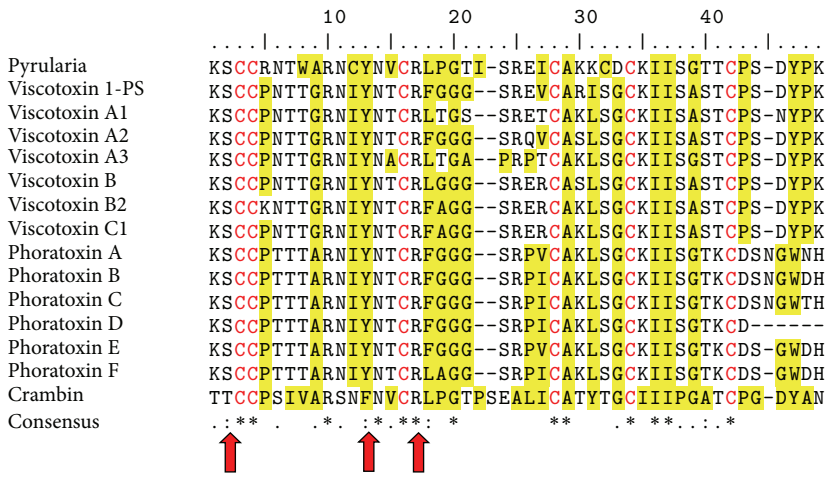

Figure 1: Alignment of amino acid sequences from thionins. The asterisk indicates amino acids conserved in all family members. The cysteine residues present in all sequences and relevant to the classification are indicated in red letters. The red arrows indicate the three residues that are essential for binding to the head regions of the membrane lipids. The hydrophobic residues are shaded in yellow. The thionin sequences included in the alignment were pyrularia (GenBank accession P07504) from *Pyrularia pubera*, viscotoxins 1-PS (GenBank accession P01537), A1 (GenBank accession 3C8P_A), A2 (GenBank accession P32880), A3 (GenBank accession VTVAA3), B (GenBank accession 1JMP_A), B2 (GenBank accession 2V9B_B), and C1 (GenBank accession P83554) from *Viscum album*, phoratoxins A (GenBank accession P01539), B, C, D, E, and F [24] from *Phoradendron tomentosum,* and crambin (GenBank accession P01542) from *Crambe hispanica*.

In summary, the cytotoxic activity of thionins is not selective; however, these peptides can be exploited for the design of new anticancer molecules. Further investigations are necessary to determine the clinical potential of this class of compounds.

4. Plant Defensins

Plant defensins are a class of plant AMPs with structural and functional properties that resemble the defense peptides produced by fungi, invertebrates, and vertebrates, called "defensins." This group of AMPs has great diversity in amino acid sequence, but its members show a clear conservation of some amino acid positions. This variation in the primary sequence is associated with the diversity of biological activities of plant defensins, which include antifungal and antibacterial activities, in addition to proteinase or amylase inhibitory activities [20]. Plant defensins can form three to four disulfide bridges that stabilize their structure [64]. Studies of the three-dimensional structure of plant defensins have shown that these peptides consist of an α-helix and three antiparallel β-sheets, arranged in the configuration $\beta\alpha\beta\beta$ [19]. These AMPs are classified into two types depending on the structure of the precursor protein from which they are derived. Type 1 defensins are the largest group, and the majority of members contain a signal peptide in the prepeptide sequence linked to the mature defensin at the N-terminus. Type 2 defensins include plant defensins for which the precursor has a signal peptide, the active domain of the defensin, and a C-terminal prodomain [20]. Recently, it was demonstrated that the C-terminal prodomain of the NaD1 defensin of *Nicotiana alata* is sufficient for vacuolar targeting and plays an important role in detoxification of the defensin [65].

Plant defensins inhibit the growth of a wide range of fungi and in a lesser extent are toxic to mammalian cells or plants [66]. The proposed mechanism of action of plant defensins is to either destabilize the cell membrane by coating its outer surface or insert themselves into the membrane to form open pores allowing vital biomolecules to leak out of the cell [34, 64].

4.1. Cytotoxic and Anticancer Activity of Plant Defensins. In addition to the antifungal activities, plant defensins exhibit anticancer and cytotoxic effects (Table 3). The first plant defensin reported with anticancer activity was the defensin sesquin from *Vigna sesquipedalis* that inhibited the proliferation of MCF-7 and leukemia M1 (2.5 mg/mL) cells [44]. Furthermore, Wong and Ng [41] reported that the defensin limenin (0.1 mg/mL), a defensin from *Phaseolus limensis*, differentially inhibited the proliferation of leukemia cells, reaching 60% inhibition for M1 and 30% inhibition for L1210 cells; however, its effect against normal cells was not evaluated. Another plant defensin with effects on cancer cell is lunatusin, a defensin purified from the seeds of the Chinese lima bean (*Phaseolus lunatus* L.), which inhibited the proliferation of MCF-7 cells (IC_{50} 5.71 μM). Unfortunately, lunatusin also possesses cell-free translation-inhibitory activity in the rabbit reticulocyte lysate system [45]. This indicates that this defensin may be cytotoxic to normal tissues and other cell types. However, from all the defensins studied, lunatusin is the only plant defensin with this effect.

Further studies identified other plant defensins that inhibit the proliferation of cancer cells, including breast and colon cancer, without cytotoxic effects on normal cells. A defensin from the purple pole bean (*Phaseolus vulgaris* cv. "Extra-long Purple Pole bean") inhibited the proliferation of the cancer cell lines HepG2, MCF-7, HT-29, and Sila (IC_{50}

Table 3: Plant defensins with anticancer and cytotoxic activity.

Name	Species	Activity against	Cytotoxic activity	Anticancer activity	Reference
Sesquin	*Vigna sesquipedalis*	MCF-7 and M1	Not tested	Yes	[44]
Limenin	*Phaseolus limensis*	L1210 and M1	Not tested	Yes	[41]
Lunatusin	*Phaseolus lunatus*	MCF-7 rabbit reticulocyte	Yes	Yes	[45]
Purple pole defensin	*Phaseolus vulgaris* cv. "Extra-long Purple Pole bean"	HepG2, MCF7, HT-29, and SiHa	No	Yes	[46]
Coccinin	*Phaseolus coccineus* cv. "Major"	HL60 and L1210	No	Yes	[47]
Phaseococcin	*Phaseolus coccineus*	L1210 and HL60	No	Yes	[48]
γ-Thionin	*Capsicum chinense*	HeLa	No	Yes	[49]
NaD1	*Nicotiana alata*	U937	Not tested	Yes	[67]
Mitogenic defensin	*Phaseolus vulgaris*	MCF-7, murine splenocytes	Yes	Yes	[68]
Vulgarinin	*Phaseolus vulgaris*	MCF-7, L1210, and M1	Not tested	Yes	[69]
Cloud bean defensin	*Phaseolus vulgaris* cv. cloud bean	L1210 and MBL2	Not tested	Yes	[70]
Nepalese	*Phaseolus angularis*	L1210, MBL2	Not tested	Yes	[71]
Gymnin	*Gymnocladus chinensis* Baill	M1, HepG2, and L1210	Not tested	Yes	[72]

4–8 μM) but did not affect human embryonic liver cells or human erythrocytes under the same conditions [46]. By contrast, coccinin from small scarlet runner beans (*Phaseolus coccineus* cv. "Major"), a peptide of 7 kDa and an N-terminal sequence resembling those of defensins, inhibited the proliferation of HL60 and L1210 cells (IC_{50} 30–40 μM); however, it did not affect the proliferation of mouse splenocytes [47]. Similarly, phaseococcin from *P. coccineus* cv. "Minor" inhibited the proliferation of HL60 and L1210 cells (IC_{50} 30–40 μM). This defensin did not affect the proliferation of mouse splenocytes or protein synthesis in a cell-free rabbit reticulocyte lysate system [48]. The lack of adverse effects of both of these defensins on the proliferation of isolated mouse splenocytes indicates that these molecules are selective. Finally, the conditioned media from bovine endothelial cells that express the cDNA of the defensin γ-thionin from *Capsicum chinense* inhibited 100% of the viability of HeLa cells but did not affect immortalized bovine endothelial cells [49]. Data from our laboratory indicate that this chemically synthetized defensin has a similar effect on both cells (data not published).

In general, the anticancer activity mechanism of plant defensins is poorly understood. However, Poon et al. [67] described the mechanism of the NaD1 defensin on the monocytic lymphoma cells U937. Interestingly, this effect was produced by a novel mechanism of cell lysis in which NaD1 acts via direct binding to the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2).

Thus, the anticancer activities of plant defensins suggest that these AMPs may be an alternative therapy for cancer treatment. The isolation and characterization of these peptides has increased, which allows for the identification of sequences that exhibit desirable characteristics against cancer cells.

5. Cyclotides

Cyclotides are macrocyclic peptides (~30 amino acids) with diverse biological activities, isolated from the Rubiaceae and Violaceae plant families. These molecules constitute a family of plant AMPs, members of which contain six conserved cysteines that stabilize the structure by the formation of disulfide bonds [74]. Cyclotides have a cystine knot with an embedded ring in the structure formed by two disulfide bonds and connecting backbone segments threaded by a third disulfide bond. These combined features of the cyclic cystine knot produce a unique protein fold that is topologically complex and has exceptional chemical and biological stability with pharmaceutical and medicinal significance for drug design [75].

Cyclotides are biosynthesized ribosomally as a precursor protein that encodes one or more cyclotide domains. The arrangement of a typical cyclotide precursor protein is an endoplasmic reticulum signal sequence, a prodomain, a mature cyclotide domain, and a C-terminal region [76]. Although the excision and cyclization processes that yield cyclic mature peptides from these precursors are not fully understood, it has been suggested that asparaginyl endoproteinase enzyme activity plays an important role in this process [77]. This hypothesis is consistent with the presence of a conserved Asn (or Asp) residue at the C-terminus of the cyclotide domain within the precursor proteins (Figure 2(a)). It is also supported by studies of the expression of mutated cyclotides in transgenic plants, in which substitution of the conserved Asn by Ala abolished the production of cyclic peptides *in planta* [78].

The main role attributable to cyclotides is host defense, and there are molecules that are expressed in large quantities in the plant (up to 1 g/kg of leaf material) [75]. Furthermore,

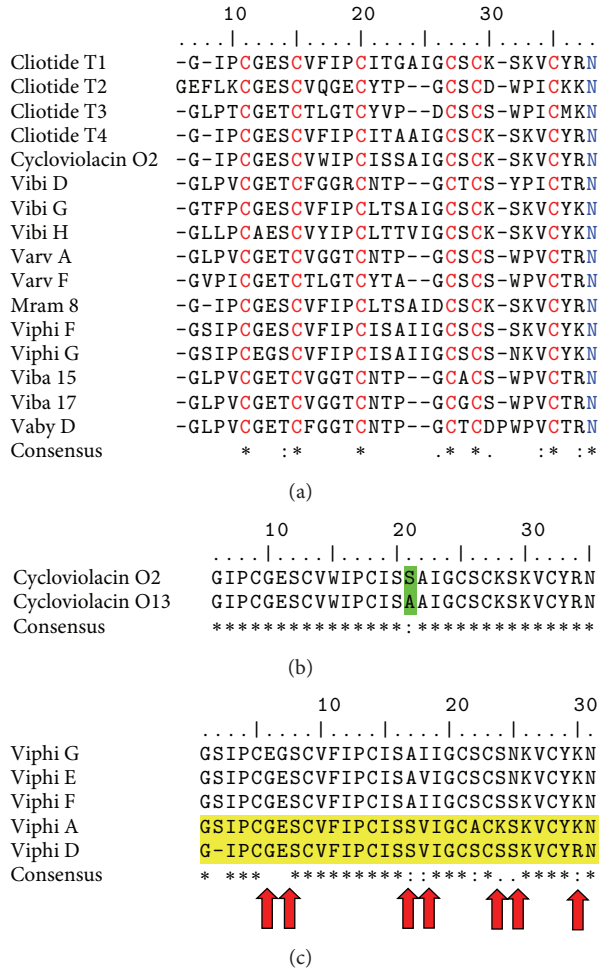

Figure 2: Alignment of amino acid sequences from cytotoxic cyclotides. (a) The cysteine residues present in all sequences and relevant to the classification are indicated in red letters. The asparagine residues present in all sequences and relevant to the cyclization process are indicated in blue letters. (b) Amino acid sequence alignment of cycloviolacins O2 and 13. The replacement of serine by alanine (shaded in green) increases the hemolytic effect by more than 3-fold. (c) Amino acid sequence alignment of Viphi G, Viphi E, Viphi F, Viphi A, and Viphi D cyclotides. Shaded in yellow are the sequences with no-toxic effects; the red arrows indicate the residues with specific variations. The sequences included in the alignment were cliotides T1 (GenBank accession AEK26402), T2 (GenBank accession AEK26403), T3 (GenBank accession AEK26404), and T4 (GenBank accession AEK26405) from *Clitoria ternatea*, cycloviolacins O2 (GenBank accession P58434) and O13 (GenBank accession Q5USNB) from *Viola odorata*, Vibi D (GenBank accession P85242), Vibi G (GenBank accession P85245), and Vibi H (GenBank accession P85246) from *Viola biflora*, Varv A (GenBank accession Q5USN7) and Varv F (GenBank accession 3E4H_A) from *Viola odorata*, Mram 8, Viphi A, Viphi D, Viphi E, Viphi F, and Viphi G [73] from *Viola philippica,* and Vaby D [55] from *Viola abyssinica*.

cyclotides display a wide range of biological and pharmacological activities, including anti-HIV, anthelmintic, insecticidal, antimicrobial, and cytotoxic effects [79]. Therefore, there is increasing interest in exploring the plant kingdom to identify new cyclotides.

5.1. Cytotoxic and Anticancer Activity of Cyclotides. One of the first activities reported for cyclotides was hemolytic activity, which only occurs in the cyclic condition. Cyclotides lose their hemolytic activity when they are linearized [80], demonstrating that the cyclic backbone is important for this activity, which also appears to be important for the other activities of cyclotides. A directed mutational analysis of cyclotide kalata B1, in which all 23 noncysteine residues were replaced with alanine, shows that both the insecticidal and hemolytic activities are dependent on a well-defined cluster of hydrophilic residues on one face of the cyclotide. Interestingly, these molecules retain the characteristic stability of the framework [73]. In addition, it has been suggested that the hemolytic activity of the cyclotides depends on the amino acid sequence. The cyclotides cycloviolacins O2 and O13 from *Viola odorata* have different hemolytic activities. Both molecules differ only in one residue (Figure 2(b)). Cycloviolacin O2 has a serine residue, whereas cycloviolacin O13 has an alanine in the same position. The loss of the hydroxyl group changes the hemolytic activity by more than 3-fold [50].

Table 4: Cyclotides with anticancer and cytotoxic activity.

Name	Species	Activity against	Cytotoxic activity	Anticancer activity	Reference
Cycloviolacin O2	*Viola odorata*	U-937, HeLa	Yes	Yes	[54]
Viphi A, Viphi F, and Viphi G	*Viola philippica*	MM96L, HeLa, BGC-823, and HFF-1	Yes	Yes	[51]
MCoTI-I	*Momordica cochinchinensis*	LNCaP and HCT116	Not tested	Yes	[81]
HB7	*Hedyotis biflora*	Capan2 and PANC1	Not tested	Yes	[82]
Vaby A and Vaby D	*Viola abyssinica*	U-937	Not tested	Yes	[83]
Cliotides T1–T4	*Clitoria ternatea*	HeLa and human erythrocytes	Yes	Yes	[84]
Psyle A, Psyle C, and Psyle E	*Psychotria leptothyrsa*	U-937	Not tested	Yes	[85]
Vibi G and Vibi H	*Viola biflora*	U-937	Not tested	Yes	[86]
Varv A and Varv F	*Viola arvensis*	10 cancer cell lines	Not tested	Yes	[87]
Viba 15, Viba 17, and Mram 8	*Viola philippica*	HFF1, MM96L, HeLa, BGC-823, and HFF-1	Yes	Yes	[51]
CT-2, CT-4, CT-7, CT-10, CT-12, and CT-19	*Clitoria ternatea*	A549	Not tested	Yes	[88]
Kalata B1 and kalata B2	*Oldenlandia affinis*	U-937 GTB HT-29 Ht116	Yes	Yes	[89]

In general, cyclotides also show anticancer activity against human cancer cells (Table 4); however, two cyclotides from *Viola philippica* (Viphi D and Viphi E) did not show activity against the human gastric cancer BGC-823 cell line [51]. These peptides have similar sequences to the cyclotides Viphi F and Viphi G (Figure 2(c)), indicating that even minimal sequence changes can significantly influence the bioactivity. It has been suggested that the potency and selectivity of cyclotides is dependent on their primary structure. For example, a single glutamic acid plays a key role in the anticancer activity of cycloviolacin O2, and when this residue is methylated, a 48-fold decrease in potency is observed [52].

Cycloviolacin O2 from *Viola odorata* is particular promising because of its selective toxicity to cancer cell lines relative to normal cells, which indicates the possibility of its use as an anticancer agent [53]. Analysis of the proposed mechanism of action of this cyclotide shows that the disruption of cell membranes plays a crucial role in the cytotoxicity of cycloviolacin O2 because the damage to cancer cells (human lymphoma) can be morphologically distinguished within a few minutes, indicating necrosis [54]. However, this activity was not detected when this cyclotide was tested in a mouse tumor model. The reasons of this discrepancy are not fully understood, although high clearance rates or poor distribution to the site of action may be involved. Cycloviolacin O2 was also lethal to mice (2 mg/kg), but no signs of discomfort to the animals were observed at 1.5 mg/kg [55]. Recently the cyclotide MCoTI-I was engineering and the resulting cyclotide MCo-PMI showed activity *in vivo* in a murine xenograft model with prostate cancer cell; treatment (40 mg/kg) significantly suppressed tumor growth [81]. In the same way, HB7 cyclotide from *Hedyotis biflora* in an *in vivo* xenograft model significantly inhibited the tumor weight and size compared to control [82]. These results suggest that cyclotides may have a good anticancer bioactivity.

With respect to the action mechanism of cyclotides, a study showed that cycloviolacin O2 and kalatas B1–B9 target membranes through binding to phospholipids containing phosphatidylethanolamine headgroups [90]. Therefore, the biological potency of these cyclotides may be correlated with their ability to target and disrupt cell membranes. The knowledge of their membrane specificity could be useful to design novel drugs based on the cyclotide framework, allowing the targeting of specific peptide drugs to different cell types.

6. Small Cationic Peptides Isolated from Plants with Anticancer Activity

In addition to plant AMPs, other small linear and cyclic peptides (2–10 aa) with anticancer activity have been reported in plants. For example, the linear peptide *Cn*-AMP1, isolated and purified from coconut water (*Cocos nucifera*), was tested against Caco-2, RAW264.7, MCF-7, HCT-116 cells, and human erythrocytes and showed a reduction of cell viability in cancer cells without causing hemolysis [91]. Other examples are the peptides Cr-ACP, isolated from *Cycas revoluta*, and the acetylated-modified Cr-AcACP1, both repressors of cell proliferation of human epidermoid cancer (Hep2) and colon carcinoma. These peptides induce cell cycle arrest at the G0-G1 phase of Hep2 cells [92]. Moreover, four small cyclic peptides, dianthins C–F, have anticancer activity against Hep G2, Hep 3B, MCF-7, A-549, and MDA-MB-231 cancer cell lines (IC_{50} 20 μg/mL) [93]. Furthermore, the cyclic heptapeptide cherimolacyclopeptide C, obtained from a methanol extract of the seeds of *Annona cherimola*, exhibited significant

in vitro cytotoxicity against KB cells (IC_{50} 0.072 μM) [94]. Other examples of small cyclic peptides are RA-XVII and RA-XVIII from the roots of *Rubia cordifolia* L., which have cytotoxicity against P-388 cells at 0.0030 μg/mL and 0.012 μg/mL, respectively; however, it was not determined whether these peptides are effective against normal cells [95]. Recently, an antiproliferative cyclic octapeptide (cyclosaplin) was purified from *Santalum album* L. The anticancer activity from this peptide was tested against human breast cancer (MDA-MB-231) cells and exhibited significant growth inhibition in a dose and time dependent manner (IC_{50} 2.06 μg/mL). Additionally, cytotoxicity on normal fibroblast cell line at concentrations up to 1000 μg/mL was not detected [56].

7. Conclusion and Future Perspectives

The identification and development of plant AMPs with anticancer properties will provide good opportunities for cancer treatment. AMPs with anticancer activities, including plant-derived peptides, show many therapeutic challenges that must be considered before they can be developed commercially. Strategies to solve their poor stability and susceptibility to proteolytic digestion, such as amino acid substitution, structural fusion of functional peptides, and conjugation with chemotherapeutic drugs, must be evaluated. Despite these limitations, AMPs are an important source of molecules useful for the design of new drugs. In this sense, cationic peptides from plants have great potential as anticancer agents, particularly because of their selectivity towards cancer cells, as has been demonstrated to coccinin and phaseococcin. The number of plant AMPs with anticancer activity is increasing and is expected to rise in the next years, particularly when the remaining plant AMP families are assessed. A crucial step in the studies of plant AMPs as anticancer agents is the identification of their mechanisms of action to discover new targets. Furthermore, the development of novel synthetic analogs of these natural molecules could enhance their activities, facilitating the development of new drugs. With the rapid development in proteomics, bioinformatics, peptide libraries, and modification strategies, these plant AMPs emerge as novel promising anticancer drugs in future clinical applications.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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10. DISCUSIÓN GENERAL

Los PAs son moléculas que forman parte del sistema inmune innato de diversos organismos [29]; sin embargo, también se les ha atribuido una actividad biológica muy diversa, dentro de la que destaca la actividad antimicrobiana, como reguladores del sistema inmune innato así como su citotoxicidad contra células cancerosas [2]. En este sentido, los PAs de origen animal y sus derivados han sido ampliamente estudiados, tanto a nivel de toxicidad como a nivel de mecanismo de acción [40]. No obstante, a pesar de que las plantas son una fuente importante de PAs, el conocimiento sobre su actividad citotóxica y los mecanismos que activan en las células cancerosas es todavía limitado [62]. En este sentido el presente trabajo evaluó la citotoxicidad de las defensinas PaDef y γ-tionina contra la línea celular proveniente de cáncer de mama MCF-7.

Las dos defensinas muestran efecto citotóxico contra las células MCF-7 de manera dependiente de la concentración y con un tiempo de tratamiento de 48 h. El rango de concentración utilizado es similar al que se encuentra reportado por otras defensinas de plantas como la NaD1, la sesquina y la limenina [76, 77, 78]. Sin embargo, a pesar de que la defensina PaDef es más efectiva (85% de inhibición) que la γ-tionina (75% de inhibición); la IC_{50} de PaDef es mayor (141.82 µg/ml) en comparación con la γ-tionina (117.29 µg/ml), lo que sugiere posibles diferencias en los mecanismos que activan. Adicionalmente, las defensinas fueron probadas contra células sanas (CEMB y PBMC) y en el caso de PaDef no mostró efecto inhibitorio contra PBMC, aunque la viabilidad de las células de CEMB si se vio afectada significativamente (datos no mostrados). Por otro lado la defensina γ-tionina no mostró citotoxicidad contra las células CEMB y PMBC. Los resultados obtenidos indican que la citotoxicidad de las defensinas puede ser selectiva hacia las células cancerosas y no dañar a ciertos tipos de células sanas, estas diferencias pueden estar relacionadas con el tipo celular, ya que se ha demostrado que existen diferencias importantes entre la estructura de la membrana de las células cancerosas y las células sanas, lo cual las vuelve más o menos

susceptibles al efecto de las defensinas [40]. Otro aspecto importante que puede definir las diferencias en la actividad de las defensinas son sus características estructurales, en este sentido; en los últimos años se ha demostrado que existen diversas características fisicoquímicas y estructurales que son clave en la actividad de los PA, entre ellos: la carga neta y la hidrofobicidad [56]. Los reportes generados al respecto apuntan a la necesidad de un balance específico de las propiedades mencionadas para obtener el máximo efecto citotóxico contra las células cancerosas y la mayor selectividad. De manera general se concluye que una elevada carga neta positiva combinada con una hidrofobicidad disminuida son características clave de PAs con alto efecto citotóxico y elevada selectividad [56]. Debido a lo anterior, se realizó un análisis estructural de la defensina PaDef y γ-tionina en busca de una posible relación estructura-actividad.

El análisis *in silico* de la γ-tionina mostró que esta posee una carga neta de +5.99 y un índice de hidrofobicidad (Kyte-Doolittle) de 0.14, además de que la distribución de los residuos de aminoácidos cargados positivamente ubicados en la hélice-α se encuentran orientados hacia la parte externa de la molécula, lo cual puede favorecer la interacción de ésta con la parte negativa de la membrana citoplasmática, y de esta manera facilitar la formación de poros transitorios y la internalización del péptido para que se activen mecanismos internos como la inducción de apoptosis (Figura 7) [58].

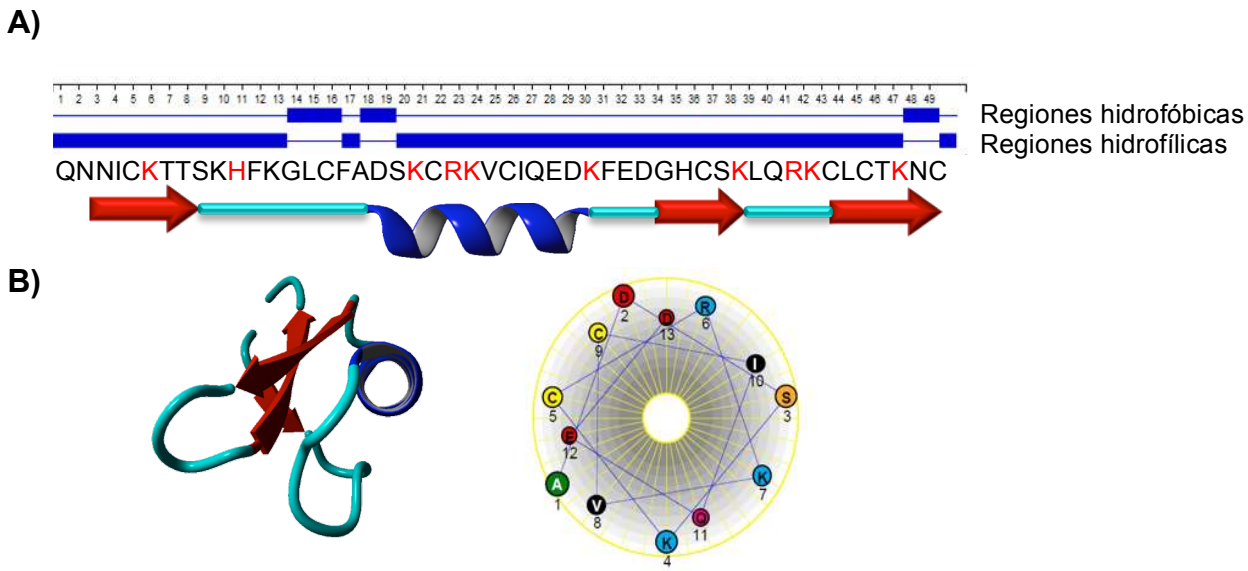

Figura 7. Análisis estructural de la defensina γ-tionina. A) Se muestra la secuencia del péptido y la distribución de las regiones hidrofóbicas e hidrofílicas; los residuos de aminoácidos cargados positivamente se marcan en rojo. **B)** Modelo de la estructura completa del péptido (izquierda) y esquema representativo de la hélice-α que muestra los aminoácidos positivos (círculos azules) orientados hacia la parte externa de la molécula. Análisis realizado con el Software DNASTAR-Lasergene/Protean.

El balance entre la carga neta positiva elevada y la hidofobicidad disminuida que tiene la γ-tionina es congruente con la actividad citotóxica y selectiva que presenta bajo las condiciones experimentales utilizadas en este trabajo; esto a diferencia de la defensina PaDef, la cual tiene una carga neta de +4.17 y un índice de hidrofobicidad aproximadamente 3 veces más elevado que la γ-tionina (0.34). En función de lo reportado, estas características corresponden con un péptido con menor selectividad tal como se observó en los ensayos de viabilidad realizados. Otro factor importante es que las regiones hidrofóbicas de PaDef se localizan principalmente en la hélice-α, lo que puede favorecer su interacción con la parte hidrofóbica de la membrana y con ello la formación de poros transmembranales más estables que permitan la liberación del citoplasma (Figura 8) [79].

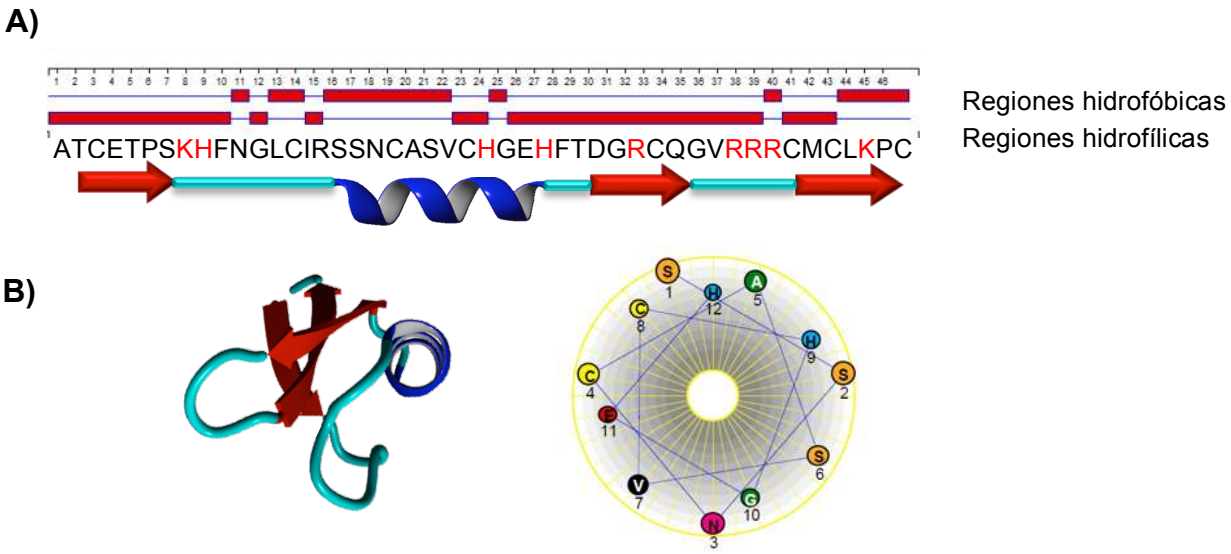

Figura 8. Análisis estructural de la defensina PaDef. A) Se muestra la secuencia del péptido y la distribución de las regiones hidrofóbicas e hidrofílicas; los residuos de aminoácidos cargados positivamente se marcan en rojo. **B)** Modelo de la estructura completa del péptido (izquierda) y esquema representativo de la hélice-α que muestra los aminoácidos positivos (círculos azules) orientados hacia la parte externa de la molécula. Análisis realizado con el Software DNASTAR-Lasergene/Protean.

Con base en las diferencias estructurales encontradas entre la defensina γ-tionina y PaDef, se puede inferir que éstas son clave en su actividad diferencial y que pueden estar activando mecanismos de citotoxicidad diferentes, no solo a nivel de estructura sino también a nivel celular.

Para elucidar el mecanismo de muerte celular que activan las defensinas de plantas en las células MCF-7, se analizó primeramente la integridad de la membrana citoplasmática la cual se relaciona con la formación de poros y ha sido reportada para otros PAs incluyendo defensinas de plantas [78]. Los resultados mostraron que ninguna de las dos defensinas modificó la integridad de la membrana por lo que se descarta la posibilidad de que este sea el mecanismo de muerte de las células cancerosas. Otro mecanismo reportado de PAs en células cancerosas es la inducción de apoptosis [45, 46]; en este trabajo se demostró que las defensinas de plantas inducen este mecanismo de manera dependiente del tiempo, iniciando el efecto a las 6 h de tratamiento sin mostrar necrosis en ninguno de los tratamientos.

Hasta el momento, este es el primer reporte de defensinas de plantas que inducen apoptosis en células cancerosas, aunque ya se ha observado este fenómeno en otros modelos como las defensinas RsAFP2 y HsAFP1 que inducen apoptosis en *Candida albicans* a través de la activación de caspasas y especies reactivas de oxígeno (ROS) [81, 82].

La apoptosis es un proceso que se activa principalmente por dos vías la extrínseca, también conocida como de receptores de muerte celular, y la intrínseca o mitocondrial [83]. Las defensinas γ-tionina y PaDef inducen la despolarización mitocondrial en las células MCF-7, este fenómeno ya se ha descrito para otros PAs como BMAP-28 [83], este resultado es el primer indicio de que la ruta intrínseca se encuentra activa. Por otro lado, el análisis de expresión de genes mostró que la defensina PaDef activa exclusivamente los genes de la ruta intrínseca así como la activación de la caspasa 9 correspondiente a esa ruta. Sin embargo, γ-tionina indujo un nivel máximo de expresión de la ruta extrínseca desde las 6 h de tratamiento en comparación con PaDef, donde la activación es dependiente del tiempo y el nivel máximo de activación se observa hasta las 24h de tratamiento. Estos resultados se correlacionan con la tasa de apoptosis que presentan cada una, ya que la defensina PaDef produjo menor activación de apoptosis (hasta 44%) con respecto a la γ-tionina (hasta 76%). Interesantemente, la γ-tionina también indujo la activación de genes de la ruta extrínseca, como la cecropina de insectos en células de carcinoma hepático [46]. No obstante, hasta nuestro conocimiento este es el primer reporte de una defensina de plantas que activa las dos rutas de señalización de apoptosis. Ya ha sido ampliamente estudiada la posibilidad de que los PAs o sus derivados tengan interacción directa con receptores proteicos y de esta manera ejercer su efecto; sin embargo, hasta el momento no hay evidencia concluyente al respecto [85]. Por lo tanto, para explicar la activación de la ruta de receptores por la γ-tionina, se propone que ésta se activa de manera indirecta, ya que se ha reportado que esta defensina tiene actividad inmunomoduladora y es capaz de inducir la expresión de TNF-α en células de epitelio mamario bovino [86]. Este factor eventualmente puede llegar a activar la ruta de receptores de muerte celular en la línea MCF-7. Otra posible

explicación es que algunos PAs, como la pardaxina, favorecen la acumulación de ROS [87], lo cual desencadena principalmente apoptosis mitocondrial; sin embargo, existe evidencia que muestra que la presencia de ROS también puede estimular directamente la activación de los receptores de muerte [88]. Sin embargo, se requieren experimentos adicionales para comprobar esta hipótesis.

11. CONCLUSIÓN GENERAL

Las defensinas de plantas PaDef (*Persea americana* var. *drymifolia*) y γ-tionina (*Capsicum chinense*) poseen efecto citotóxico contra la línea celular de cáncer de mama MCF-7, con una IC_{50} de 141.62 µg/ml y 117.29 µg/ml, respectivamente. Este efecto no está relacionado con el daño directo a la membrana. Sin embargo, favorecen la inducción de apoptosis de manera diferencial. La defensina PaDef induce solo la vía mitocondrial o intrínseca, mientras que la defensina γ-tionina activa ambas rutas en función del tiempo, en las primeras horas se activa la vía intrínseca y al final del tratamiento solo la vía extrínseca. Esta diferencia puede estar relacionada con las características fisicoquímicas de las defensinas como la cationicidad e hidrofobicidad que son importantes de considerar para futuras aplicaciones de estos PAs como alternativas terapéuticas.

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