UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

BIOMEDICINA

POTENCIAL ANTIGENOTÓXICO DE INHIBIDORES DE CITOCROMO P4501A1 (CYP1A1)

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

REBECA SANTES PALACIOS

TUTOR PRINCIPAL: **DR. JESÚS JAVIER ESPINOSA AGUIRRE**
INSTITUTO DE INVESTIGACIONES BIOMÉDICAS, UNAM

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UNAM
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COORDINACIÓN

OFICIO CPCB/356/2019

Asunto: Oficio de Jurado para Examen de Grado.

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

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día 28 de enero del 2019, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la alumna **SANTES PALACIOS REBECA** con número de cuenta **40808913-7** con la tesis titulada **"Potencial antigenotóxico de inhibidores de Citocromo P4501A1 (CYP1A1)** realizada bajo la dirección del **DR. ESPINOSA AGUIRRE JESÚS JAVIER:**

Presidente:	DR. LUIS ANTONIO MÉNDOZA SIERRA
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Suplente:	DRA. ANA MARÍA SALAZAR MARTÍNEZ

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

A T E N T A M E N T E
"POR MI RAZA HABLARA EL ESPIRITU"
Cd. Universitaria, Cd. Mx., a 20 de marzo del 2019.

POSGRADO EN CIENCIAS BIOLÓGICAS
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DR. ADOLFO GERARDO NAVARRO SIGÜENZA
COORDINADOR DEL PROGRAMA

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ÍNDICE

LISTA DE ABREVIATURAS

2AA	2-aminoantraceno
2AF	2-aminofluoreno
AKR	Aldo-ceto reductasa
ANF	α-naftoflavona
BaP	Benzo[*a*]pireno
BG	Bergamotina
CYP	Citocromo P450
CYP1A1	Citocromo P4501A1
DiMeIQx	2-amino-3,4,8-dimetilimidazo [*4,5-f*] quinoxalina
DMBA	7,12-Dimetilbenzo[*a*]antraceno
EH	Epóxido hidrolasa
GMT	Glutatión metiltranferasa
GST	Glutatión *S*-transferasa
HAP	Hidrocarburos aromáticos policíclicos
MeIQx	2-amino-3,8-dimetilimidazo [*4,5-f*] quinoxalina
NAR	Naringenina
NAT	*N*-acetiltransferasa
PhIP	2-amino-1-metil-6-fenilimidazo [*4,5-b*] piridina
SRS	Sitios de reconocimiento de sustrato
SULT	Sulfotransferasa
S9	Homogenado de tejido obtenido por centrifugación a 9000 xg

RESUMEN

El citocromo P4501A1 (CYP1A1) es una de las enzimas más importantes que participa en la activación de procarcinógenos. Utilizando diferentes modelos experimentales, se ha demostrado su papel en la carcinogénesis y la inhibición de su actividad catalítica ha sido utilizada como estrategia para la búsqueda de compuestos quimiopreventivos. Sin embargo, los datos experimentales derivados de modelos animales no son fácilmente extrapolables para su uso en humanos, en parte por el supuesto de que los compuestos interactúan con la enzima humana de la misma manera en que lo hacen con la enzima de otras especies, además de que no se consideran factores como la regulación genética, la actividad enzimática y la afinidad de las enzimas, los cuales influyen en las diferencias entre especies.

Este trabajo se centró en el estudio cuantitativo de las diferencias interespecies en la interacción de seis promutágenos y dos inhibidores con CYP1A1 humano y de rata utilizando las mismas condiciones experimentales. Se evaluó la activación de los mutágenos por CYP1A1 recombinante humano o de rata mediante la prueba de Ames, así como el efecto antimutagénico de los inhibidores. Con esto se determinó que la mutagenicidad y la antimutagenicidad dependen tanto del grupo químico como de la especie que lleva a cabo la activación: las aminas aromáticas y los hidrocarburos aromáticos policíclicos mostraron mayor mutagenicidad cuando se activaron por la enzima humana, mientras que las aminas heterocíclicas fueron más mutagénicas en presencia de la enzima de rata, de la misma manera que los compuestos del jugo de toronja inhibieron en mayor medida a CYP1A1 de rata. También se evaluó a través de un análisis *in silico* la unión de los mutágenos e inhibidores al sitio catalítico del CYP1A1 humano y de rata, identificando los posibles residuos involucrados en el reconocimiento del ligando, por ejemplo, la fenilalanina podría participar en la estabilización de los compuestos a través de interacciones π-π. Además, se propuso que las variaciones en las conformaciones tridimensionales y las distancias hacia el grupo hemo pueden contribuir a las diferencias entre especies en la interacción con CYP1A1.

En conclusión, las diferencias entre CYP1A1 humano y de rata tienen impacto en la mutagenicidad y antimutagenicidad, por lo que deben considerarse al momento de extrapolar los resultados de los modelos animales para la evaluación del riesgo en seres humanos.

ABSTRACT

One of the most important enzymes participating in procarcinogen activation is cytochrome P4501A1 (CYP1A1). Using different experimental models, the role of this enzyme in carcinogenesis has been demonstrated and the inhibition of its catalytic activity has been applied as a strategy for the search of chemopreventive compounds. Nevertheless, experimental data derived of animal models are often misused in human risk assessment due to the assumption that compounds interact with the human enzyme in the same way as with another species' enzyme. In this matter, it is important to consider factors as genetic regulation, enzymatic activity and enzyme's affinity, which play an important role in differences between species.

Here, we focused on the quantitative study of the interspecies differences in interaction of selected mutagens and inhibitors with human and rat CYP1A1 using the same experimental conditions. We evaluated bacterial mutagenicity (Ames test) resulting from mutagen activation by recombinant human or rat CYP1A1, as well the antimutagenic effect of CYP1A1 inhibitors. Mutagenicity and antimutagenicity depend on both the chemical group and species-specific activation: aromatic amines and polycyclic aromatic hydrocarbons showed higher mutagenic activity when activated by the human enzyme, whereas heterocyclic amines were more mutagenic in the presence of the rat enzyme, in the same way that grapefruit juice compounds inhibited mutagen activation by rat CYP1A1. In addition, we analyzed through an *in silico* methodology, the binding of mutagens and inhibitors to human and rat CYP1A1 catalytic site, identifying residues expected to participate in ligand recognition. A phenylalanine residue was involved in CYP-mutagen stabilization through π-π stacking. Variations in the three-dimensional conformations and distances to the heme groups may contribute to differences between human and rat CYP-ligand interactions. In conclusion, CYP1A1 shows significant differences between species, in terms of mutagen activation and antimutagenic effect, which should be considered in the context of human risk assessment.

1. INTRODUCCIÓN

Los citocromos P450 (CYP) son una superfamilia de hemoproteínas ampliamente distribuidas en la naturaleza que participan en el metabolismo de compuestos endógenos como hormonas, ácidos biliares y colesterol; también de xenobióticos como fármacos y contaminantes ambientales [1].

El papel de los CYP en el metabolismo de xenobióticos es controversial. Por un lado, la mayoría de las reacciones llevadas a cabo por los P450 involucran la introducción de grupos polares para facilitar la excreción de las moléculas. Sin embargo, algunos compuestos como los hidrocarburos aromáticos policíclicos, aminas aromáticas y otros procarcinógenos son biotransformados a metabolitos altamente reactivos que ocasionan daño celular [2]. En este proceso de bioactivación, las isoenzimas CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2E1 y CYP3A4 participan en al menos el 77% de los casos [3].

1.1 Citocromo P4501A1: ligandos

El citocromo P4501A1 (CYP1A1) es una proteína con actividad monoxigenasa expresada en diversos órganos y tejidos como pulmón, piel, tracto gastrointestinal y placenta [4]. El CYP1A1 es particularmente estudiado en la carcinogénesis ambiental ya que un aumento de su actividad enzimática se ha relacionado con una mayor susceptibilidad al desarrollo de cáncer cervical, de mama, ovario y pulmón por exposición a compuestos procarcinógenos [5–8]. Por eso, la búsqueda de moléculas moduladoras de esta isoenzima ha cobrado gran relevancia en la quimioprevención [9–12].

La enzima humana consta de 512 aminoácidos de los cuales los primeros treinta de la región *N*-terminal permiten la asociación de la proteína a las membranas mitocondriales y del retículo endoplásmico, así como la interacción con la NADPH-CYP reductasa [13–15]. La estructura tridimensional de CYP1A1 está conformada por 12 α-hélices (A-L), 3 láminas β (β1-β3) y 4 regiones helicoidales cortas (A', B', F' y G') que en conjunto forman seis secuencias de reconocimiento de

sustrato (Figura 1), permitiendo la unión de moléculas planas con dimensiones de aproximadamente 12.3 Å de largo y 4.6 Å de ancho [4,16].

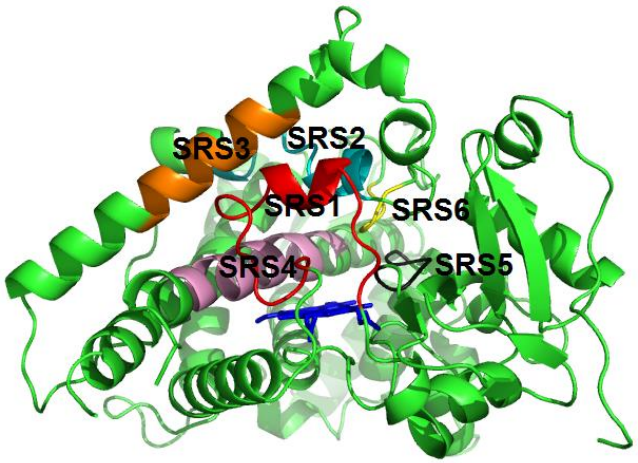


Figura 1. Sitios de reconocimiento de sustrato (SRS) de CYP1A1 humano [1]. Imagen diseñada en PyMOL versión 1.3.

En general, los ligandos de CYP1A1 (sustratos e inhibidores) son moléculas con anillos aromáticos o heterocíclicos que son esenciales para la formación de enlaces π-π en el sitio activo de la proteína, principalmente con la fenilalanina de la posición 224 de la hélice F, dando mayor estabilidad al complejo enzima-ligando [17–19]. Algunos ejemplos de ligandos con estas características son las aminas aromáticas, aminas heterocíclicas, hidrocarburos aromáticos policíclicos y algunos compuestos de origen natural como los flavonoides y furanocumarinas, entre otros.

1.1.1 Aminas aromáticas

Las aminas aromáticas se utilizan en la elaboración de colorantes sintéticos, pesticidas, herbicidas, plásticos y combustibles; aunque también pueden ser encontradas en el humo del cigarro, como productos de combustión del diesel y en la materia orgánica carbonizada. En mamíferos y peces se han demostrado sus propiedades genotóxicas y carcinogénicas, siendo el 2-aminoantraceno (2AA) uno de los controles positivos de mutagenicidad más utilizados [20,21].

Una vez que las aminas aromáticas ingresan al organismo, los CYP1A llevan a cabo la *N*-hidroxilación, donde los productos *N*-hidroxilamina logran activarse aún más por acción de las *N*-acetiltransferasas (Figura 2), dando como resultado la producción de compuestos altamente reactivos que se unen al DNA [22].

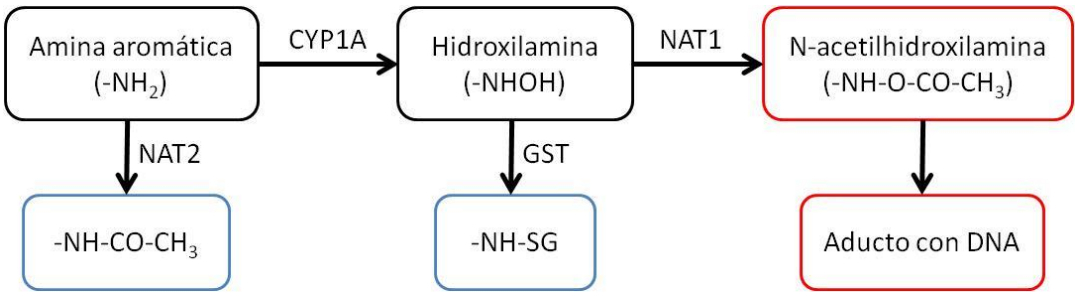


Figura 2. Esquema simplificado del metabolismo de las aminas aromáticas. En recuadros azules se muestran los metabolitos no reactivos, mientras que el recuadro rojo hace referencia a los compuestos altamente reactivos [23]. NAT: *N*-acetiltransferasa, GST: Glutatión *S*-transferasa.

En general, el metabolismo de las aminas aromáticas induce daño alquilante al DNA (O^6-alquilguanina), estrés oxidante y producción de iones diazonio, que pueden contribuir al proceso carcinogénico si las lesiones provocadas al DNA, lípidos o proteínas no son eficientemente reparadas [24].

1.1.2 Aminas heterocíclicas

Las aminas heterocíclicas son compuestos derivados del proceso de cocción de la carne a altas temperaturas, siendo el 2-amino-1-metil-6-fenilimidazo [*4,5-b*] piridina (PhIP) y el 2-amino-3,8-dimetilimidazo [*4,5-f*] quinoxalina (MeIQx) dos de los compuestos más abundantes, con un alto potencial carcinogénico en múltiples tejidos y especies [25].

La principal enzima hepática involucrada en la activación de las aminas heterocíclicas es CYP1A2. A pesar de que la enzima de rata y humano muestran actividades catalíticas casi idénticas con el sustrato 7-metoxirresorufina y comparten un porcentaje de identidad en su secuencia de aminoácidos del 75%, CYP1A2 humano tiene casi 10 veces mayor eficiencia catalítica que CYP1A2

de rata en la activación tanto de PhIP como de MeIQx [26]. A su vez, el nivel de CYP1A2 varía considerablemente entre los humanos. Al evaluar la genotoxicidad de MeIQx en presencia de microsomas hepáticos humanos, se encontró que las muestras de algunos individuos mostraban mayor bioactivación que los microsomas de rata, cuyo CYP1A2 se había inducido con 3-metilcolantreno o bifenilos policlorados [26].

En tejidos extrahepáticos, la bioactivación de las aminas heterocíclicas se da principalmente por acción del CYP1A1, siendo la *N*-hidroxilación la principal reacción de activación, seguida de una *O*-acetilación catalizada por la *N*-acetiltransferasa 2 [27,28] (Figura 3). El daño genotóxico producido por los metabolitos de las aminas heterocíclicas comprende rupturas al DNA de cadena sencilla, aberraciones cromosómicas y aductos con DNA en regiones ricas en guanina (en la posición N2 o C8 de la guanina) [24].

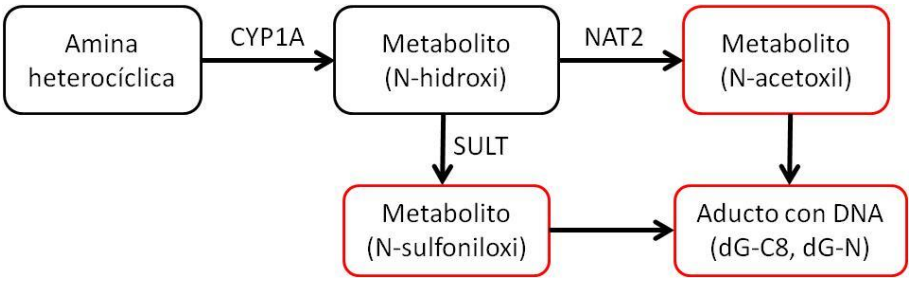


Figura 3. Esquema simplificado del metabolismo de las aminas heterocíclicas. En recuadros rojos se muestran los compuestos altamente reactivos [29]. NAT: *N*-acetiltransferasa, SULT: Sulfotransferasa.

1.1.3 Hidrocarburos aromáticos policíclicos

Los hidrocarburos aromáticos policíclicos (HAP) son compuestos presentes en las emisiones de combustibles fósiles, alimentos carbonizados, humo de tabaco y otros productos resultantes de la combustión incompleta de la materia orgánica [30]. Después de ingresar al organismo son metabolizados y transformados en agentes reactivos que pueden unirse a ácidos nucleicos, proteínas o lípidos [31].

En el área experimental, los modelos animales han contribuido a vislumbrar los mecanismos carcinogénicos de los HAP, siendo el benzo[*a*]pireno (BaP) y el 7,12-dimetilbenz[*a*]antraceno (DMBA) dos de los compuestos prototipo [32,33]. Se ha demostrado que la carcinogenicidad de éstos depende de la conformación de sus sistemas de anillos, es decir, los HAP que albergan una "región bahía" o una "región fiordo" son carcinógenos más potentes, ya que debido a razones estructurales, sus metabolitos reactivos (dihidrodiol epóxidos producidos por acción de CYP1 y la epóxido hidrolasa) son eliminados con menor eficacia [34]. En cuanto a la bioactivación de BaP mediada por CYP1A1, el metabolito benzo[*a*]pireno-4,5-dihidrodiol es específico de la especie, pues es formado únicamente por la enzima de rata y no por la de humano [35,36].

Además de la vía de activación de HAP ya señalada, se han postulado dos más. Una de ellas involucra la formación de radicales catión de HAP catalizada por la peroxidasa P450; mientras que la segunda implica la formación de *O*-quinonas por acción de dihidrodiol deshidrogenasas (Figura 4) [37].

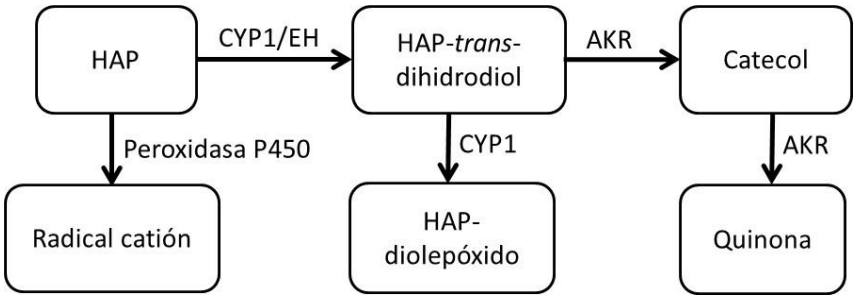


Figura 4. Esquema simplificado del metabolismo de los hidrocarburos aromáticos policíclicos. EH: Epóxido hidrolasa, AKR: Aldo-ceto reductasa [38].

Una vez que los metabolitos de los HAP han sido producidos, algunos de éstos pueden unirse covalentemente al DNA, generando aductos en la posición *N2* de la guanina o *N3* de la adenina, entre otros [24]. Además del daño a DNA, el estrés oxidante y la desregulación de vías de transducción de señales involucradas en la homeostasis del calcio y proliferación celular son otros mecanismos asociados a la carcinogénesis inducida por exposición a HAP [39,40].

1.1.4 Inhibidores

Los ratones *knock out* han mostrado la importancia de CYP1A1 en la carcinogénesis química inducida por la exposición a contaminantes ambientales [41,42], así que la búsqueda de moléculas candidatas de origen sintético o derivados de productos naturales como inhibidores de CYP1A1 ha sido empleada como estrategia para el desarrollo de agentes quimiopreventivos en el cáncer derivado de la exposición a xenobióticos. Particularmente, la α-naftoflavona se ha convertido en el prototipo de los inhibidores de CYP1 debido a su potente efecto de inhibición, dando paso a la búsqueda de análogos estructurales con una mayor actividad biológica [43]. Entre éstos, los compuestos presentes en el jugo de toronja como la naringenina y la bergamotina han sido de especial interés para el desarrollo de terapias contra el cáncer ya que poseen una gran diversidad de actividades biológicas como antioxidantes, reguladores de transportadores, inhibidores de CYP, entre otras [44–47]. Previamente se ha demostrado que estos compuestos tienen una potencia de inhibición sobresaliente sobre CYP1A1 humano y de rata [48]; sin embargo, se desconoce si estas diferencias tienen impacto en el potencial antigenotóxico de ambas moléculas.

1.2 Consideración de las diferencias entre especies en el metabolismo mediado por CYP1A1

Existen varios modelos experimentales que han permitido evaluar la participación de CYP1A1 en la bioactivación de procarcinógenos; por ejemplo, ensayos *in vitro* empleando fracción S9 hepática, microsomas de origen humano o animal, enzimas recombinantes, cultivo celular, entre otros; además de modelos *in vivo* de tumorigénesis o animales *knock out*, con un uso generalizado de especies como peces o roedores [49–51].

De igual forma, con la aplicación de herramientas computacionales se han podido evidenciar posibles determinantes estructurales necesarias para la interacción con los ligandos y optimizar la búsqueda de inhibidores de CYP1A1 [10,52–54]. Sin embargo, estos modelos son limitados y dificultan la extrapolación de los resultados para su uso en humanos; por ejemplo, en ocasiones se omite el hecho de que existen diferencias en la composición de estas fracciones celulares que

dependen del organismo de origen, como es el caso de los homogenados obtenidos por centrifugación a 9000 xg (S9) de hígado humano, rata y ratón, donde se determinó que comparten aproximadamente el 58% de las proteínas totales [55]. Estas mismas diferencias interespecies se han documentado en la actividad enzimática [56–58].

A nivel industrial, la expresión de CYP1A1 se ha realizado empleando organismos genéticamente modificados como *E. coli, S. cerevisiae, S. typhimurium,* células de insecto infectadas con virus y células de mamífero [59–61]. La mayor problemática con estos sistemas de expresión consiste en evitar la formación de cuerpos de inclusión, coexpresar otras proteínas importantes en la catálisis (NADPH óxido reductasa, citocromo b_5), obtener un plegamiento tridimensional similar al de anclaje a membrana y actividad enzimática cuantificable de la proteína de interés [62]. No obstante, al utilizarse generalmente para expresar la enzima humana, estas estrategias de expresión de proteínas recombinantes impiden comparar los datos experimentales en diferentes especies.

Otro punto a considerar concerniente a las diferencias interespecies es que la abundancia relativa de la enzima varía en cada tejido y los mecanismos de regulación genética son distintos cuando se habla de órganos y especies diferentes [63,64]; por ejemplo, las regiones codificantes de los genes de CYP1 están dispuestas de manera similar en mamíferos, pero cambia en especies no mamíferas como los peces y anfibios [65]. Como consecuencia, las redes de regulación pueden ser ligeramente distintas a las de humano, haciendo más susceptibles al efecto de los procarcinógenos a unas especies con respecto a otras. Tal es el caso de los peces, los cuales son empleados para monitorear concentraciones pequeñas de contaminantes ambientales en agua ya que CYP1 es altamente inducible en estos organismos [66,67].

Para asegurar una mayor actividad de CYP, los modelos animales son administrados con inductores, lo cual los hace difícilmente comparables con muestras provenientes de humanos. En estos últimos, donde los inductores a los que están expuestos no están completamente caracterizados, también se encuentran diferencias interindividuales en las enzimas que dificultan una adecuada comparación [68–70].

Otros parámetros como las tasas de absorción, distribución, metabolismo y excreción difieren entre especies [71]. Empleando microsomas de hígado, se ha demostrado que en ratones es más alta la depuración, seguida de humanos y por último, ratas [72,73].

Además, las variaciones interindividuales de enzimas de fase II como *N*-acetiltransferasas (NAT), glutatión-*S*-transferasas (GST) y glutatión metiltransferasas (GSM), que modifican la bioactivación de los xenobióticos, deben ser consideradas [74].

Como se ha mencionado, los productos de la activación de los contaminantes ambientales por CYP1A1 son los principales compuestos que interactúan con DNA, proteínas o lípidos. Algunos de estos metabolitos son especie-específicos, no se forman o se encuentran en concentraciones variables [35,75]. Por ejemplo, las células de codorniz tratadas con la misma dosis de policlorobifenilos presentaron un mayor número de aductos con DNA en comparación de las células humanas y de rata [56]. Aunado a lo anterior, la estabilidad y reactividad de los metabolitos generados pueden variar considerablemente; incluso entre aquéllos del mismo grupo químico pues los tiempos de vida media van desde segundos hasta horas, ocasionando daño celular que puede restringirse a la célula en la que se produce la activación o distribuirse a otros órganos susceptibles.

2. PLANTEAMIENTO DEL PROBLEMA

Un problema en Toxicología y Farmacología preclínica es que la mayoría de los experimentos se realizan en modelos animales y los resultados deben extrapolarse a los humanos, así que la elección de las especies animales más apropiadas es crítica. Sin embargo, las diferencias entre especies a nivel de regulación genética, concentración de proteína y actividad enzimática de CYP1A1 hacen que las aproximaciones obtenidas en el modelo animal no sean fácilmente extrapolables a otras especies. Si bien se considera que los compuestos podrían interactuar de manera parecida con las enzimas debido a su similitud estructural, se ha reportado que las diferencias entre especies pueden tener implicaciones en la susceptibilidad de los organismos a algunos compuestos, ya sea por los metabolitos derivados del proceso de bioactivación de procarcinógenos o el efecto de los inhibidores enzimáticos.

3. HIPÓTESIS

Las diferencias a nivel estructural entre CYP1A1 humano y de rata tendrán implicaciones en el daño genotóxico inducido por contaminantes ambientales, así como en la interacción con los inhibidores enzimáticos, reflejándose en potencias mutagénicas y antimutagénicas distintas entre especies.

4. OBJETIVOS

4.1 Objetivo general

Determinar si existen diferencias interespecies en la interacción de seis promutágenos y dos inhibidores con CYP1A1 humano y de rata utilizando las mismas condiciones experimentales.

4.2 Objetivos particulares

- Determinar las diferencias entre CYP1A1 humano y de rata para la activación de diversos promutágenos.
- Determinar el efecto antigenotóxico de los inhibidores de CYP1A1, naringenina y bergamotina, sobre el daño provocado por los mutágenos.
- Determinar las diferencias en interacción y reconocimiento de los ligandos a través de ensayos de acoplamiento molecular asistido por computadora.

5. MATERIALES Y MÉTODOS

5.1. Reactivos

El benzo[*a*]pireno (BaP), 7,12-dimetilbenz[*a*]antraceno (DMBA), 2-aminoantraceno (2AA), 2-aminofluoreno (2AF), α-naftoflavona (ANF), naringenina (NAR), bergamotina (BG) y el NADPH fueron adquiridos en Sigma-Aldrich (Darmstadt, Alemania) con una pureza mayor al 98%. Los mutágenos 2-amino-3,4,8-trimetilimidazo [*4,5-f*] quinoxalina (DiMeIQx) y 2-amino-3,8-dimetilimidazo [*4,5-f*] quinoxalina (MeIQx) fueron comprados a Santa Cruz Biotechnology (Dallas, TX, USA) y United States Biological (Salem, MA, USA), respectivamente.

Los reactivos para la preparación de medios de cultivo bacterianos fueron de Becton Dickinson (Estado de México, México), Sigma-Aldrich y Oxoid (Lenexa, KS, USA), mientras que las enzimas recombinantes de CYP1A1 humano y de rata (Supersomes®) fueron adquiridas de Corning (Corning, NY, USA).

5.2. Estrategia experimental

Empleando CYP1A1 recombinante humano y de rata como activadores metabólicos, se evaluó la mutagenicidad inducida por dos aminas aromáticas (2AA y 2AF), dos aminas heterocíclicas (DiMeIQx y MeIQx) y dos hidrocarburos aromáticos policíclicos (BaP y DMBA) a través de la prueba de Ames. En todos los casos se realizaron experimentos preliminares para que a partir de curvas concentración-efecto se calculara la potencia mutagénica que corresponde a la pendiente de la porción lineal de la curva. Comparando estos datos se determinó cuál de las dos enzimas, de humano o de rata, es más eficiente en la activación de los promutágenos seleccionados.

Posteriormente, a una concentración fija de mutágeno (2AA y DiMeIQx), se evaluó la antimutagenicidad de los inhibidores de CYP1A1 (NAR y BG) para cuantificar las diferencias entre especies en la antimutagenicidad.

Finalmente, a través de un ensayo de acoplamiento molecular se determinaron las características estructurales de CYP1A1 que podrían influenciar en el reconocimiento de los ligandos y, por ende, tener alguna repercusión en la mutagenicidad y antimutagenicidad de los compuestos evaluados.

5.3. Caracterización de las cepas para la prueba de Ames

La prueba de Ames emplea cepas de la bacteria *Salmonella typhimurium* genéticamente modificadas para la detección de compuestos mutagénicos. En estos ensayos se utilizaron las cepas YG1024 y TA100, siendo la cepa YG1024 altamente sensible al efecto de las aminas aromáticas y heterocíclicas, mientras que la cepa TA100 lo es al tratamiento con hidrocarburos aromáticos policíclicos [76,77]. Antes de cada experimento, las cepas fueron caracterizadas fenotípicamente para evidenciar que aún poseen las mutaciones genéticas inducidas para la detección de compuestos mutagénicos: presencia de los plásmidos pKM101 y pYG109 (resistencia a ampicilina y/o tetraciclina), alteración de la permeabilidad celular (sensibilidad al cristal violeta), reparación deficiente de DNA (sensibilidad a la luz UV), requerimiento de histidina y frecuencia de la reversión espontánea [78].

5.4. Prueba de Ames

La mezcla de reacción (2 pmol de CYP1A1 recombinante disueltos en Tris-HCl 50 mM + $MgCl_2$ 25 mM pH 7.6, 0.15 mM de NADPH, 100 µL del cultivo de *Salmonella typhimurium* YG1024 o TA100 a una densidad de 1-2x10^9 bacterias/mL y 5 µL de distintas concentraciones de mutágeno disuelto en DMSO) en un volumen final de 700 µL, fue preincubada durante 30 min a 37 °C en agitación constante (150 rpm) para después ser vertida en 2 mL de agar de superficie precalentado e incorporada en cajas Petri con medio mínimo de Vogel-Bonner [78,79].

Las concentraciones de los mutágenos evaluados fueron de 5-52 pmol/caja de 2AA, 55-2759 pmol/caja de 2AF, 2-4400 pmol/caja de DiMeIQx, 0.2-14.1 nmol/caja de MeIQx, 40-396 nmol/caja de BaP y 2-20 nmol/caja de DMBA.

Las cajas se dejaron solidificar a temperatura ambiente y posteriormente fueron incubadas a 37 °C durante 48 h. Pasado el tiempo de incubación, se determinó el número de colonias revertantes obtenidas con cada tratamiento. La reversión espontánea es el número de colonias generadas en ausencia de mutágeno, por lo que un compuesto se considera mutagénico si el número de colonias contadas es al menos el doble de la reversión espontánea [80,81]. A su vez, las cajas fueron revisadas bajo el microscopio óptico para detectar anormalidades en el crecimiento bacteriano de fondo, que junto con una disminución en el número de colonias revertantes, son un reflejo de la toxicidad inducida por el compuesto evaluado [78].

Las potencias mutagénicas y antimutagénicas fueron determinadas a partir del análisis por regresión lineal de las curvas de concentración-efecto (valor de la pendiente de la recta) y se expresaron como el número de revertantes incrementadas (potencia mutagénica) o disminuidas (potencia mutagénica) por pmol o nmol de compuesto [70].

Todos los ensayos fueron realizados por triplicado y los resultados se muestran como promedios con su respectiva desviación estándar.

5.5. Ensayos computacionales

Las estructuras químicas de los ligandos fueron tomadas de la base de datos ChemSpider de la Real Sociedad de Química. Posteriormente, se revisaron y optimizaron con el programa AutoDock Tools (ADT) versión 1.5.6 [82].

La estructura tridimensional del CYP1A1 humano se obtuvo de la base de datos Protein Data Bank (PDB, 4I8V.pdb) y fue modificada empleando el programa PyMOL versión 1.3 para eliminar las moléculas de agua, los iones y el ligando con el que se cocristalizó [48]. Por otro lado, dado que no existe una estructura del CYP1A1 de rata obtenida por cristalografía de rayos X, se procedió a realizar su modelado por homología con CYP1A1 humano empleando el servidor ModWeb [83,84]. El grupo hemo fue añadido después por superposición del modelo de CYP1A1 de rata con la estructura cristalográfica del CYP1A1 humano.

La incorporación de todos los átomos de hidrógeno no polares y la asignación de las cargas Gasteiger a las proteínas y a los ligandos fue realizada con el programa ADT para generar los

archivos pdbqt correspondientes. A partir de éstos, empleando el programa AutoDock Vina [85], se llevó a cabo la obtención de los archivos que contienen las conformaciones tridimensionales de los ligandos con las macromoléculas, donde se eligieron aquéllas que presentaron las energías de unión calculadas más negativas. Finalmente, las interacciones proteína-ligando se determinaron con ADT y Maestro (Schrödinger, LLC, Nueva York, NY).

Las figuras representativas de la conformación tridimensional fueron realizadas en PyMOL, donde también se llevó a cabo la medición de las distancias (Å) entre los ligandos y el grupo hemo de las proteínas evaluadas. Mientras que los esquemas de interacción 2D se obtuvieron con la opción *Ligand Interaction Diagram* del programa Maestro.

6. RESULTADOS

6.1. Bioactivación de promutágenos por CYP1A1

La prueba de Ames permitió evaluar si existen diferencias interespecies entre CYP1A1 humano y de rata en la activación de seis promutágenos de diferentes grupos químicos. Empleando las mismas concentraciones de CYP1A1 recombinante humano y de rata como activadores metabólicos, se evaluó la mutagenicidad inducida en la cepa de *S. typhimurium* YG1024 por dos aminas aromáticas (2AA y 2AF) y dos aminas heterocíclicas (DiMeIQx y MeIQx); mientras que la mutagenicidad de dos hidrocarburos aromáticos policíclicos (BaP y DMBA) fue evaluada en la cepa de *S. typhimurium* TA100. Se realizaron una serie de experimentos preliminares donde se evaluaron amplios intervalos de concentraciones de los promutágenos con el propósito de establecer los intervalos de concentración donde la mutagenicidad observada tiene una respuesta lineal sin ser tóxicos para la bacteria.

Para las aminas aromáticas evaluadas, únicamente con 2AA se observa una mayor mutagenicidad en presencia de CYP1A1 humano, mientras que con 2AF no hay una diferencia marcada entre especies en la activación del mutágeno (Figura 5).

Figura 5. Mutagenicidad inducida en la cepa de *S. typhimurium* YG1024 por acción de las aminas aromáticas en presencia de 2 pmol de CYP1A1. Las barras representan el promedio de tres experimentos independientes con su desviación estándar. La línea punteada indica el valor de la reversión espontánea. Donde RE: Reversión espontánea, 2AA: 2-aminoantraceno, 2AF: 2-aminofluoreno, hCYP1A1: CYP1A1 recombinante humano, rCYP1A1: CYP1A1 recombinante de rata.

Por el contrario, el DiMeIQx y el MeIQx fueron más eficientemente activados por el CYP1A1 de rata en comparación con la enzima humana (Figura 6). Adicional a esto, se determinó que concentraciones mayores a 4.7 nmol/caja de MeIQx en presencia de CYP1A1 de rata son tóxicas para la bacteria pues hubo disminución en el número de colonias revertantes (Figura 6) y alteraciones en el crecimiento de fondo (datos no mostrados).

Figura 6. Mutagenicidad inducida en la cepa de *S. typhimurium* YG1024 por acción de las aminas heterocíclicas en presencia de 2 pmol de CYP1A1. Las barras representan el promedio de tres experimentos independientes con su desviación estándar. La línea punteada indica el valor de la reversión espontánea. Donde RE: Reversión espontánea, DiMeIQx: 2-amino-3,4,8-trimetilimidazo [*4,5-f*] quinoxalina, MeIQx: 2-amino-3,8-dimetilimidazo [*4,5-f*] quinoxalina, hCYP1A1: CYP1A1 recombinante humano, rCYP1A1: CYP1A1 recombinante de rata.

En cuanto a la mutagenicidad inducida por BaP y DMBA, cuando se emplearon 4 pmol de CYP1A1 en el ensayo de Ames, no se observó un efecto dependiente de la concentración para la activación de ambas moléculas (Figura 7). Dado lo anterior, se decidió incrementar la concentración de enzima a 8 pmol para determinar las diferencias en mutagenicidad a una concentración fija de mutágeno (40 nmol para BaP y 4 nmol para DMBA). En estas condiciones experimentales, la enzima humana es más eficiente en la activación de los HAP (Figura 8).

B a P **D M B A**

Figura 7. Mutagenicidad inducida en la cepa de *S. typhimurium* TA100 por acción de los hidrocarburos aromáticos policíclicos en presencia de 4 pmol de CYP1A1. Las barras representan el promedio de tres experimentos independientes con su desviación estándar. La línea punteada indica el valor de la reversión espontánea. Donde RE: Reversión espontánea, BaP: benzo[*a*]pireno, DMBA: 7,12-dimetilbenz[*a*]antraceno, hCYP1A1: CYP1A1 recombinante humano, rCYP1A1: CYP1A1 recombinante de rata.

B a P **D M B A**

Figura 8. Mutagenicidad inducida en la cepa de *S. typhimurium* TA100 por acción de los hidrocarburos aromáticos policíclicos en presencia de 8 pmol de CYP1A1. Las barras representan el promedio de tres experimentos independientes con su desviación estándar. La línea punteada indica el valor de la reversión espontánea. Donde RE: Reversión espontánea, BaP: benzo[*a*]pireno, DMBA: 7,12-dimetilbenz[*a*]antraceno, hCYP1A1: CYP1A1 recombinante humano, rCYP1A1: CYP1A1 recombinante de rata.

Una vez que se establecieron los intervalos de concentración de mutágeno donde se obtienen curvas concentración-efecto (Figuras 5 y 6), se realizó el cálculo de la potencia mutagénica, la cual corresponde al valor de la pendiente resultante del análisis por regresión lineal del número de colonias revertantes en función de la concentración de mutágeno. Comparando estos datos se estimaron cuantitativamente las diferencias entre las dos enzimas,

CYP1A1 de humano o de rata, para la activación de los promutágenos seleccionados. Para el 2AA, hay 3 veces más mutagenicidad en presencia de la enzima humana en comparación con el CYP1A1 de rata. Sin embargo, la mutagenicidad inducida por 2AF es la misma con los dos citocromos. A su vez, se determinó que el DiMeIQx es 75 veces más mutagénico en presencia de CYP1A1 de rata, mientras que para el MeIQx la diferencia con respecto a la enzima humana es de 5 veces. Finalmente, con 8 pmol de CYP1A1, la enzima humana es 5 a 7 veces más eficiente en la activación de los HAP (Cuadro 1).

Cuadro 1. Potencia mutagénica de promutágenos activados por 2 pmol de CYP1A1.

Grupo	Mutágeno	Potencia mutagénica	
		CYP1A1 humano	CYP1A1 de rata
Aminas aromáticas[a]	**2AA**	4.4 ± 0.2 revertantes/pmol	1.4 ± 0.1 revertantes/pmol
	2AF	66 ± 1 revertantes/nmol	65 ± 7 revertantes/nmol
Aminas heterocíclicas[a]	**DiMeIQx**	0.09 ± 0.01 revertantes/pmol	6.6 ± 0.4 revertantes/pmol
	MeIQx	25 ± 4 revertantes/nmol	133 ± 8 revertantes/nmol
Hidrocarburos aromáticos policíclicos[b]	**BaP**	547 ± 65 revertantes/40 nmol	114 ± 6 revertantes/40 nmol
	DMBA	873 ± 44 revertantes/4 nmol	129 ± 18 revertantes/4 nmol

a. Mutagenicidad inducida en *S. typhimurium* YG1024.

b. Mutagenicidad inducida en *S. typhimurium* TA100, se emplearon 8 pmol de CYP1A1 en el ensayo.

6.2. Antimutagenicidad de los inhibidores de CYP1A1

Para evaluar las diferencias entre especies en la inhibición de CYP1A1 humano y de rata, se determinó la potencia antimutagénica de dos componentes del jugo de toronja (naringenina y bergamotina) sobre el daño inducido por el 2AA y el DiMeIQx en *S. typhimurium* YG1024.

Previamente se estableció que 517 pmol por caja de 2AA son suficientes para tener el mismo número de colonias revertantes con ambos CYP1A1 y no son tóxicos para la bacteria, por

lo que se empleó esta cantidad de mutágeno en ensayos posteriores. En cambio, con el DiMeIQx se usaron concentraciones diferentes de compuesto para inducir la misma mutagenicidad (3.3 nmol/caja de DiMeIQx en presencia de CYP1A1 humano y 110 pmol/caja de DiMeIQx con CYP1A1 de rata). Así, independientemente del mutágeno usado, se determinó que la enzima de rata es notoriamente más sensible al efecto de la naringenina en comparación con el CYP1A1 humano (Figura 9).

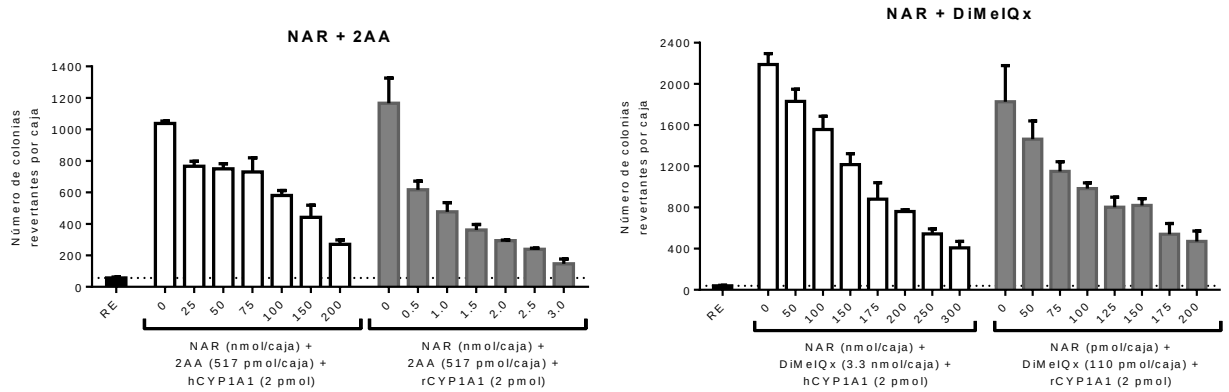


Figura 9. Efecto antimutagénico de la naringenina sobre el daño inducido por 2-aminoantraceno y DiMeIQx en la cepa de *S. typhimurium* YG1024. Se emplearon 2 pmol de CYP1A1 como activador metabólico. Las barras representan el promedio de tres experimentos independientes con su desviación estándar. La línea punteada indica el valor de la reversión espontánea. Donde RE: Reversión espontánea, NAR: naringenina, 2AA: 2-aminoantraceno, DiMeIQx: 2-amino-3,4,8-trimetilimidazo [*4,5-f*] quinoxalina, hCYP1A1: CYP1A1 recombinante humano, rCYP1A1: CYP1A1 recombinante de rata.

De igual manera a lo que sucede con la naringenina, la bergamotina posee mayor efecto antimutagénico en presencia de CYP1A1 de rata (Figura 10).

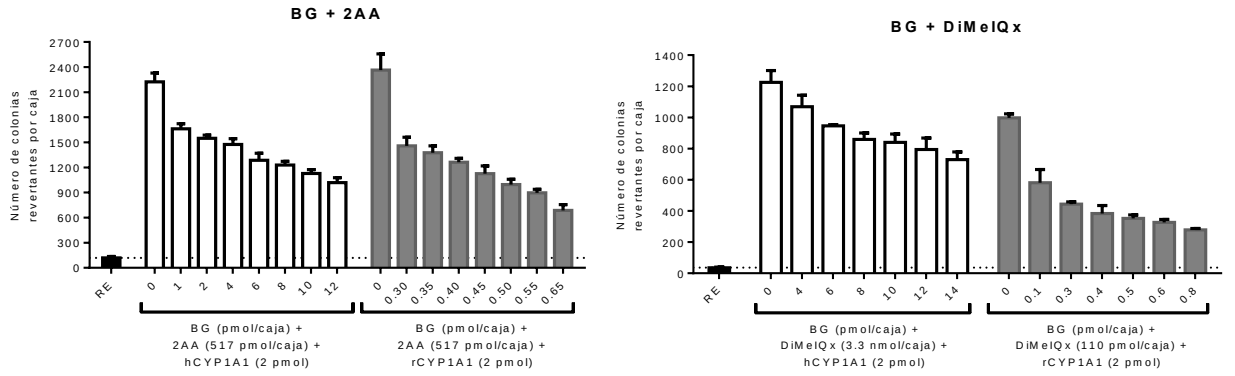


Figura 10. Efecto antimutagénico de la bergamotina sobre el daño inducido por 2-aminoantraceno y DiMeIQx en la cepa de *S. typhimurium* YG1024. Se emplearon 2 pmol de CYP1A1 como activador metabólico. Las barras representan el promedio de tres experimentos independientes con su desviación estándar. La línea punteada indica el valor de la reversión espontánea. Donde RE: Reversión espontánea, BG: bergamotina, 2AA: 2-aminoantraceno, DiMeIQx: 2-amino-3,4,8-trimetilimidazo [*4,5-f*] quinoxalina, hCYP1A1: CYP1A1 recombinante humano, rCYP1A1: CYP1A1 recombinante de rata.

En resumen, la naringenina es de 60 hasta casi 1000 veces más antimutagénica cuando interactúa con CYP1A1 de rata a diferencia de cuando interactúa con CYP1A1 humano. Mientras que con la bergamotina se disminuye de 13 a 40 veces más la activación de los mutágenos en presencia de la enzima de rata en comparación con la enzima humana (Cuadro 2).

De los dos compuestos analizados, la bergamotina es más antimutagénica que la naringenina, requiriéndose menor cantidad de compuesto para disminuir notablemente la mutagenicidad del 2AA y el DiMeIQx (Cuadro 2).

Cuadro 2. Potencia antimutagénica de inhibidores de CYP1A1.

Inhibidor	Mutágeno	Potencia antimutagénica	
		CYP1A1 humano	CYP1A1 de rata
NAR	2AA	3 ± 0.3 revertantes/nmol	179 ± 12 revertantes/nmol
	DiMeIQx	6.1 ± 0.4 revertantes/nmol	6.2 ± 0.5 revertantes/pmol
BG	2AA	57 ± 3 revertantes/pmol	2278 ± 128 revertantes/pmol
	DiMeIQx	31 ± 4 revertantes/pmol	423 ± 50 revertantes/pmol

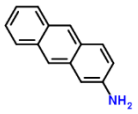
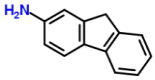
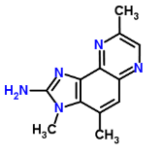
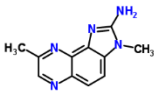
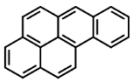
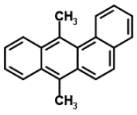
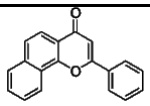
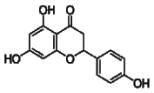
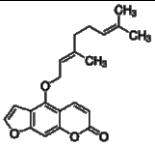
Mutagenicidad inducida en *S. typhimurium* YG1024, se emplearon 2 pmol de CYP1A1 en el ensayo.

6.3. Ensayos de acoplamiento molecular

A partir de un análisis *in silico* se determinó si existen diferencias en la interacción de los ligandos con ambas enzimas mediante la comparación de los parámetros de energía de unión y la determinación de los residuos con los que hay interacción (Cuadro 3). Los ensayos computacionales sugieren que el sitio catalítico del CYP1A1 humano podría tener una mayor afinidad por las aminas aromáticas, los HAP y ANF al contar con un mayor número de interacciones y distancias más cortas hacia el grupo hemo; mientras que CYP1A1 de rata sería más afín por las aminas heterocíclicas, NAR y BG considerando los parámetros ya mencionados (Figura 11).

Además, en ambas enzimas parece mantenerse la naturaleza hidrofóbica de las interacciones, ya que la mayoría de los residuos involucrados en éstas son fenilalaninas (F123, F224 y F258 en humano; F228 y F316 en rata).

Cuadro 3. Interacciones de mutágenos e inhibidores con CYP1A1.

Grupo	Ligando	CYP1A1 de humano		CYP1A1 de rata	
		Energía de unión (kcal/mol)	Aminoácidos que participan en la interacción[a]	Energía de unión (kcal/mol)	Aminoácidos que participan en la interacción[a]
Aminas aromáticas	 2AA	-11.5	F224, F258, G316, F319, D320	-11.4	F228, F262, F316, G320, F323, D324
	 2AF	-10.7	F224, G316, A317, F319	-10.7	F127, F228, F262, F316, G320, A321, F323
Aminas heterocíclicas	 DiMeIQx	-10.4	S116, F224, L312, D313, G316, T321	-9.5	F127, F228, G320, A321, F323, D324, T325, I390, HEM
	 MeIQx	-9.3	F123, F224, G316, A317	-9.1	T126, F127, F228, F316, G320, A321, HEM
Hidrocarburos aromáticos policíclicos	 BaP	-15.3	F123, F224, F258, G316, A317, D320, T321	-15.0	T126, F127, F228, F316, G320, A321, F323
	 DMBA	-15.1	F123, F224, D313, G316, A317, F319, D320	-15.1	F127, F228, F316, G320, A321, F323, D324
Inhibidores	 ANF	-14.5	F123, F224, F258, L312, G316, D320, T321	-13.0	T87, F189, F264, F277, D278, G281, A282, D285, I351, T462, HEM
	 NAR	-11.1	S122, F123, F224, L254, F258, G316, A317, I386, HEM	-10.0	T87, F88, F189, D278, G281, A282, V347, I351, L461, T462, HEM
	 BG	-11.4	F123, F224, N255, F258, L312, G316	-11.8	F189, D278, G281, A282, F284, L461, HEM

a. Los residuos de la proteína que interactúan con el ligando fueron determinados con el programa AutoDock Tools [82]. Están subrayados aquellos aminoácidos que presentan interacciones π-π con el ligando empleando el programa Maestro (Schrödinger, LLC, Nueva York, NY).

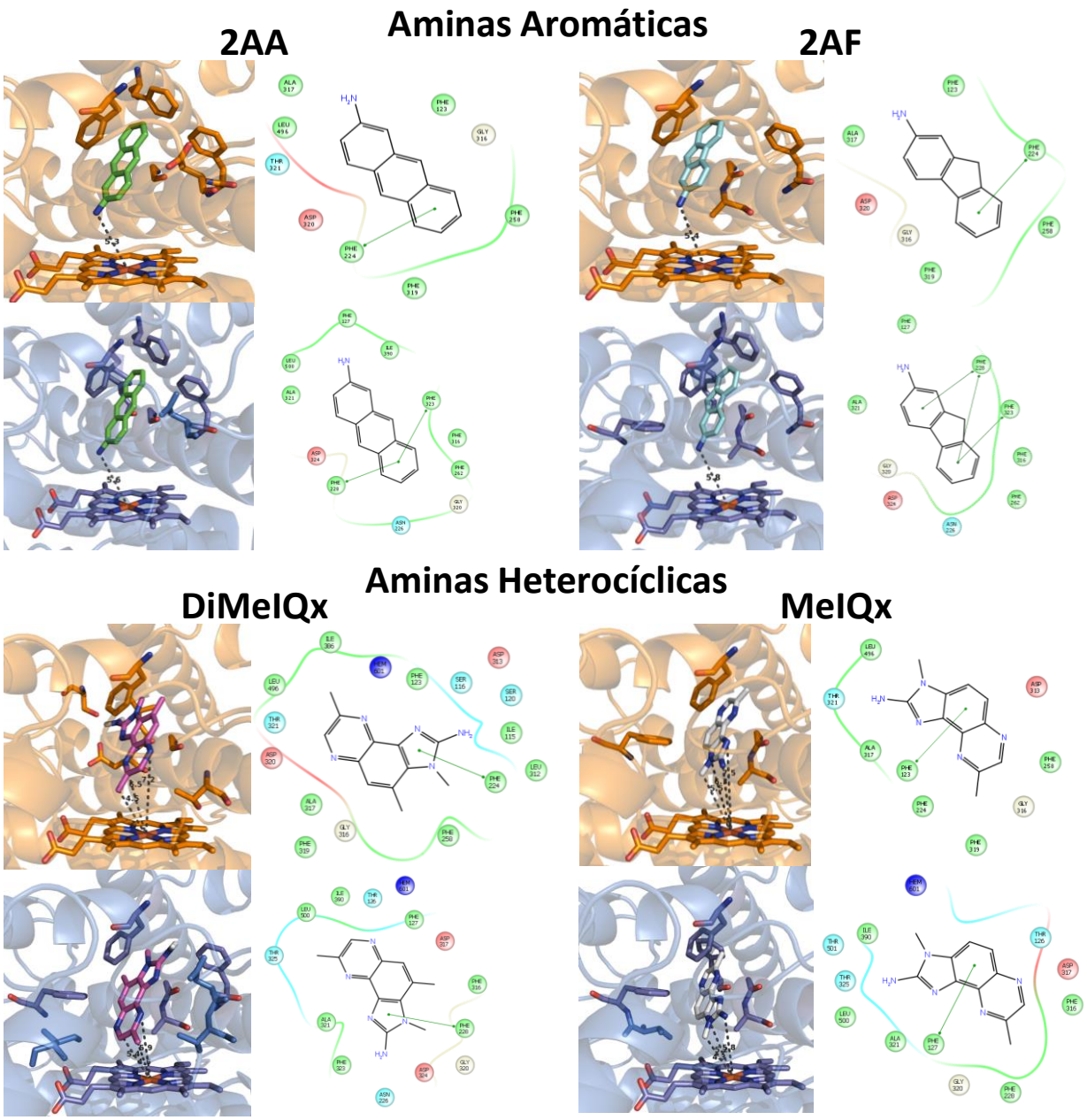


Figura 11. Conformaciones tridimensionales y esquemas simplificados de la interacción de los ligandos con CYP1A1. Se representa CYP1A1 humano en color naranja y CYP1A1 de rata en color azul. Las líneas negras discontinuas muestran la distancia (Å) del átomo correspondiente del ligando hacia el grupo hemo de la proteína medidas con PyMOL. En líneas verdes se indican los apilamientos π-π determinados en el acoplamiento molecular con el programa Maestro (Schrödinger, LLC, Nueva York, NY).

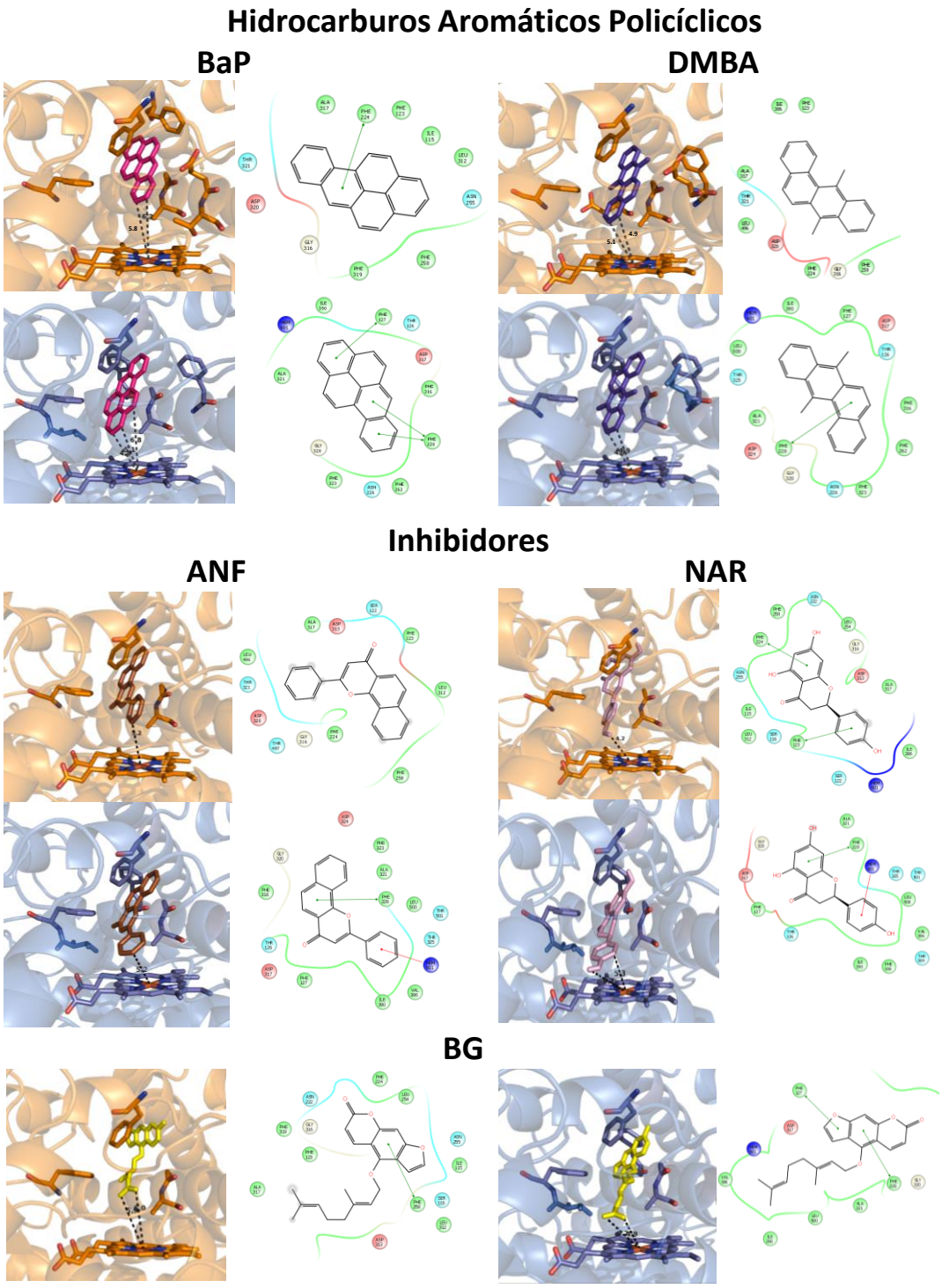


Figura 11 (Continuación). Conformaciones tridimensionales y esquemas simplificados de la interacción de los ligandos con CYP1A1. Se representa CYP1A1 humano en color naranja y CYP1A1 de rata en color azul. Las líneas negras discontinuas muestran la distancia (Å) del átomo correspondiente del ligando hacia el grupo hemo de la proteína medidas con PyMOL. En líneas verdes se indican los apilamientos π-π determinados en el acoplamiento molecular con el programa Maestro (Schrödinger, LLC, Nueva York, NY).

7. DISCUSIÓN

Durante el proceso de bioactivación, los compuestos precarcinogénicos son biotransformados en metabolitos altamente reactivos, donde la magnitud del daño está relacionada con la actividad de las enzimas responsables de la activación y la concentración de los metabolitos producidos. A su vez, los mecanismos de regulación y expresión de estas enzimas difiere entre especies, haciendo más susceptibles a algunos organismos con respecto a otros al efecto del mismo compuesto y dificultando la extrapolación de los resultados obtenidos en modelos animales para su uso en seres humanos [86–90]. Particularmente, CYP1A1 humano y de rata tienen un porcentaje de identidad en su secuencia de aminoácidos del 79% [48]; sin embargo, se demostró en este trabajo que las diferencias tienen un efecto notable en la interacción de ambas enzimas con los compuestos evaluados, reflejándose en potencias mutagénicas y antimutagénicas distintas entre especies.

Con respecto al 2AA, la mutagenicidad fue mayor en presencia del CYP1A1 humano. Esta misma tendencia ha sido observada utilizando una mezcla más compleja (S9 humano) como activador metabólico [70]. Para que se produzca una reacción de óxido-reducción específica del sustrato, el ligando también requiere estar orientado con su grupo reactivo hacia el grupo hemo [91]. Dicha conformación fue sugerida por el ensayo de acoplamiento molecular, donde el grupo amino del 2AA y 2AF se encuentra orientado hacia el hemo de CYP1A1 humano y de rata (Figura 11). Sin embargo, el análisis *in silico* no evidencia grandes diferencias interespecies en la interacción de estas proteínas con las aminas aromáticas, pues se determinaron energías de unión similares (Cuadro 3).

Reportes previos muestran que MeIQx es débilmente activado por cepas de *E. coli lac Z* que expresan CYP1A1 humano y NADPH-citocromo P450 reductasa [27]. Contrario a esos hallazgos, en este trabajo se evidenció que concentraciones menores a 4.7 nmol/caja del mismo compuesto son altamente mutagénicas en *S. typhimurium* YG1024 cuando se metabolizan por CYP1A1 de humano y de rata. A pesar de que los autores también utilizan a una bacteria como organismo modelo para la evaluación de la mutagenicidad, la sobreexpresión de la enzima *O*-

acetiltransferasa en *S. typhimurium* YG1024 permite una mejor detección de la mutagenicidad inducida por las aminas heterocíclicas en esta cepa en comparación con *E. coli* [76].

A diferencia de la bioactivación de las aminas aromáticas mediada por CYP1A2, en presencia de CYP1A1, la enzima de rata es más eficiente en la activación de las aminas heterocíclicas en comparación con la enzima humana [92]. De igual forma, con los ensayos computacionales se determinó una menor distancia entre las aminas heterocíclicas y el grupo hemo, lo cual correlaciona con los hallazgos *in vitro* y otros reportes de interacciones CYP-ligando [4,93–95]. Aún falta realizar la caracterización y cuantificación de los metabolitos reactivos para determinar si son los mismos que se producen en ambos casos o si existen algunos que sean específicos de la especie.

Con los HAP se obtuvo una mayor mutagenicidad en presencia de la enzima humana, lo cual también ha sido demostrado al comparar la afinidad de microsomas hepáticos humanos y de rata por BaP [72]. A su vez, la orientación tridimensional de BaP y DMBA con CYP1A1 son consistentes con el sitio preferido de metabolismo mediante la vía de formación de diol epóxidos [34]; mientras que con CYP1A1 de rata, los HAP están en una conformación no productiva, donde las moléculas se encuentran giradas 180^{o} de la posición preferente para el metabolismo (Figura 11).

Los patrones de oxidación de BaP con el mismo sistema de expresión de los CYP1A1 recombinantes de humano y de rata empleado en este trabajo (células de insecto infectadas con baculovirus, comercialmente denominadas Supersomes®) han mostrado la formación de metabolitos que son especie-específicos, además de la contribución de la actividad de otras enzimas como NADPH:óxido-reductasa y citocromo b_5 [35,36,96], por eso sería importante esclarecer si las diferencias entre especies en la mutagenicidad observada en la prueba de Ames están relacionadas con el aumento de los metabolitos mutagénicos o con los productos de eliminación.

El DMBA es uno de los HAP más ampliamente utilizados en modelos de tumorigénesis; sin embargo, existen pocos estudios enfocados en las diferencias entre especies para su bioactivación, la cual ha sido similar en hepatocitos humanos y de rata [97,98]. En presencia de CYP1 y epóxido hidrolasa, el metabolito carcinogénico formado es el 3,4-dihidrodiol-1,2-epóxido [99]. En ausencia de epóxido hidrolasa, hay formación de metabolitos fenoles que contribuyen a

la formación de tumores en modelos animales [100]. De acuerdo con estos reportes, es probable que otras vías de activación hayan participado en el metabolismo de DMBA en presencia de Supersomes®, los cuales carecen de epóxido hidrolasa, con la posibilidad de que los intermediarios fenoles de DMBA pudieran haber sido sustratos de CYP1A1 dando como resultado metabolitos mutagénicos.

Para los inhibidores como la ANF, las interacciones con el sitio activo de CYP1A1 humano (Cuadro 3) fueron consistentes con hallazgos previos donde se documenta que el apilamiento π-π entre los anillos aromáticos del compuesto y F224 genera conformaciones más estables [4]. El favorecimiento de este tipo de interacciones π-π entre las fenilalaninas del CYP1A1 y el ligando debe ser abordado para el diseño de inhibidores potentes de la enzima como agentes protectores contra el daño generado por exposición a contaminantes ambientales.

Previamente se reportaron las diferencias entre especies en la inhibición de CYP1A1 por NAR, siendo el CYP1A1 de rata casi 3000 veces más sensible al efecto del inhibidor en comparación con la enzima humana [48]. Con los ensayos de antimutagenicidad se observó la misma preferencia de inhibición de la enzima de rata, de 60 a 1000 veces mayor potencia antimutagénica que en presencia de CYP1A1 humano. Sin embargo, los resultados de acoplamiento molecular sugieren mayor interacción con la enzima humana. Al respecto, hay que puntualizar que el análisis computacional tiene ciertas limitaciones ya que está basado en modelos matemáticos que tratan de explicar los factores que tienen influencia en la interacción de la proteína con el ligando. A modo de ejemplo, las interacciones entre NAR y CYP1A1 de rata obtenidas en el acoplamiento molecular (Cuadro 3) sugieren que la NAR es un inhibidor competitivo de la enzima al unirse a ciertos residuos que forman parte del sitio catalítico, pero experimentalmente se demostró que en realidad se trata de un inhibidor de tipo mixto [48], siendo ésta una condición importante que no es considerada en el análisis computacional.

La reciente determinación de la estructura tridimensional del CYP1A1 humano en complejo con la BG por cristalografía de rayos X ha permitido validar los hallazgos *in silico,* donde se sugiere que el anillo psoraleno está orientado de forma distal al grupo hemo [101] (Figura 11). Además, la evidencia de formación de cuatro metabolitos de BG por CYP1A1 humano debe ser considerada para su identificación en la catálisis mediada por CYP1A1 de rata [101]. La notable actividad

antimutagénica de la BG demostrada en este trabajo (Cuadro 2), la convierte en una molécula con alto potencial para su uso como agente quimiopreventivo.

Por otro lado, las diferencias estructurales entre especies no sólo son importantes para la unión del sustrato, sino que también deben considerarse las variaciones individuales en la estructura del CYP1A1. Si bien éstas no fueron evaluadas en este trabajo, se sabe que existen correlaciones entre los polimorfismos de un solo nucleótido en el CYP1A1 humano y la susceptibilidad al cáncer [102–105], pero pocos estudios han examinado los efectos de éstos sobre la actividad enzimática. Por ejemplo, las variantes alélicas en residuos que no pertenecen al sitio catalítico de CYP1A1 humano, como CYP1A1.2 (Ile462Val), CYP1A1.4 (Thr461Asn) y CYP1A1*2C (rs1048943) mostraron diferencias considerables en el perfil de metabolitos y eficiencias catalíticas del metabolismo del estrógeno en comparación con la enzima *wild type* [106,107], así que valdría la pena evaluar la frecuencia de estas variantes en poblaciones expuestas a contaminantes ambientales como los HAP y las aminas heterocíclicas.

Finalmente, este trabajo destaca la importancia de considerar las diferencias interespecies en la interacción de la enzima con el ligando al momento de extrapolar los resultados de modelos animales para su uso en asesoramiento de riesgo en seres humanos. También proporciona evidencia para proponer a la naringenina y la bergamotina como agentes protectores contra el daño asociado a la bioactivación de procarcinógenos mediada por CYP1A1.

8. CONCLUSIONES

- El BaP, DMBA y 2AA se activaron con mayor eficacia por el CYP1A1 humano a compuestos mutagénicos, mientras que las aminas heterocíclicas se activaron principalmente por el CYP1A1 de rata. No hubo diferencias en la activación de 2AF por CYP1A1 humano y de rata.
- En el caso de los inhibidores NAR y BG, hubo mayor antimutagenicidad de los componentes del jugo de toronja en presencia del CYP1A1 de rata.
- Los resultados *in silico* de interacción de los HAP, ANF y BG con CYP1A1 correlacionaron con los hallazgos experimentales.

- Con el ensayo de acoplamiento molecular, se evidenció el apilamiento π-π como la principal forma de interacción del ligando con la proteína.

9. PERSPECTIVAS

- Cuantificar las diferencias en la concentración de metabolitos mutagénicos generados por la bioactivación mediada por CYP1A1 humano y de rata.
- Determinar si existen diferencias en el perfil de metabolitos generados por la inhibición de CYP1A1 con naringenina y bergamotina.
- Explorar la influencia de las enzimas NADPH:óxido-reductasa y citocromo b_5 en las diferencias entre especies del metabolismo mediado por CYP.
- Determinar el efecto de las diferencias entre especies en diversos niveles de regulación, incluida la actividad enzimática, los mecanismos de daño y reparación, la farmacocinética, el perfil de metabolitos, la concentración de aductos y los espectros mutacionales, así como la contribución de las enzimas de fase II del metabolismo.

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ANEXOS

La mayoría de los resultados de esta tesis doctoral forman parte de la siguiente publicación:

- **Santes-Palacios R**, Camacho-Carranza R, Espinosa-Aguirre J J. (2018). Bacterial mutagenicity of selected procarcinogens in the presence of recombinant human or rat cytochrome P4501A1. Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 835: 25-31. doi: 10.1016/j.mrgentox.2018.09.001.

Además, durante mis estudios de doctorado, participé en la publicación de 3 artículos.

- **Santes-Palacios R**, Ornelas-Ayala D, Cabañas N, Marroquín-Pérez A, Hernández-Magaña A, Olguín-Reyes S, Camacho-Carranza R, Espinosa-Aguirre JJ. (2016). Regulation of human cytochrome P4501A1 (hCYP1A1): A plausible target for chemoprevention? BioMed Research International, Vol. 2016, Article ID 5341081, 17 pp. doi:10.1155/2016/5341081.

- **Santes-Palacios R**, Romo-Mancillas A, Camacho-Carranza R, Espinosa-Aguirre J J. (2016). Inhibition of human and rat CYP1A1 enzyme by grapefruit juice compounds. Toxicol. Lett. 258: 267-275. doi:10.1016/j.toxlet.2016.07.023.

- Rodeiro I, Olguín S, **Santes R**, Herrera J A, Pérez C L, Mangas R, Hernández Y, Fernández G, Hernández I, Hernández-Ojeda S, Camacho-Carranza R, Valencia-Olvera A, Espinosa-Aguirre J J. Gas chromatography-mass spectrometry analysis of ulva fasciata (Green Seaweed) extract and evaluation of its cytoprotective and antigenotoxic effects. (2015). Evidence-Based Complementary and Alternative Medicine, Vol. 2015, Article ID 520598, 11 pp. doi:10.1155/2015/520598.

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Bacterial mutagenicity of selected procarcinogens in the presence of recombinant human or rat cytochrome P4501A1

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ABSTRACT

Cytochrome P4501A1 (CYP1A1) is an important enzyme of procarcinogen activation. We have studied bacterial (Ames test) mutagenicity resulting from mutagen activation by recombinant human or rat CYP1A1. Mutagenicity depends on both the chemical group and species-specific activation: polycyclic aromatic hydrocarbons showed higher (5-7-fold) mutagenic activity when activated by the human enzyme, whereas heterocyclic amines were more mutagenic (5-75-fold) in the presence of the rat enzyme. With regard to the two aromatic amines tested, only 2-aminoanthracene showed a clear species preference, activated 3-fold more effectively by human than by rat CYP1A1. We also analyzed in silico the binding of these compounds to the human and rat enzyme catalytic sites, identifying residues expected to participate in ligand recognition. A phenylalanine residue was involved in CYP-mutagen stabilization through π-π stacking. Variations in the three-dimensional conformations and distances to the heme groups may contribute to differences between human and rat CYP-substrate interactions. In conclusion, CYP1A1 shows significant differences between species, in terms of mutagen activation, which should be considered in the context of human risk assessment.

1. Introduction

Cytochrome P4501A1 (CYP1A1) is expressed in extrahepatic tissues and is one of the most important enzymes involved in procarcinogen activation [1]. In this process, substrates - mainly planar and hydrophobic, such as polycyclic aromatic hydrocarbons (PAH), aromatic amines (AA), and heterocyclic amines (HA) - are converted into highly reactive metabolites [2] which can react with DNA, lipids, or proteins, resulting in carcinogenesis initiation [3].

The identification and development of CYP1A1 modulating substances is relevant to the study of carcinogenesis [4–6]. Because of limitations on the use of human materials, several alternative experimental models, such as liver S9 fraction or microsomes, recombinant enzymes, and cell lines, are used as *in vitro* systems for metabolism studies. *In vivo* models of tumorigenesis and knockout models (fish, rats, mice) have been developed [7–9]. Recently, new strategies have been proposed for toxicological assessment, including the establishment of dual human-rodent activation models, "humanized" animals for the prediction of pharmacokinetic profiles, mathematical models of toxicokinetics, molecular dynamic assays, and other computational methods [10–15]. However, it is often difficult to extrapolate data from animal models to human metabolism and health. For example, CYP inducers are typically used to study xenobiotic activation in animal models, resulting in enzyme activity levels higher than those in most human samples. Humans are exposed to multiple lifestyle-related inducers, which are difficult to monitor [16]. Gene regulation also differs among species. The promoter of the CYP1A1 gene is well-conserved in mammals, but differs in fish and amphibians [17]. When cultures of human, rat, and quail cells were treated with the widely used P450 inducer aroclor 1524, enzymatic activity of rat CYP1A1 was induced 55-fold, whereas only 3-fold induction was observed with the human and quail cells [18]. Human liver microsomes showed the lowest

Abbreviations: CYP1A1, cytochrome P4501A1; BaP, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; 2AA, 2-aminoanthracene; 2AF, 2-aminofluorene; DiMeIQx, 2-amino-3,4,8-dimethylimidazo[4,5-*f*]quinoxaline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PAH, polycyclic aromatic hydrocarbon; AA, aromatic amine; HA, heterocyclic amine

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ethoxyresorufin O-deethylase activity, when compared to rat, monkey, mouse, and hamster liver microsomes [19].

Human enzymes may interact differently with substrates, compared to enzymes of other species. Structural studies of CYP1A1 have shown the importance of substrate-enzyme recognition for enzyme activity [5]. For example, substitution of human CYP1A1 residue Ala62 lowers the enzyme's affinity for ethoxyresorufin. The corresponding residue is valine in cows; serine in zebrafish, mice, and rats [20]. Amino acid residues in the distal regions of the CYP1A1 catalytic site play an important role in the metabolism of dioxins in rodents [21,22].

For risk assessment of human exposure to environmental and dietary toxicants, some international organizations have proposed the use of *in vitro* assays [23]. The bacterial reversion assay (Ames test) is one of the most widely used short-term genotoxicity assays, with results that correlate well with animal carcinogenicity tests [24]. The use of specific bacterial strains can provide insight into the molecular mechanisms of mutagenesis [24–26].

Here, we have evaluated two AA, two HA, and two PAH as CYP1A1 substrates, noting that different activation pathways lead to the ultimate carcinogens that form DNA adducts [27–32]. We focused on determining how interspecies differences in activation affect genotoxicity in the Ames test with metabolism by recombinant human or rat CYP1A1. Patterns of activation were related both to the chemical groups and the species. Additionally, using computational tools, we analyzed differences in substrate-enzyme orientation and recognition.

2. Materials and methods

2.1. Materials

Benzo[*a*]pyrene (BaP), 7,12-dimethylbenz[*a*]anthracene (DMBA), 2-aminoanthracene (2AA), 2-aminofluorene (2AF), and NADPH were purchased from Sigma-Aldrich (Darmstadt, Germany). Supplies for the preparation of bacterial culture media were purchased from Becton Dickinson (Estado de México, México), Sigma-Aldrich, and Oxoid (Lenexa, KS, USA). 2-Amino-3,4,8-dimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) was acquired from Santa Cruz Biotechnology, Dallas, TX, USA. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) was purchased from United States Biological (Salem, MA, USA). Recombinant human and rat CYP1A1 (Supersomes®) were acquired from Corning (Corning, NY, USA).

2.2. Bacterial mutagenicity assay

The Ames test with a preincubation period was employed, using recombinant human or rat CYP1A1 and *Salmonella typhimurium* strains YG1024 or TA100 [33–36]. These strains were kindly donated by Dr. Takehiko Nohmi (National Institute of Health Sciences, Tokyo, Japan) and Dr. Bruce Ames (University of California, Berkeley, CA, USA).

The incubation (final volume, 700 µL) consisted of overnight culture (100 µL), mutagen, dissolved in DMSO (5 µL), P450 enzyme (2 pmol) in buffer, 500 µL (50 mM Tris-HCl + 25 mM $MgCl_2$, pH 7.6), and NADPH (0.15 mM). Mutagen doses per plate were: 1–10 ng 2AA, 10–500 ng 2AF, 0.5–1000 ng DiMeIQx, 0.05–3 µg MeIQx, 10 µg BaP, and 1 µg DMBA.

After incubation, 30 min at 37 °C with shaking (150 rpm), top agar was added and the incubations were poured onto minimal medium plates [33,34]. Plates were incubated for 48 h at 37 °C and the numbers of revertant colonies were scored. Spontaneous revertant yield is the number of revertant colonies in the absence of mutagen. A dose of a compound is considered mutagenic when the number of revertant colonies is at least twice the spontaneous yield [37–39]. Plates were examined under the microscope to detect thinning of the background lawn which, in conjunction with a decreased number of revertant colonies, is a sign of toxicity induced by the test article [33].

When a dose-response effect was obtained, the mutagenic potency was calculated by linear regression of the linear portion of the dose-effect curve and was reported as the number of revertant colonies per ng or µg of mutagen [16,40,41]. Preliminary experiments were performed with DiMeIQx (250, 500, and 750 ng/plate) and MeIQx (0.75, 1.0 and 1.5 µg/plate) in strain YG1024 to determine the appropriate dose range to be tested in the presence of human or rat recombinant CYP1A1 (Supplementary Table S1). Based on those results, we chose doses between 25–1000 ng/plate and 0.5–25 ng/plate DiMeIQx when using human and rat CYP1A1, respectively. For MeIQx, we tested doses 0.25–3.0 µg/plate with human CYP1A1 and 0.05–1.25 µg/plate with rat CYP1A1. When PAH mutagenicity was tested, one dose (according to OCDE guidelines recommendations for bacterial mutagenicity test positive controls) was used, 10 µg/plate BaP and 1 µg/plate DMBA [42]. All experiments were performed in duplicate with three internal replicates and results are shown as mean values with standard deviations.

2.3. Computational studies

2.3.1. Proteins and ligands

The three-dimensional structure of human CYP1A1 was obtained from Protein Data Bank (PDBID: 4I8V) [43] and prepared for docking assay according to previous reports by removing the ligand (α-naphthoflavone), counter-ions, and crystallographic waters [44]. In the absence of a crystallographic structure of rat CYP1A1, a model was built through the homology modeling approach using the ModWeb server [45,46] and was validated with Procheck tools [47]. For ligands, the structures of 2AA, 2AF, DiMeIQx, MeIQx, BaP, and DMBA were downloaded from the ChemSpider database (Royal Society of Chemistry) and prepared with AutoDock tools [48].

2.3.2. Molecular docking

AutoDock 4 software was employed for molecular docking based on conditions described in previous work [44]. Protein-ligand interactions were determined with AutoDock tools and Maestro (Schrödinger, LLC, New York, NY). Figures of the resulting conformations were designed using PyMOL [49].

3. Results

3.1. Ames test

The bacterial mutagenicity assay was performed to evaluate differences in mutagen activation in the presence of recombinant human vs rat CYP1A1. For AA and HA, a dose-dependent effect was observed and the mutagenic potency was calculated (Table 1). When compared to that of the rat enzyme, a higher activation of 2AA (3-fold) was observed with human CYP1A1. In Table 1, similar values of mutagenic potency indicate that there was no enzyme preference in 2AF activation. In contrast, DiMeIQx and MeIQx were activated more efficiently by rat than by human CYP1A1 (75-fold and 5-fold, respectively) (Table 1).

Nonetheless, when a single dose of each PAH was evaluated in the presence of 8 pmol human CYP1A1, the enzyme more efficiently activated BaP (5-fold) and DMBA (7-fold) than did the same amount of rat CYP1A1 (Table 2).

3.2. Molecular docking

To probe differences in mutagen-enzyme interactions, a computational study was performed. The binding energies, the residues involved in the interactions, and the distances to the heme group are presented in Table 3.

In general, there were species differences in the interactions between enzymes and chemical groups, with human CYP1A1 showing greater affinity for AA and PAH, and the rat enzyme for HA (greater numbers of interactions and shorter distances to the heme group). Human CYP1A1 residues F224, G316, and A317 participate in at least four ligand interactions. For the rat enzyme, the participating residues were F127, F228, F316, G320, A321, and F323. The most favorable

Table 1
Mutagenicity of aromatic and heterocyclic amines in YG1024 *Salmonella typhimurium* strain. Human or rat CYP1A1, 2 pmol, was used. The number of YG1024 spontaneous revertant colonies was 36 ± 2. Toxicity was observed with doses > 1 μg MeIQx per plate in the presence of rat CYP1A1. MP: mutagenic potency.

Mutagen		Revertants		Mutagen		Revertants		Mutagen		Revertants	
		Human	Rat			Human	Rat			Human	Rat
2AA	0	31 ± 5	26 ± 5	DiMeIQx	0	32 ± 6	32 ± 6	MeIQx	0	26 ± 1	26 ± 1
(ng/plate)	1	86 ± 12	64 ± 11	(ng/plate)	0.5	–	40 ± 2	(μg/plate)	0.05	–	77 ± 4
	2	92 ± 2	70 ± 5		1.0	–	78 ± 13		0.10	–	140 ± 16
	4	156 ± 16	89 ± 5		2.5	–	114 ± 24		0.25	139 ± 10	214 ± 16
	6	186 ± 12	108 ± 9		5.0	–	180 ± 2		0.50	202 ± 22	400 ± 64
	8	241 ± 27	119 ± 6		7.5	–	234 ± 34		0.75	–	516 ± 95
	10	287 ± 23	126 ± 8		10	–	342 ± 54		1.00	253 ± 11	408 ± 67
	MP	22.9 ± 1.2	7.3 ± 0.6		15	–	409 ± 29		1.25	–	341 ± 41
2AF	0	36 ± 2	36 ± 2		25	109 ± 10	444 ± 39		1.50	297 ± 17	–
(ng/plate)	10	39 ± 1	39 ± 5		50	158 ± 8	–		2.00	298 ± 22	–
	100	73 ± 15	88 ± 4		100	222 ± 33	–		2.50	312 ± 12	–
	250	122 ± 4	151 ± 11		200	251 ± 26	–		3.00	313 ± 31	–
	500	218 ± 20	219 ± 8		400	310 ± 26	–		MP	119 ± 19	623 ± 36
	MP	0.36 ± 0.008	0.36 ± 0.039		600	313 ± 11	–				
					800	326 ± 23	–				
					1000	312 ± 51	–				
					MP	0.39 ± 0.060	29.2 ± 1.9				

Table 2
Mutagenicity of polycyclic aromatic hydrocarbons in strain TA100. Human or rat CYP1A1, 8 pmol, was used.

Mutagen (μg/plate)		Revertants	
		Human	Rat
BaP	0	89 ± 10	89 ± 10
	10	547 ± 65	114 ± 6
DMBA	0	89 ± 10	89 ± 10
	1	873 ± 44	129 ± 18

conformations are shown in Fig. 1. The ligand orientation for PAH only was found to differ between human and rat CYP1A1.

4. Discussion

Species differ in sensitivity to carcinogens, and the molecular mechanisms of regulation and expression of P450 enzymes may explain some of these differences [17,18,50]. Procarcinogens are transformed into reactive metabolites and the amount of damage is related to the activities of activating enzymes. Here, we determined rat vs human

Table 3
Interactions of substrates with CYP1A1.

Chemical group	Ligand	Human CYP1A1		Rat CYP1A1	
		Binding energy (kcal/mol)	Distance to heme group (Å)/ interactions	Binding energy (kcal/mol)	Distance to heme group (Å)/ interactions
Aromatic amines	 2AA	−11.5	5.3 F224, F258, G316, F319, D320	−11.4	5.6 F228, F262, F316, G320, F323, D324
	 2AF	−10.7	5.4 F224, G316, A317, F319	−10.7	5.8 F127, F228, F262, F316, G320, A321, F323
Heterocyclic amines	 DiMeIQx	−10.4	4.5/6.5/7.2 S116, F224, L312, D313, G316, T321	−9.5	4.6/5.4/5.9 F127, F228, G320, A321, F323, D324, T325, I390, HEM
	 MeIQx	−9.3	5.5/5.8/7.5 F123, F224, G316, A317	−9.1	4.3/5.2/5.8 T126, F127, F228, F316, G320, A321, HEM
Polycyclic aromatic hydrocarbons	 BaP	−15.3	5.8/6.2 F123, F224, F258, G316, A317, D320, T321	−15.0	4.8/5.1/6.8 T126, F127, F228, F316, G320, A321, F323
	 DMBA	−15.1	4.9/5.1 F123, F224, D313, G316, A317, F319, D320	−15.1	4.7/4.9 F127, F228, F316, G320, A321, F323, D324

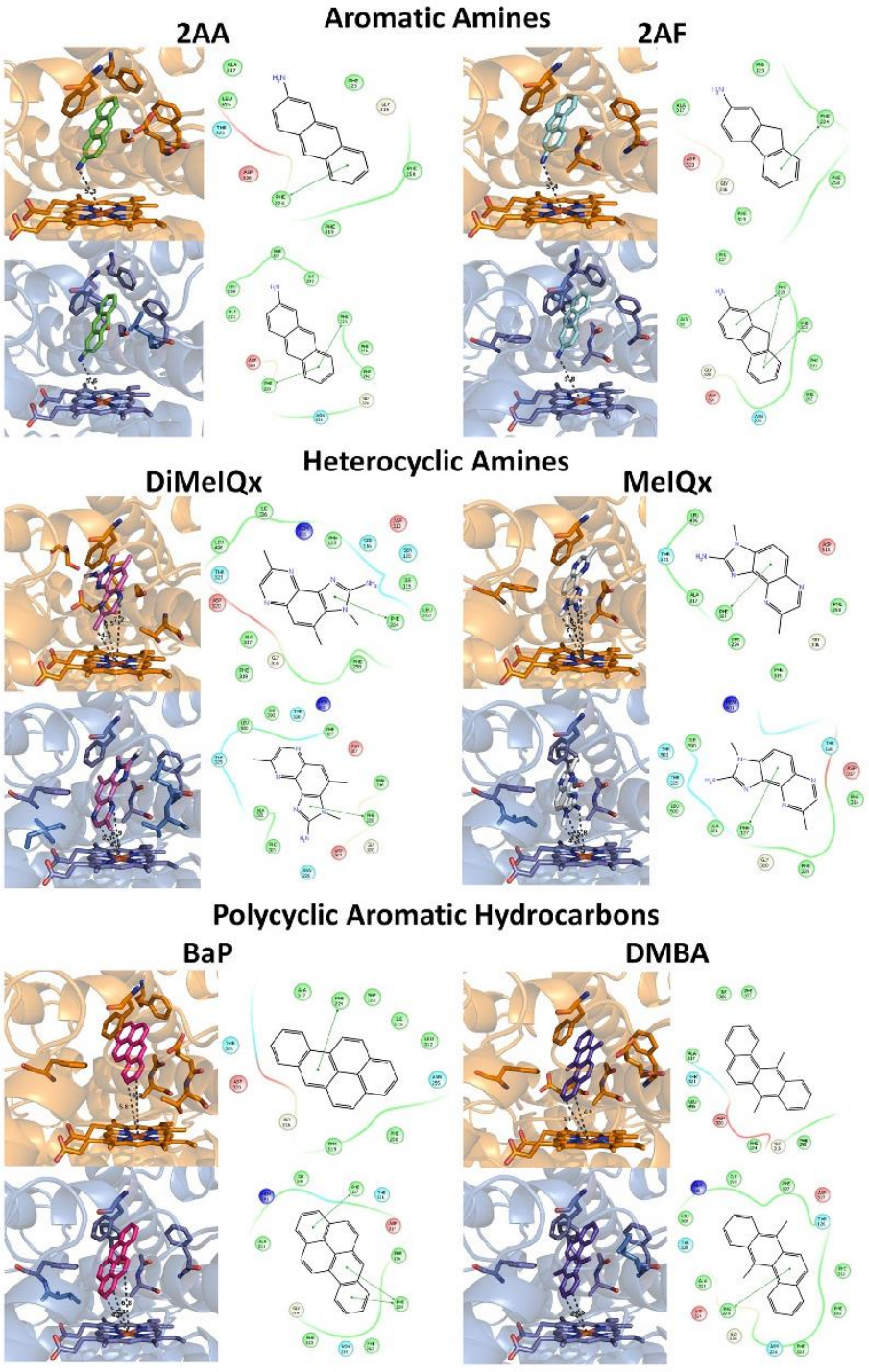

Fig. 1. Three-dimensional conformations of CYP1A1 with mutagenic compounds. Enzymes are represented as cartoons with human CYP1A1 in gold and rat CYP1A1 in blue. Numbers and discontinuous lines indicate the distance (Å) to heme group.

differences in the enzyme-substrate interactions and the activation of six mutagens by recombinant P450 1A1, through *in vitro* and *in silico* methods. Since Ames test studies typically use rat liver S9 fraction as a source of activation enzymes, relevance to human health may be questionable [51]. In this work, the tests were performed with the same amount of recombinant CYP1A1 from each species. The human and rat P450 1A1 sequences share 77.5% amino acid identity and 88% similarity, and differ in length by 12 residues overall [44]. Despite this similarity, the interspecies differences in substrate-enzyme interactions may play a major role in determining mutagenic potency.

It has been reported that MeIQx is weakly activated by *Escherichia coli lacZ* strains co-expressing recombinant human CYP1A1 and NADPH-cytochrome P450 reductase [52]. However, we found that HA are activated at doses < 1 μg/plate by both human and rat CYP1A1 (Table 1). Turesky et al. determined the catalytic efficiencies of CYP1A2 for MeIQx metabolism (recombinant human and rat enzymes). Human CYP1A2 was 10-19-fold more efficient at N-oxidation of MeIQx than was the rat enzyme [53]. Subsequently, the same group reported that human CYP1A2 catalyzes the formation of 2-amino-3-methylimidazo [4,5-*f*]quinoxaline-8-carboxylic acid (IQx-8-COOH), a detoxication product, and that this metabolite is not produced by rat CYP1A2 [54]. Although the differences between human and rat metabolism of MeIQx have already been described, we highlight that the preference of HA mutagenic activation by CYP1A1 is the opposite of that of MeIQx metabolism by CYP1A2, with 75- and 5-fold higher activation of DiMeIQx and MeIQx, respectively, by rat CYP1A1 (Table 1).

Further study is needed to compare the levels of reactive metabolites formed by human and rat CYP1A1, and to test whether the pathway of HA activation is the same as for CYP1A2. Binding energies were slightly more favorable for human CYP1A1 in the docking assay, but, with the rat enzyme, there were more HA interactions and the HA are positioned closer to the heme group (Fig. 1). Previous *in silico* reports have established that a shorter distance between the external atom of a ligand (non-hydrogen) and the Fe atom of the heme group is important for strong protein-ligand interaction [55]. Our *in silico* results (Table 3) revealed distances 4.3–7.5 Å, similar to other CYP-ligand docking assays [56–58]. Taken together, the computational results suggest a stronger interaction of HA with rat CYP1A1 and corroborate our experimental findings.

We did not observe a simple dependence of AA mutagenicity on the chemical structure. In strain YG1024, higher 2AA mutagenicity was obtained with human CYP1A1 than with the rat enzyme. This is the same species preference reported previously, using strain TA100 + S9 [16]. In addition, docking assays suggest that human CYP1A1 has a higher affinity for AA and confirm F224 as a key amino acid participating in π-π stacking [57].

Not only are interspecies structural differences important for substrate binding, but individual variations in CYP1A1 structure must also be considered. Correlations between SNPs in human CYP1A1 and cancer susceptibility have been reported [59–63], but few studies have examined the effects of these polymorphisms on enzyme activity. The allelic variants CYP1A1.2 (Ile462Val), CYP1A1.4 (Thr461Asn), and CYP1A1*2C (rs1048943) showed considerable differences in metabolite profiles and catalytic efficiencies of estrogen metabolism when compared to the wildtype enzyme [64,65].

More revertants were obtained for PAH in the presence of the human enzyme (Table 2). In agreement with previous studies, when the affinities of human and rat hepatic microsomes for BaP were compared, the former had 25-fold lower Km than the latter [66]. *In silico* assays suggested that differences in BaP and DMBA mutagenicity may result from differences in molecular recognition by human and rat CYP1A1. The dihydrodiol epoxide, radical cation, and *o*-quinone pathways represent the main routes of PAH metabolic activation [67]. In the first of these pathways, dihydrodiol epoxides are formed at the bay or fjord regions and interact with DNA [68]. The orientations of BaP and DMBA bound to human CYP1A1 (Fig. 1) are consistent with the preferred sites of metabolism. In contrast, these orientations are flipped 180° into a nonproductive conformation with rat CYP1A1.

The oxidation patterns of BaP with human and rat CYP1A1 Supersomes® revealed the formation of species-specific metabolites, such as BaP-4,5-dihydrodiol, a compound that was not detected with the human enzyme [69]. Our results suggest that the major activated products were produced in low amounts by rat CYP1A1, because mutagenicity was lower than with human Supersomes®. The participation of other enzymes involved in BaP metabolism, such as epoxide hydrolase, was not considered in our experimental model, and the potential contribution of such enzymes cannot be ruled out. In the absence of epoxide hydrolase, other enzymes, such as NADPH:P450 oxidoreductase and cytochrome b5 have been reported to participate in BaP bioactivation. When BaP was oxidized with human CYP1A1 Supersomes® reconstituted with NADPH:P450 oxidoreductase in the presence of DNA, a 9-hydroxy-BaP-4,5-epoxide-DNA adduct is formed in the absence of epoxide hydrolase [70]. Moreover, the addition of cytochrome b5 into the incubation mixture containing recombinant human CYP1A1 increases the oxidation of BaP [71].

Although DMBA administration in animals is used as a model for human tumorigenesis, few studies have focused on species-specific differences, and DMBA activation was found to be similar in human and rat hepatocytes [72,73]. In contrast, our findings demonstrate that DMBA is preferentially activated by human CYP1A1. DMBA is metabolized by CYP1 and epoxide hydrolase following the bay region epoxidation pathway, in a similar way to other PAH, with the formation of 3,4-dihydrodiol-1,2-epoxide as the ultimate carcinogen [74]. In epoxide hydrolase-null mice, phenol metabolites were generated via hydrolysis of the epoxides, and tumor formation by an epoxide hydrolase independent pathway was described [75]. Based on these findings, it is likely that other activation pathways participated in the metabolism of DMBA in our experimental system. The concept that DMBA phenol intermediates could act as substrates for further oxidation mediated by CYP1A1, leading to carcinogenic metabolites, merits further exploration.

5. Conclusions

Although interspecies differences in CYP1A1-mediated metabolism have been addressed previously [76–79], we aimed to determine how these differences translate quantitatively into mutagenicity, in order to improve extrapolation from animal models to humans. BaP, DMBA, and 2AA were more efficiently activated by human CYP1A1 to mutagenic compounds, suggesting that humans may be more susceptible to the effects of these molecules, whereas rats are likely more affected by HA exposure. Interspecies differences at various levels of regulation, including enzymatic activity, damage and repair mechanisms, pharmacokinetics, metabolite profile, concentration of adducts, and mutational spectra, as well as the contribution of conjugating enzymes, must be explored further to improve human risk assessment.

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Conflict of interest

None.

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Appendix A. Supplementary data

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Review Article

Regulation of Human Cytochrome P4501A1 (hCYP1A1): A Plausible Target for Chemoprevention?

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Human cytochrome P450 1A1 (hCYP1A1) has been an object of study due to its role in precarcinogen metabolism; for this reason it is relevant to know more in depth the mechanisms that rule out its expression and activity, which make this enzyme a target for the development of novel chemiopreventive agents. The aim of this work is to review the origin, regulation, and structural and functional characteristics of CYP1A1 letting us understand its role in the bioactivation of precarcinogen and the consequences of its modulation in other physiological processes, as well as guide us in the study of this important protein.

1. Introduction

Cytochrome P450 (CYP) is a superfamily of hemoproteins, with monooxygenase activity, which are spread into the three domains of life. They are biological catalysts that metabolize endogenous compounds such as hormones, bile acids, cholesterol, and xenobiotics like environmental pollutants and drugs. The hCYP1A1 is an enzyme of biomedical and toxicological interest, which catalyzes the biotransformation of polycyclic aryl hydrocarbons (PAHs), aromatic amines, and polychlorinated biphenyls into polar compounds, which can be conjugated to soluble compounds suitable for excretion by urine or bile. Nevertheless, under specific circumstances, this enzyme catalyzes the bioactivation of compounds capable of reacting with macromolecules, such as DNA, leading to the start of mutagenic process.

Every day, we are exposed to compounds that are substrates of CYP1A1, through environmental pollution, food, and, particularly, cigarette smoke. The importance of this protein in chemical carcinogenesis induced by PAHs has been demonstrated in *CYP1* knockout mice, in which the lack of this protein shows less formation of adducts PAH-DNA [1, 2]. In addition, rodent exposition to CYP1A1 inhibitors diminished the number of tumors induced by PAHs [3, 4].

Epidemiologic studies focused on the relationship among PAH exposition, PAH-DNA adducts level, and cancer incidence in humans demonstrate an increased risk in colon adenocarcinoma [5], breast cancer [6], and lung cancer [7] in those individuals with higher levels of adducts.

This data suggests that imbalance between detoxification and bioactivation of carcinogens, independence of enzyme catalysis, regulation of gene expression of *CYP1A1*, and cellular environment are crucial factors at the beginning of chemical carcinogenesis process. Because of this, several questions are still to answer; we propose that a global view of the function and regulation of this enzyme would help to answer these questions; thus, the aim of this work is to integrate the knowledge that has been generated until now about the origin, regulation, and structural characteristics of hCYP1A1.

2. Some Aspects of CYP1A1 Evolution

CYPs constitute a superfamily of ancient genes encoding to heme-thiolate proteins that catalyze the monooxygenation of

endogenous and exogenous substrates in bacteria, archaea, eukaryotes, and viruses [8, 9]; therefore these proteins must descend from a prokaryotic common ancestor ~3 billion years ago, before the oxygenation of the atmosphere and emergence of eukaryotic cells [10, 11].

The first CYP proteins were involved in the biosynthesis of compounds required for the formation and maintenance of cell structures and then following CYP proteins coevolved as defense mechanisms in plants and insects and more recently a set of these enzymes evolved in response to xenobiotics [12, 13].

CYPs belonging to families 1–4 are the main mediators of exogenous metabolism; however, cytochromes from family 1 are of particular biomedical and toxicological interest because of their affinity to halogenated polycyclic, aromatic amines, aromatic hydrocarbons, and endogenous compounds, whose metabolites can be toxic, mutagenic, or carcinogenic [14–16].

CYP genes of family 1 are grouped into six subfamilies: CYP1A, CYP1B, CYP1C, CYP1D, CYP1E, and CYP1F, from these 1E and 1F are found in urochordates; 1A, 1B, 1C, and 1D are found in fish and amphibians; in mammals the subfamilies that are mainly distributed are 1A and 1B and in some cases 1D [9, 17].

CYP1A and CYP1B diverged from a common ancestor ~450 million years ago (Ma); thus, CYP1A appears early in aquatic vertebrates, as a single copy, which has been identified in teleost fish, while mammals and birds have paralogous genes of CYP1A: CYP1A1, CYP1A2, in mammals, and CYP1A4, CYP1A5 in birds, which emerged ~250 Ma from a duplication event and one inversion, common for both lineages [15, 18, 19] (Figure 1).

In humans, the CYP1A1 gene consists of 6069 bp and is located at the CYP1A1_CYP1A2 locus on chromosome 15q24.1, sharing a regulatory region of 23306 bp with the CYP1A2 gene that is oriented in opposite direction. The 5′ flanking region is shared by both genes and contains a bidirectional promoter and DNA motifs, known as response elements, that activate and regulate the expression of these genes [20, 21].

The participation of multiple signaling pathways in the regulation of the hCYP1A1 transcription has been reported. Next, an overview about the pathways involved in this regulation is reviewed.

3. Upregulation of CYP1A1

The constitutive *hCYP1A1* gene has low level of expression in extrahepatic tissues of adult humans. However, liver and extrahepatic expression of this enzyme can be induced by many substrates through multiple pathways. The aryl hydrocarbon receptor (AHR) pathway has been widely studied and it appears to be the main protein receptor that influences *CYP1A1* induction. The AHR is a cytosolic ligand-activated transcription factor associated with two heat shock proteins of 90 kDa (Hsp90), a hepatitis B virus X-associated protein (XAP2), and a chaperone of 23 kDa (p23). This receptor is activated by endogenous ligands and several xenobiotics such as polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines, and halogenated biphenyls [22]. After ligand activation, AHR undergoes conformational changes that promote its translocation into the nucleus, via β importin, where it is dissociated from the chaperone proteins (Hsp90, XAP2, and p23), and binds to the nuclear translocator AHR (ARNT) [23, 24]; then the formed AHR-ARNT complex (AHRC) binds to xenobiotic responsive elements (XRE) (5′-TNGCGTG-3′) located at the enhancer element [25].

Thirteen XRE have been identified in the regulatory region of human *CYP1A1* [25]. It has been speculated that they are located at the major grooves of the DNA and they would be exposed during nucleosomal movements, allowing the AHRC binding. In turn, this promotes the recruitment of chromatin remodeling proteins such as p300, SRC1/2, and BRG1 [26], subsequent hyperacetylation of lysines 9 and 14 in histone 3 (H3K9ac and H3K14ac), and methylation of lysine 4 in histone 3 (H3K4me) (from dimethylation to trimethylation) at the promoter; meanwhile hyperacetylation of lysine 16 in histone 4 (AcH4K16) and increased phosphorylation of serine 10 in histone 3 (pH3S10) take place at the enhancer element. The increase of acetylation marks at the promoter region of mouse CYP1A1 (mCYP1A1) is consistent with the releasing of a basal repressive complex, which is composed of histone deacetylase 1 (HDAC1) and DNA methyltransferase 1 (DNMT1). It has been suggested that marks at the enhancer could stabilize the open chromatin state to allow the AHRC-mediated transcriptional loop [27–29]. Finally, this AHR-dependent pathway has target genes such as *CYP1A1*, *CYP1A2*, and *CYP1B1* and aldehyde dehydrogenase 3A1 (*ALDH3A1*) [30, 31]. Figure 2 shows some regulatory mechanisms involved in *CYP1A1* regulation.

A number of pathways also modulate *CYP1A1* transcription through binding to the promoter, interactions with AHR, or both mechanisms. Next, we briefly describe some of them.

The canonical Wnt/β-catenin signaling pathway is involved in the adult tissue homeostasis regulation, embryonic development, and tumorigenesis. It has also been implicated in the induction of some CYPs, including m*Cyp1a1*. In mice, this was demonstrated by the specific loss of *CTNNB1* that encodes β-catenin and leads to a decrease of *mCyp1a1* induction by AHR agonists such as 3-methylcholanthrene (3-MC), β-naphthoflavone (β-NF), and butylated hydroxyanisole. Additionally, it has been observed that maximum *mCyp1a1* induction was obtained when β-catenin acted as coactivator of AHR, although this protein also binds to the transcription factor TCF, which has a binding site in *mCyp1a1* promoter, suggesting a different mode of action [32–34]. Similarly, in rat hepatoma, it has been observed that the interaction between AHR and hypophosphorylated retinoblastoma protein (pRb) aids maximum induction of rat *CYP1A1* by 2.3, 7.8 tetrachlorodibenzo-p-dioxin (TCDD); pRb plays an important role in cell cycle control and it has been proposed that it could also act as a coactivator of AHR [35, 36].

Furthermore, several nuclear receptors are involved in the upregulation of *hCYP1A1*; for example, the constitutive androstane receptor (CAR) [37] which is also a regulator of the expression of the CYP2A, 2B, 2C, and 3A subfamilies is activated by drugs; the liver X receptor α (LXRα) that

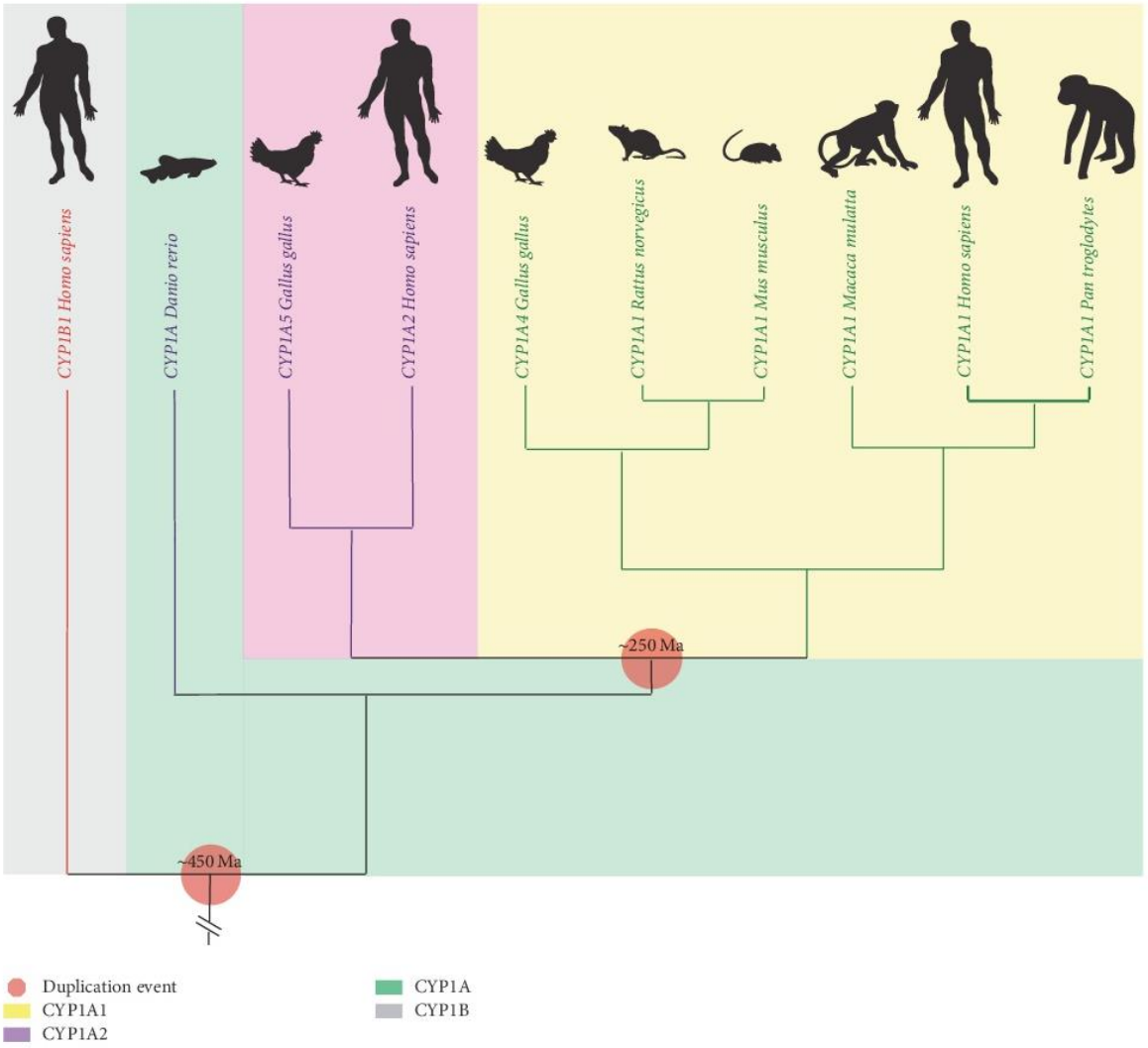

FIGURE 1: Phylogenetic tree of CYP1A subfamily through different species to human. Amino acid sequences and accession numbers of different species CYP were obtained from the Uniprot database, and with them phylogenetic tree was built in *phyloT*: *a tree generator* and visualized with *ITOL v3 Interactive Tree Of Life*. Silhouettes, background colors, and symbols were added to the image using Adobe Illustrator CC 2015.0.0 program.

is involved in lipid homeostasis is activated by oxysterols [38, 39]; and the peroxisome proliferator-activated receptor α (PPARα), is activated by fibrates, phthalates, arachidonic acid, and its derivatives [40, 41]. These receptors bind to their specific responsive elements located in the gene promoter, activate the transcription, and potentiate the induction of *hCYP1A1*. The crosstalk amongst signaling pathways involved in regulating the expression of *CYP1A1* could have implications for drug-drug, drug-toxic, and drug-food interactions.

4. Downregulation of CYP1A1

The tight regulation of *CYP1A1* is highly necessary due to the known harmful effects of electrophilic compounds produced by the enzymatic activity of CYP1A1; a number of CYP1A1 downregulation mechanisms have been described; for example, the AHR repressor protein (AHRR) is a target gen of the transcriptional activity of AHR and competes with AHR for binding to XREs. AHRR has been described as a negative tissue-specific regulator of *mCYP1A1* expression [43, 44]. Its overexpression in transgenic mice suppresses the *mCYP1A1* induction in lung, spleen, and adipose tissue [45]. Moreover, it has been suggested that rat CYP1A1 regulates its own expression because it catalyzes the removal of AHR agonists and thus decreases the activation of this pathway [46, 47].

Hypoxia inducible factor participates as a negative regulator of *hCYP1A1* expression through the competition with AHR for the binding to ARNT. Under hypoxia conditions,

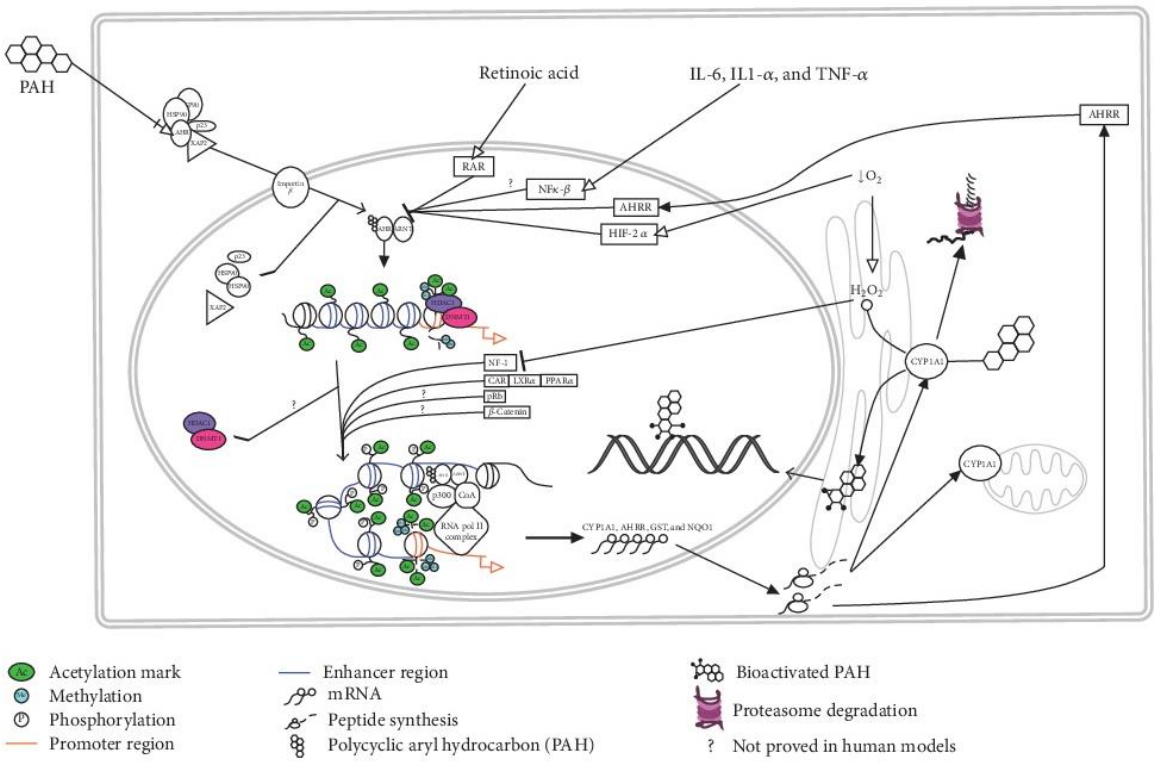

Figure 2: Mechanisms involved in the CYP1A1 regulation. Pathways implicated in up- and downregulation of CYP1A1 are shown, as well as changes in epigenetic marks upon the induction of this gene. The "?" symbol means pathways that had not been proved in human models, specified along the text. Image was created using PathVisio program [42] and edited with Adobe Illustrator CC 2015.0.0 program.

basal *hCYP1A1* expression decreases [48] and induction by AHR ligands is inhibited [49, 50].

Moreover, the retinoic acid receptor pathway (RAR) is also implicated in the regulation of *hCYP1A1* expression through two mechanisms. In the first one, RAR modulates the transcriptional expression of this protein through its binding to a retinoic acid responsive element (RARE) located in the *hCYP1A1* promoter [51, 52]. In the second one, the corepressor SMRT (silencing mediator for retinoid and thyroid receptors), which is attached to RAR, is released upon activation of RAR by retinoic acid; subsequently released SMRT can interact with AHR and reduce *hCYP1A1* induction [53].

Another protein involved in the downregulation of *hCYP1A1* induction is the nuclear factor I (NFI). NFI activates the expression by binding to promoter of *hCYP1A1* and it is sensitive to oxidative stress [54]. It has been demonstrated that increased activity of hCYP1A1 generates reactive oxygen species, which in turn can lead to the oxidation of the single cysteine residue on NFI and then it is released from the *hCYP1A1* promoter, thus decreasing the expression of this gene [55, 56].

The presence of a glucocorticoid responsive element in the intron one of the *CYP1A1* gene in several species has been reported. The activity of the glucocorticoid receptor potentiates the effect of activated AHR in rat hepatocytes unlike human hepatocytes where dexamethasone (glucocorticoid analog) decreases the *hCYP1A1* protein but not mRNA induced by 3-MC [57, 58]. However, additional studies are needed to clarify the effect of glucocorticoids on *CYP1A1* gene and protein levels.

Gut-enriched Kruppel like factor (KLFG or KLF4) is a regulator of cell proliferation, differentiation, apoptosis, and cellular reprogramming and has been identified as a negative regulator of rat *CYP1A1* transcription in a dependent way of its binding to the basic transcription element (BTE); moreover, this effect might also be part of the interaction between KLFG and Sp1, an *CYP1A1* transcriptional activator [59].

Another kind of downregulation is through the action of proinflammatory cytokines IL-1β and IL-6, TNF-α, and lipopolysaccharides; these cytokines decrease constitutive CYP1A1 expression and AHR-mediated induction in human and mouse hepatocytes [60–64].

5. Epigenetic, Posttranscriptional, and Posttranslational Regulation of CYP1A1

Until now, several modes of action have been reported for the regulation of human CYP1A1. In essence, transcriptional expression has been reviewed, but there is another kind of gene regulation that involves epigenetic mechanisms

such as methylation, acetylation, histone ubiquitination, or DNA methylation and hydroxylation. In this regard, to explore the role of these mechanisms on the regulation of *hCYP1A1* expression studies were conducted using the DNMTs inhibitor, 5-aza-2-deoxycytidine (5AzadC), and HDACs inhibitors, trichostatin A (TSA) and sodium butyrate. Table 1 summarizes the effects of these inhibitors on *CYP1A1* expression. Such effects are species-specific and depend on whether the tissue is derived from healthy or cancerous donations. This review focuses mainly on hCYP1A1 regulation and just on enriching the data presented; Table 1 shows results from studies conducted in human, mouse, or rat cell lines primary cultures.

According to the results it is not possible to conclude whether *hCYP1A1* has a DNA methylation dependence regulation or not. It seems that tissue and temporal issues might have been involved in this regard as well as the tumor state. We cannot rule this, but tumor or cancer state allows an increased DNA methylation in *hCYP1A1* regulatory region, at least in prostate [27] and lung [65, 69]; thus, in these models this gene has no constitutive expression which is activated by exposition to 5AzadC.

There is another type of *hCYP1A1* regulation, which is through posttranscriptional modulation. Some in silico studies have been conducted in order to determine a possible regulation of CYP1A through noncoding RNAs. Based on web databases analyses, six putative micro RNAs (miRNAs), hsa-miR-125b-2, hsa-miR-488, hsa-miR-657, hsa-miR-892a, hsa-miR-511, and hsa-miR-626, with one or more binding sites to the 3′UTR region of *hCYP1A1* were identified [21]. Following the same strategy, an additional study used five different bioinformatics programs and predicted 332 miRNAs to target *hCYP1A1* UTRs, from which 12% were predicted in at least 2 programs [110].

Interestingly, in a study performed in human breast cancer cell line MCF-7 exposed to BaP leads to diminish miR-892a expression and function. This miRNA binds to 515–535 nucleotides of 3′-UTR of human *CYP1A1* and acts as translational repressor of this transcript. The putative effect of miR-892a was previously predicted by an in silico study [111]. Another study conducted in normal human liver tissues ($n = 92$) searched for a correlation between the protein level of CYP1A1 and the expression of miRs and a negative correlation was found for miR-200a ($r_s = -0.36$), miR-142-3p ($r_s = -0.36$), and miR-200b ($r_s = -0.36$) [112]. Nevertheless, another study with healthy human liver tissues from individuals of different ages determined that upregulation of miR-125b-5p was related to downregulation of CYP1A1 from fetal and pediatric samples. The effect of this miRNA was also previously predicted [113].

At this point we realize that the protein expression of CYP1A1 is tissue-, health- and age-specific; thus, it is not strange to expect that also the mechanisms and factors involved in its expression would be specific as we can observe from the previous data where two miRNAs were predicted in silico and confirmed in vivo, but none of them were found repeatedly among the studies reviewed here. It would be obvious that if there are differences in miRNAs found among results with human CYP1A1, there could be much more differences between human and other species models. This assumption is supported by a report conducted in mice fetal thymocytes where miR-31 was found as a negative regulator of *mCyp1a1* translation after exposition of cells to TCDD. Furthermore, miR-31 has matched with 3′-UTR of the transcript of this protein [114].

There are some studies reporting indirect regulation of CYP1A1 through the regulation of AHR by small noncoding RNAs, as in the case of the Sprague-Dawley rats treated during 2 weeks with an antagonist of the corticotrophin releasing factor I. Results show that rat liver CYP1A1 expression was increased through an atypical pathway different from AHR ligand and suggest the involvement of miR-29a-5p, miR-680, and miR-700 which were negatively expressed 10-, 6- and 8.6-fold, respectively. Whether these miRNAs could act through rCYP1A1 direct binding or not is still unknown because the first two had binding sites in the 3′-UTR region of both rCYP1A1 and AHR [115]. More information about hCYP1A1 regulation through its 3′UTR region shall be discovered in the near future to achieve this objective; also more tissues and health conditions are needed to be studied.

Until this point we covered evolutionary origin of CYP1A1 and its transcriptional and posttranscriptional regulation, but once the CYP1A1 protein is formed its cellular lifetime is regulated too. The half-life time of this protein is of ~2.8 hours; this suggests a mechanism of protein degradation and the studies prompted to proteasomal degradation pathway. In fact, treatment with ubiquitin-proteasome inhibitor MG132 keeps the levels of CYP1A1, while lysosomal inhibitors do not [116–118]. In spite of these experiments, there are no reports that could help us figure out the mechanism of degradation of CYP1A1.

Another possible regulation of CYP1A1 is through the degradation of its heme group, which has been explored in human hepatoma cell line HepG2 exposed to different heavy metals. Here an increase in hemooxygenase 1 was found; this enzyme is involved in the metabolism of the heme group. Its increased levels found after heavy metals exposition correlate with diminished activity of CYP1A1, while protein level and gene expression remain unchanged [117, 119, 120].

6. Structural Characteristics of Human CYP1A1 and Its Ligands

Human CYP1A1 has a molecular weight of 58.16 kDa and consists of 512 amino acids of which the first thirty of the N-terminal region allow the association of the protein with the mitochondrial membrane and the disordered region of the smooth endoplasmic reticulum rich in unsaturated fatty acids, unlike the human CYP1A2 which is located in the sorted regions rich in cholesterol, sphingomyelin, and saturated fatty acids. Moreover, these thirty residues would also be mediating the interaction with NADPH-CYP reductase [121–124].

Directed mutagenesis in the residues of the human protein showed altered kinetic parameters and demonstrates the importance of certain amino acids like Phe123, Phe224, Glu256, Asp313, Gly316, Ala317, Thr321, Val382, and Ile386 (Table 2) in the recognition, binding, and affinity for the

Table 1: Effect of DNA methyltransferases and histone deacetylases inhibition on CYP1A1 expression.

DNMT inhibitor dosing schedule	Cell type or specie	PAH type	Effect	DNA methylation status	Source
	Human cell adenocarcinoma, A549 Human bronchial epithelium cell line, Beas-2B	BaP 1 nM, 100 nM, and 10 uM	*hCYP1A1* expression started with 10 μM. *hCYP1A1* expression started with 100 nM.	35% complete methylation 11% complete methylation.	[65]
5AzadC, 5 uM, 96 h	Human breast carcinoma cell line, MCF-7 Human cervical adenocarcinoma cell line, HeLa	10 nM TCDD lasts 24 hours	*hCYP1A1* expression increased 2-3-fold in Aza versus ctrl but did not change in Aza-TCDD versus TCDD. *hCYP1A1* expression increased 4-fold in Aza versus ctrl and 7-fold in Aza-TCDD versus ctrl.	Both cell lines: highly methylated at CpG sites in enhancer region. Low methylated at CpG sites in promoter region.	[66]
5AzadC, 0, 0.25, and 1 uM	Human prostatic epithelial cell line, PWR1-E Human prostatic epithelial cell line, RWPE-1 Human prostate adenocarcinoma cell line, LNCaP	TCDD, 10 nM	*hCYP1A1* expression increased in both PWR1 and RWPE1 treated with AzadC but not in the induction by TCDD. LNCaP increased their *hCYP1A1* induction by TCDD in a dose dependence of AzadC	RWP1 low methylated than LNCaP at enhancer region. No methylation at promoter.	[27]
5AzadC, 2 uM, 72 h (each 12 h)	Mouse hepatoma cell line, Hepa1c1c7	5 uM BaP, 8 h	Aza does not change *mCYP1A1* expression versus control Aza-BaP does not change *mCyp1a1* induction versus BaP	ND	[28]
5AzadC, 5 uM, 3 days	Mouse hepatoma cell line, Hepa1c1c7 Mouse embryonic fibroblast, C3H10T1/2	10 nM TCDD, 48 h	Nonincrease *mCyp1a1* expression in Aza-TCDD induced versus TCDD. C3H10T1: *mCyp1a1* expression increased in Aza-TCDD induced versus TCDD.	ND	[67]
5AzadC, 5 uM, 72 h	Human breast cancer cell line, MCF7 Human hepatic cancer cell line, HepG2	TCDD last 24 h of 5AzadC treatment	MCF7, no differences. HepaG2. no differences.	ND	[29]
5AzadC 1, 5, 10, 50, 250, and 500 uM, 72 hours after EGF treatment	Primary rat hepatocytes (Sprague-Dawley rats)		rCYP1A1 protein increases in dose dependence of AzadC	ND	[68]
5AzadC, 0.5 uM, 5 days	Primary normal human bronchial epithelial cells, NHBE ($n = 12$). Human bronchial epithelial cell lines (HBEC $n = 3$). Human lung adenocarcinoma cell lines (HLAC $n = 9$)		AzadC increased *hCYP1A1* expression in HLAC	NHBE and HBEC were low methylated than HLAC at enhancer region.	[69]

Table 1: Continued.

DNMT inhibitor dosing schedule	Cell type or specie	PAH type	Effect	DNA methylation status	Source
5AzadC, 5 uM, 7 days (with culture media changed on day four). On day 6 cells were split into 60 mm dishes in culture media with AzadC. Day 7, media were changed.	Human cervical adenocarcinoma cell line, HeLa		AzadC increased *hCYP1A1* expression versus ctrl.	HeLa and HepG2 were equally methylated at promoter.	[70]
5AzadC, 5 μM, 5 days 5 μM RG108, 5 days	Human primary hepatocytes (hPH) Human embryonic stem cells derived hepatocytes (hESC.Hep)		hESC-Hep: increased *hCYP1A1* expression in both 5AzadC and RG108 treatments.	hPH: no methylated hESC-Hep: high methylated.	[71]
HDAC inhibitor dosing schedule	Cell line type	AHR ligand	Effect		Source
TSA (200 ng/mL), 30 min prior to TCDD	Mouse hepatoma cell line, Hepa1c1c7	TCDD, 1 pM	No effect on EROD basal enzyme activity Increased TCDD, concentration dependence induction of EROD enzyme activity and CYP1A1 protein		[72]
TSA, 100 ng/mL, 24 h	Human breast carcinoma cell line, MCF-7 Human cervical adenocarcinoma cell line, HeLa	TCDD 10 nM (after TSA), 24 h	Increased basal *hCYP1A1* expression, but TSA had no effect on TCDD induced mRNA. Increased basal and TCDD induced *hCYP1A1* mRNA		[66]
SAHA (0.2–4.0 μM),12 and 24 h TSA (0.2–4.0 μM), 12 and 24 h	Human breast carcinoma cell line, MCF-7	BaP, 4 μM	Increased BaP induced EROD activity and basal *hCYP1A1* mRNA No effects on BaP induced *hCYP1A1* mRNA Increased BaP induced EROD activity and basal *hCYP1A1* mRNA Decreased BaP induced *hCYP1A1* mRNA		[73]
TSA (25 μM), 2, 4, and 7 days	Primary rat hepatocytes (Sprague Dawley)	None	Increased EROD activity at day 7. Increased rCYP1A1 protein at all days tested. Increased *rCYP1A1* mRNA at days 4 and 7.		[74]
Sodium butyrate (NaB), 2 mM,16 h	Mouse hepatoma cell line, Hepa1c1c7	BaP, 5 μM, 8 h	No changes on basal and induced m*Cyp1a1* mRNA		[28]
TSA, 100 nM, 24 h	Mouse hepatoma cell line, Hepa1-OT Mouse embryonic fibroblast cell line, C3H10T1/2	TCDD, 10 nM, 24 h	Increased TCDD induced *mCyp1a1* mRNA Increased TCDD induced *mCyp1a1* mRNA		[67]
AN-8 (1–5 μM), 72 h	Primary hepatocytes culture	None	Increased CYP1A1 protein level		[68]
TSA 250 nM,16 h	Human cervical adenocarcinoma cell line, HeLa	PCB, 136 3 μM (after TSA), 6 h	Increased basal and PCB induced *hCYP1A1* mRNA		[70]

ND: nondetermined. All increases or decreases in DNA methylation, mRNA, or protein were significantly different with respect to the respective control. For more information about this, references to the original work are provided.
EROD: Ethoxyresorufin *O*-deethylation CYP1A1 enzyme activity.

substrates. However, the spatial orientation of these residues was known until the three-dimensional structure of human CYP1A1 was resolved by X-ray crystallography at a resolution of 2.6 Å [125].

The protein crystallization of human CYP1A1 allowed us to know that this protein is comprised by twelve α-helices (A–L), three β-sheets (β1–β3), and four helical short regions (A′, B′, F′, and G′) forming six sequences as putative substrate

Table 2: Effect of mutations in the amino acid sequence of human CYP1A1 on the kinetic parameters of this enzyme.

Amino acid	Position	Amino acid type	Mutation	Amino acid type	Effect	Reference
Gly	45 loop A′	Nonpolar, aliphatic	Asp	Negatively charged	K_m and V_{max} are decreased by 42.9% and 75.1%, respectively	[75]
Ala	62 helix A	Nonpolar, aliphatic	Pro	Nonpolar, aliphatic	K_m is increased by 84% and V_{max} is decreased by 21%	[76]
Ser	116 helix B′	Polar, uncharged	Ala	Nonpolar, aliphatic	K_m and V_{max} do not change	[77]
Ser	122 loop B′-C	Polar, uncharged	Thr	Polar, uncharged	Activity is increased by 25%	[78]
			Ala	Nonpolar, aliphatic	K_m and V_{max} are increased by 74% and 2-fold, respectively	[79]
Phe	123 loop B′-C	Aromatic	Ala	Nonpolar, aliphatic	Without activity. K_m is increased by 12.8-fold and V_{max} is decreased by 42.5%	[77, 79]
Glu	161 helix D	Negatively charged	Lys	Positively charged	K_m is decreased by 39% and V_{max} does not change	[77]
Glu	166 helix D	Negatively charged	Gln	Nonpolar, aliphatic	K_m and V_{max} are increased by 3.7-fold and 24%, respectively	[77]
Val	191 helix E	Nonpolar, aliphatic	Met	Polar, uncharged	K_m and V_{max} do not change	[77]
Asn	221 helix F	Nonpolar, aliphatic	Thr	Polar, uncharged	Activity is decreased to 28%	[78]
Phe	224 helix F	Aromatic	Ala	Nonpolar, aliphatic	V_{max} and K_m are decreased by 11.4-fold and 75%, respectively	[79]
Gly	225 helix F	Nonpolar, aliphatic	Val	Nonpolar, aliphatic	Activity is decreased to 19%	[78]
Val	228 helix F	Nonpolar, aliphatic	Thr	Polar, uncharged	K_m and V_{max} do not change	[77]
Glu	256 helix G	Negatively charged	Lys	Positively charged	K_m is decreased by 70% and V_{max} does not change	[77]
Tyr	259 helix G	Aromatic	Phe	Aromatic	K_m is increased by 2.7-fold and V_{max} does not change	[77]
Asn	309 helix H	Nonpolar, aliphatic	Thr	Polar, uncharged	K_m and V_{max} do not change	[77]
Leu	312 helix I	Nonpolar, aliphatic	Asn	Nonpolar, aliphatic	Activity is decreased to 42%	[78]
			Phe	Aromatic	K_m is increased by 89% and V_{max} does not change	[77]
Asp	313 helix I	Negatively charged	Ala	Nonpolar, aliphatic	K_m and V_{max} are increased by 21-fold and 28%, respectively	[77]
			Asn	Nonpolar, aliphatic	K_m is increased by 24.5-fold and V_{max} is decreased by 37.5%	[77]
Gly	316 helix I	Nonpolar, aliphatic	Val	Nonpolar, aliphatic	K_m is increased by 17-fold and V_{max} is decreased by 30%	[77]

Table 2: Continued.

Amino acid	Position	Amino acid type	Mutation	Amino acid type	Effect	Reference
Ala	317 helix I	Nonpolar, aliphatic	Tyr	Aromatic	Without activity	[79]
			Gly	Nonpolar, aliphatic	K_m is increased by 30-fold and V_{max} is decreased by 25%	[77]
Asp	320 helix I	Negatively charged	Ala	Nonpolar, aliphatic	K_m is increased by 2.7-fold and V_{max} is decreased by 35%	[77]
Thr	321 helix I	Polar, uncharged	Gly	Nonpolar, aliphatic	K_m is increased by 30% and V_{max} is decreased by 70%	[79]
			Pro	Nonpolar, aliphatic	K_m is increased by 6.2-fold and V_{max} does not change	[77]
			Ser	Polar, uncharged	K_m and V_{max} are increased by 7.6-fold and 2-fold, respectively	[77]
Val	322 helix I	Nonpolar, aliphatic	Ala	Nonpolar, aliphatic	K_m is increased by 67% and V_{max} does not change	[77]
Val	382 helix K/ loop β1–4	Nonpolar, aliphatic	Ala	Nonpolar, aliphatic	Activity is decreased to 66%	[78]
			Leu	Nonpolar, aliphatic	Activity is decreased to 7%	[78]
Ile	386 helix K/ loop β1–4	Nonpolar, aliphatic	Gly	Nonpolar, aliphatic	Without activity	[79]
			Val	Nonpolar, aliphatic	K_m and V_{max} are increased by 87% and 58%, respectively	[77]
Ile	458 helix L	Nonpolar, aliphatic	Pro	Nonpolar, aliphatic	K_m is increased by 44% and V_{max} does not change	[77]
			Val	Nonpolar, aliphatic	K_m and V_{max} are decreased by 55% and 21%, respectively	[77]
Thr	497 loop β4	Polar, uncharged	Ser	Polar, uncharged	K_m is increased by 3-fold and V_{max} does not change	[77]

recognition sites (SRS) important for ligand selectivity of this enzyme [125, 126], which are shown in Figure 3 and listed as follows.

(i) SRS1 corresponds to the amino acid region 106–124 of loop between helix B and helix B′ and portion of loop between helix B′ and helix C. In turn, it forms part of the wall of the active site and it is proposed as a site for the input and output of ligands that influence the regioselectivity for the oxidation of substrates [127, 128].

(ii) SRS2 is part of the helices E and F, as well as of the residues 217–228, in the loop that connects these regions. Its role is similar to SRS1 participating in the ligand orientation [129, 130].

(iii) SRS3 is found in helix G from amino acid 251 to amino acid 262 [126].

(iv) SRS4 corresponds to helix I (residues 309–324) [126].

(v) SRS5 goes from residue 381 to residue 386 and connects helix J to the beta sheet. In other CYPs this region has been associated with the entry of the ligand due to its high flexibility [130].

(vi) SRS6 is the shortest region and is located in the loop near the β3 sheet [126].

The human CYP1A1 structure allows binding planar molecule with ~12.3 Å in length and ~4.6 Å in width, conformed by aromatic, polyaromatic, and heterocyclic rings which are essential for the formation of π-π stacking in the protein active site, mainly with Phe-224 at helix F, conferring stability to the enzyme-substrate complex [43, 80, 81, 131–135]. Nevertheless, for specific substrate redox reaction to be produced (Table 3), ligand also requires to be oriented with its reactive group facing the heme group [136, 137].

Table 3: Reactions carried out by the human CYP1A1 depending on the type of substrate.

Origin	Category compound	Type of reaction	Source
Synthetic compounds	Polycyclic aromatic hydrocarbons	Oxidation Epoxidation	[80, 81]
	Nitrosamides	Nitroreduction	[82, 83]
	Arylamines	*N*-hydroxylation Oxidation	[80, 81]
	Benzotriazole	Oxidation	[84]
	Heterocyclic amines	*N*-hydroxylation Oxidation	[80, 81]
	Nitroarenes	Nitroreduction	[85]
	Azoaromatic amines	Oxidation	[80, 81]
Natural compounds	Difuranocumarin	Epoxidation Oxidation	[86]
	Nefrotoxin	Hydroxylation	[87]
	Flavonoid	Hydroxylation *O*-demethylation	[88, 89]
Drugs	Ellipticin	Oxidation	[90]
	Omeprazol	ND	[91]
	Oltipraz	ND	[92]
Endogenous substrates	Arachidonic acid	Hydroxylation	[93]
	Melatonin	Hydroxylation	[94]
	Eicosapentaenoic acid	Epoxidation	[93]
	Stradiol	Hydroxylation	[95]

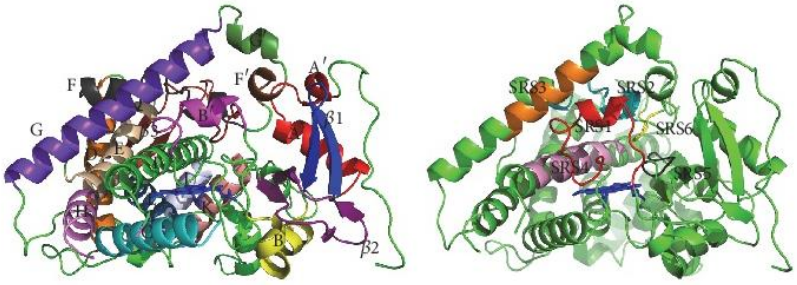

Figure 3: Three-dimensional structure and substrate recognition sites (SRS) of human CYP1A1. Figure was created with PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.

7. CYP1A1 through Development

Besides its importance in the metabolism of xenobiotics, CYP1A1 is also involved in the metabolism of endogenous compounds, such as arachidonic acid, eicosapentaenoic acid [93], 17β-estradiol [95], and melatonin [94].

Arachidonic acid and eicosapentaenoic acid are biotransformed by this enzyme to products such as 14, 15-epoxyeicosatrienoic acid and 17, 18-epoxyeicosatetraenoic acid, which influence cardiovascular pressure [93]. This attribute highlighted the importance of the association between heart diseases and CYP1A1 polymorphisms [138–140].

Treatment with the CYP1A inhibitor, α-naphthoflavone, shows that the activity of CYP1A1 is important for the proper development of the embryo's cardiovascular system [141–143]. However, so far there is not enough information about the impact of this isoform in the endogenous metabolism, so it is essential to conduct more studies that can help us to understand the mechanisms of these processes and their impact on the human health.

The use of different animal models has proved that activity and basal expression of CYP1A1 during embryonic development are organ-stage-specific (Table 4), where the liver and cardiovascular tissues have the highest expression. In the chicken, exposure to CYP1A1 inducers causes an increase in heart size and weight, while, in fish, edema in pericardium as well as modifications in the normal shape of the organ has been reported [141, 142, 144–148].

Searching whether the function of CYP1A1 is crucial for life, a line of knockout mice for this gene was produced [149]. These animals show decreased liver, kidney, and heart weight, as well as increased blood pressure and lower heart rate compared to wild type mice, thus demonstrating the importance of CYP1A1 in the cardiovascular system [150].

In adulthood, the human CYP1A1 expression is low and is found particularly in tissues of the respiratory system such

Table 4: Basal expression and activity of CYP1A1 in different animal models.

Animal model	Development stage	Spatial localization	Detection method	Reference
Human	16–36 gestation weeks	Not determined-	PCR	[96]
	50–60 gestation weeks	Hepatic tissue	BZROD (microsomes) (8.8 ± 2.1 pmol/mg of protein/min^{-1})	[97]
	74–145 gestation days	Day 87: kidney Days 55, 70, 101, and 112: lung Days 45, 70, and 85: liver	PCR: southern blot	[98]
Mouse	E17	Not determined-	PCR	[96, 99]
	E7-E14	E7: extraembryonic ectoderm and mesoderm E8.5: myocardial cells in ventricular chamber E10: left and right heart ventricle Dorsal aorta and neuroepithelial cells of midbrain E12: myocardial cells of both heart ventricles and midbrain E13: dorsal aorta, heart, and epithelium of midbrain E14: dorsal aorta, both heart ventricles, and atrium Epithelium of midbrain and trigeminal ganglion.	*lacZ* reporter with the promoter of *CYP1A1*	[100]
Rat	15–29 gestation days	D15: liver D29: lung and liver	PCR Southern blot	[98]
Chicken	4–15 incubation days	D4–D7: embryonic pool D9–D15: liver D4–D15: yolk sac	EROD (microsomes) (<1 pmol/mg of protein/min^{-1}) (>300 <1100 pmol/mg of protein/min^{-1}) (>20 <400 pmol/mg of protein/min^{-1})	[101]
	17 incubation days	Liver	Run-on transcription assay	[102]
	18 incubation days	Liver kidney	EROD (microsomes) (35 ± 6 pmol/mg of protein/min^{-1}) (25 ± 9 pmol/mg of protein/min^{-1})	[103]
	10 incubation days	Liver	q-PCR	[104]
Zebra Fish	8–128 hours after fertilization (hpf)	8 hpf: germs layers 32–80 hpf: cardiovascular system 104–128 hpf: cardiovascular system, liver, intestine, urinary tract, and kidney	EROD in vivo (>0.08 <0.5 pmol/mg of protein/min^{-1})	[105]
	48–120 hpf	Embryonic pool	q-PCR EROD in vivo (0.0107–0.0184 pmol/mg of protein/min^{-1})	[106]
	4–8 days after fertilization	Not determined	EROD in vivo (50–100 fmol h^{-1} larva^{-1})	[107]
Medaka fish	8 hpf	Not determined	EROD in vivo (arbitrary units)	[108]
	50–245 hpf	Gallbladder	EROD in vivo (arbitrary units)	[109]

as trachea and lungs, but after induction, it is also detected in other organs such as liver, adrenal gland, bladder, heart, kidney, ovary, placenta, prostate, testis, thyroid, salivary gland, and spleen [96, 151]. Among these organs, different levels of the protein are detected [152].

8. Concluding Remarks

CYP1A1 is a relevant enzyme for biotransformation of environmental compounds into mutagenic metabolites; this fact has a strong effect on worldwide population; therefore, the knowledge of its tridimensional structure as well as its ligands allows us to the rationale search and development of inhibitors that would become chemopreventive agents for diseases related to exposure to CYP1A1 activated carcinogens.

On the other hand, the presence of CYP1A1 among several species forces us to choose biological models that share with humans similar CYP1A1 characteristics in order to obtain results able to be extrapolated. The animals frequently used for this purpose are rats and mice, in which some of the regulatory mechanisms and other data, reported here, have been described. Moreover, as already mentioned in the "upregulation of CYP1A1" Section, several pathways could be involved like the recently reported WNT-β catenin, RAR, or CAR pathways that regulate *CYP1A1* expression by direct interaction with its gene promoter or with that of *AHR* or both. However, these alternative pathways are poorly described and more studies in this regard are required to know how and what are the factors involved as well as the specific conditions necessary for their action on *CYP1A1* expression, like the tissue and its microenvironment or culture cell type used just to mention two of them. The discoveries of pathways that converge in CYP1A1 regulation are opportunities for the selection of new therapeutic targets that allow drug development for chemoprevention.

For the study of CYP1A1, we need to take into account that impairment of gene expression or enzyme activity could lead to adverse effects because it is involved in endogenous metabolism, an issue discussed in "CYP1A1 through development," with particular interest in cardiotoxicity.

The integration of data generated about CYP1A1, factors, and mechanisms that play a role in carcinogen bioactivation will help us to rise up strategies that improve our life quality. In this context, some key questions that need to be addressed are written below.

It will be worth to continue the searching for chemopreventive agents that inhibit CYP1A1 even if it seems to be involved in the normal development of the heart. It is a good strategy to improve chemopreventive agents acting on different regulating CYP1A1 pathways at the same time; meanwhile they have fewer side effects. What is the real contribution of CYP1A1 in the process of carcinogen bioactivation knowing that it shares regulatory elements with additional CYPs of the same family? Do the cardiotoxicity effects produced in the lack of CYP1A1 activity be a window for searching new therapeutic targets for cardiovascular diseases? What is the biological relevance of reactive oxygen species production by CYP1A1? Why do tissues have differences on CYP1A1 expression? Is the tissue-specific, or even cell-specific, expression of CYP1A1 explained by differences in endogenous metabolism requirements or by alternative modulation of a particular set of AHR co-activators? Do the specific CYP1A1 expression and induction play a role in the development of a particular cancer ligand related?

Competing Interests

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

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Inhibition of human and rat CYP1A1 enzyme by grapefruit juice compounds

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HIGHLIGHTS

- Interspecies differences on inhibition of CYP1A1 were found.
- Rat CYP1A1enzyme is more sensible to inhibition than human CYP1A1.
- Naringenin showed different mechanism of inhibition of CYP1A1.
- Molecular modeling suggested differences in interactions of inhibitors with CYP1A1.

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ABSTRACT

Cytochrome P4501A1 is involved in the metabolism of carcinogenic polycyclic aromatic hydrocarbons; therefore, its inhibition interferes with the carcinogenesis process induced by these compounds in rats. The human and rat CYP1A1 differ by 21% in amino acid sequence, including the active site of the enzyme; this difference may be an important factor when results obtained using animal models are interpolated to humans. Based on its previously reported CYP inhibitory properties, we studied the effects of two molecules contained within grapefruit juice, naringenin and 6′,7′-dihydroxybergamottin, on human and rat CYP1A1 activity. For this purpose, the kinetics of inhibition as well as computational simulations were used. Naringenin and 6′,7′-dihydroxybergamottin were found to be competitive inhibitors of human and rat CYP1A1. Additionally, naringenin exerted a mixed type inhibition effect on rat CYP1A1. Computational docking showed that inhibitors might block the oxidation of 7-ethoxyresorufin by binding to the CYP1A1 active site. Our results demonstrate the differences in CYP inhibitory mechanisms for the same molecule when CYP from different species are considered.

1. Introduction

Cytochrome P450 (CYP) enzymes are ubiquitous in living organisms and constitute a family of proteins involved in the metabolism of a large number of compounds (Gonzalez and Gelboin, 1994; Nelson et al., 1996). Of major importance is the involvement of these enzymes in toxicant biotransformation and drug metabolism (Zanger and Schwab, 2013). CYP1A1 is a subfamily of highly conserved enzymes in humans and other mammalian organisms. For example, human CYP1A1 (512 amino acid residues) is 12 amino acid smaller than its rat equivalent (524 residues), with which it exhibits 79% identity (Ioannides, 2005). These differences between species may affect its structure, activity, and selectivity; however, to the best of our knowledge, not enough attention has been paid to this matter.

CYP1A1 plays an important role in the metabolic activation of chemical carcinogens (Rendic and Guengerich, 2012). The enzyme oxidizes benzo[a]pyrene and other polycyclic aromatic hydrocarbons to their toxic derivatives (Shou et al., 1996; Shimada and

Abbreviations: ER, 7-ethoxyresorufin; NAR, naringenin; DHB, 6′,7′-dihydroxybergamottin.

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Fujii-Kuriyama, 2004). Available selective substrates and inhibitors of CYP1A1 will add to the study of the metabolic role of this enzyme in cancer and drug toxicity and may provide a basis for the rational design of inhibitors as CYP1A1-targeted cancer chemopreventive agents. However, species-specific differences make it difficult to interpolate the results obtained using animal models to humans. Previously, biochemical inhibition parameters for naringenin were calculated by Ubeaud et al. (1999). Using human and rat liver microsomal preparations as CYP source and simvastatin as substrate, they reported similar inhibitory constants (Ki) for naringenin and concluded that no species-specific differences occur in this metabolic interaction. Nevertheless, the Ki value for bergamottin, another CYP inhibitor, was 174 ± 36 μM in rat liver microsomes and 34 ± 5 μM in human liver microsomes, indicating a higher sensitivity of human CYP to bergamottin (Le Goff-Klein et al., 2003). Furthermore, tangeretin, a flavone present in citrus fruits, is an inhibitor of rat CYP1A2 with an IC_{50} of 0.8 μM and 16 μM in human microsomes (Obermeier et al., 1995). Thus, although differences have been reported, no general rule exists to help predict CYP inhibition potency of a chemical when interpolating result from one species to another. Here, we examine the binding of two compounds present in grapefruit juice to human and rat CYP1A to identify possible structural determinants associated with its inhibition profile through kinetic analyses and computational simulations. Kinetic studies revealed that DHB and naringenin are competitive inhibitors for human CYP1A1. On the other hand, DHB competitively inhibited rat CYP1A1 activity while naringenin was a mixed-type inhibitor. Docking of enzyme inhibitors in the active site allowed us to identify amino acid residues that might be important for inhibition. Based on the structure of human CYP1A1 (Walsh et al., 2013), we constructed a homology model of rat CYP1A1. Molecular modeling showed that DHB and naringenin were tightly coordinated within the active site of human and rat CYP1A1 enzyme, which may underlie the inhibitory mechanism.

2. Materials and methods

2.1. Materials

Resorufin, 7-ethoxyresorufin (ER), naringenin (NAR), 6′,7′-dihydroxybergamottin (DHB) and NADPH were purchased from Sigma Chemicals Co. (St. Louis MO, USA).

Microsomes from a Baculovirus expression system of insect cells (Supersomes®) expressing rat or human CYP1A1 were purchased from BD-Gentest (Woburn, MA, USA), and microsomes prepared from Sf9 insect cells infected with control baculovirus (Supersomes® Sf9 insect cell control) were from Corning (New York, USA). Pooled human liver S9 was from Moltox (Boone, NC, USA).

2.2. Animals

Male Wistar rats (200 g, Instituto de Investigaciones Biomédicas, UNAM) were used. The animals were kept on a 12 h light/dark cycle. Rat liver was removed for preparation of the S9 fraction (Maron and Ames, 1983) in 0.15 M KCl solution.

2.3. Preparation of microsomes

In order to obtain liver microsomes, human and rat S9 fractions were processed according to the procedure described previously (Olguín-Reyes et al., 2012).

2.4. 7-Ethoxyresorufin O-deethylation assay

7-Ethoxyresorufin *O*-deethylation activity (EROD) was determined by a continuous spectrofluorometric method as described previously (Burke et al., 1994), with minor modifications. An incubation mixture containing human or rat microsomes, substrate (5 μM 7-ethoxyresorufin), and buffer pH 7.6 (50 mM Tris–HCl and 25 mM $MgCl_2$) was incubated for 3 min at 37 °C, in the presence and absence of inhibitors (NAR and DHB). The concentration of DMSO (vehicle for inhibitors and substrate) in the incubation mixture was ≤1%. The reaction was started with the addition of NADPH (0.5 mM), and the incubation was carried at 37 °C with constant shaking in a 96-well plate. Fluorescence readings were recorded every 20 s for 10 min at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Calibration curves were constructed with resorufin, and linear regression analysis was used to calculate the amount of resorufin formed in each incubation sample.

Inhibitor concentrations that produce 50% inhibition in EROD activity (IC_{50}) were calculated by linear regression of Log [inhibitor] vs% EROD activity.

2.5. Kinetic inhibition of CYP1A1

CYP1A1 Supersomes® and different concentrations of ER were used to determine CYP1A1 enzymatic kinetics. A typical Michaelis–Menten curve was constructed. The final reaction mixture (0.2 mL) contained 10 pmol/mL CYP1A1 (Supersomes®), 0.5 mM NADPH, and different concentrations of ER and inhibitor.

Comparable protein concentrations of Sf9 insect cells infected with baculovirus control (Supersomes® Sf9 insect cell control) were used as a control. EROD activity from the expression system non-transfected with CYP1A1 was not detected (data not shown).

Kinetic constants were obtained by a nonlinear regression analysis of experimental data fitted to the Michaelis–Menten equation with competitive or mixed-type inhibition (Software GraphPad Prism version 6). Kinetic analysis was also determined by using the Lineweaver–Burk and Dixon plot. The secondary replot of slope or the Y-intercept from the Lineweaver–Burk graph vs. Inhibitor concentration was linearly fitted assuming a single inhibition site.

2.6. Computer modeling and docking studies

2.6.1. Proteins

The crystallographic structure for human CYP1A1 (PDB: 4I8V, Walsh et al., 2013) was downloaded from the Protein Data Bank (Berman et al., 2000). Crystallographic waters, counter-ions, and ligands except the heme group were removed and the residues' protonation states were adjusted with the Protein Preparation Wizard of the Maestro Suite v9.4 (Schödinger LLC, version 9.6). With this wizard, the correct protonation states for residues were adjusted to pH 7.0.

Since no crystallographic structure from rat CYP1A1 has been reported, a homology model was constructed using ModWeb, which is a web-based server using Modeller (Eswar et al., 2006; Fiser et al., 2000; Marti-Renom et al., 2000; Sali and Blundell, 1993), using the human CYP1A1 structure as template. Using the Maestro Suite, heme group was added and the correct protonation states for residues were adjusted to pH 7.0.

2.6.2. Molecular dynamics simulations

The proteins previously prepared were minimized for 500 steps using the steepest descent optimization algorithm prior to the generation of MD trajectory. The solvent-protein contacts were optimized by a simulation lasting 200 ps, with harmonic constraints in the heavy atoms of the protein (all, except for hydrogen). Afterwards, an unrestrained trajectory of 10 ns was generated. The simulation was performed using a 2 fs time step. The pressure was set at 1 bar using a Berendsen barostat (Berendsen et al., 1984), and

the temperature was kept constant at 300 K by temperature coupling using velocity rescaling with a stochastic term (Bussi et al., 2007). The linear constraint solver algorithm was employed to remove bond vibrations (Hess et al., 1997). The particle mesh Ewald method (Darden et al., 1993; Essmann et al., 1995) coupled to periodic boundary conditions was used to simulate long-range electrostatic interactions using a direct space cutoff of 1.2 nm and a grid spacing of 0.12 nm. Because periodic boundary conditions can produce periodic artifacts, especially if combined with Ewald methods (Essmann et al., 1995), the minimum distance between protein molecules in adjacent boxes was calculated as a function of time. The simulation box size was adequate for the system, based on the cutoff used for electrostatic and short-range contributions to the potential function. The van der Waals interactions were computed using periodic boundary conditions coupled to a spherical cutoff of 1.4 nm. The MDS was performed using GROMACS 4.6.1 (Hess et al., 2008), and the four most relevant conformations for each protein were extracted for use in docking calculations.

2.6.3. Ligands

Using MGLTools (Scripps Research Institute, version 1.5.6), Gasteiger-Marsili atomic charges were assigned, and the non-polar hydrogens fused for the structures were obtained from molecular dynamic simulations. The files were saved in the proper format for use in Autodock (Scripps Research Institute, version 4.2, Morris et al., 2009). Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined.

2.6.4. Molecular docking

Docking calculations were carried out on the four most relevant conformations derived from MD simulations for each protein. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDockTools (Morris et al., 1998). Affinity (grid) maps of 70 grid points and 0.375 Å spacing were generated using the Autogrid program (Morris et al., 1998) and centered on the catalytic site of each protein model. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively.

Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method (Solis and Wets, 1981). Initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 20 different runs that were set to terminate after a maximum of 5000000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

3. Results

3.1. Effects of NAR and DHB on EROD activity in liver microsomes

IC_{50} values were calculated using 5 μM ethoxyresorufin and showed differences in the potency of inhibitors. Enzymes present in rat liver microsomes were about 1000 times more sensitive to inhibition than human liver microsomes (Table 1).

3.2. Kinetic analysis of the inhibition of CYP1A1 by NAR and DHB

To study the inhibition of CYP1 enzymes by NAR and DHB, 7-ethoxyresorufin was used as the common substrate for rat and human CYP1A1. Kinetic analysis was performed using non-linear regression analysis. Human CYP1A1-catalyzed EROD activity had Km and Vmax values of 0.14 ± 0.01 μM and 93 ± 1 pmol/min/mg protein, respectively (Table 2). On the other hand, rat CYP1A1-catalyzed EROD activity had Km and Vmax values of 0.36 ± 0.03 μM and 6089 ± 133 pmol/min/mg protein, respectively (Table 2).

Table 1
IC_{50} values of EROD inhibition by NAR and DHB.

Compound	Human liver microsomes	Rat liver microsomes
NAR	8.31 ± 1.20 mM	5.18 ± 1.22 μM
DHB	1.43 ± 0.11 mM	11.14 ± 0.19 μM

Data are expressed as mean ± SE for three independent experiments.

NAR and DHB showed different inhibition kinetics for human and rat CYP1A1. NAR caused a competitive inhibition of human CYP1A1 (Fig. 1A) with a Ki of 489 ± 22 μM. The same molecule also caused a mixed type inhibition of rat CYP1A1 with Ki and K'i values of 0.17 ± 0.02 μM and 0.39 ± 0.02 μM, respectively (Fig. 1B). DHB inhibited human and rat CYP1A1 activity in a competitive manner (Fig. 2); the Ki value for DHB-mediated human CYP1A1 inhibition (55 ± 2 μM) was higher than that for rat CYP1A1 (1.73 ± 0.03 μM, Table 2).

3.3. Molecular modeling of human and rat CYP1A1

To obtain meaningful structures of both human and rat CYP1A1, molecular dynamics simulations were performed with the crystallographic data of human CYP1A1 and in a homology model of rat CYP1A1. Thermodynamic data of the simulations shows acceptable stability of the simulations. Fluctuations in the catalytic site are similar in both models (Table 3), although to a greater extent for rat CYP1A1, and the heme group shows little changes in position, reflected with low RMSF (0.496 Å for human model and 0.526 Å for rat model). The binding sites 2 and 3, shown in Table 3, are considered the same owing to overlapping volume values.

Regarding conformational behavior, 12 (human) and 17 (rat) conformations clusters were found by using the root-mean-square deviation (RMSD) of 1.5 Å of protein backbone according to the algorithm (Daura et al., 1999) available in GROMACS. Of those clusters, the sum of the first five clusters equals more than 85% of the conformations obtained in the simulation and are distributed along the 10 ns of the MD simulation (Fig. 3, Table 4). Due to rapid conformational changes at the beginning of the simulation, only the relevant clusters after 1 ns were used to perform molecular docking of NAR and DHB.

3.4. Docking of NAR and DHB into CYP1A1

To help identify crucial amino acid residues involved in the inhibition of CYP1A1, computer modeling of the binding of NAR and DHB to CYP1A1 enzymes was performed. After analyzing the

Table 2
Enzyme kinetic analysis of CYP1A1 inhibition by NAR and DHB.

Compound	Parameter	Human CYP1A1	Rat CYP1A1
ER (Substrate)	Vmax (pmol/min/mg protein)	93 ± 1	6089 ± 133
	Km (μM)	0.14 ± 0.01	0.36 ± 0.03
NAR (Inhibitor)	Type of inhibition	Competitive	Mixed
	Ki (μM)	489 ± 22	0.17 ± 0.02
	K'i (μM)		0.39 ± 0.02
DHB (Inhibitor)	Type of inhibition	Competitive	Competitive
	Ki (μM)	55 ± 2	1.73 ± 0.03

Vmax and Km for 7-ethoxyresorufin O-deethylation by recombinant CYP1A1 and Ki for the inhibition of these enzymes by NAR and DHB were determined as described in the Materials and Methods. Data are expressed as mean ± SE for three independent experiments.

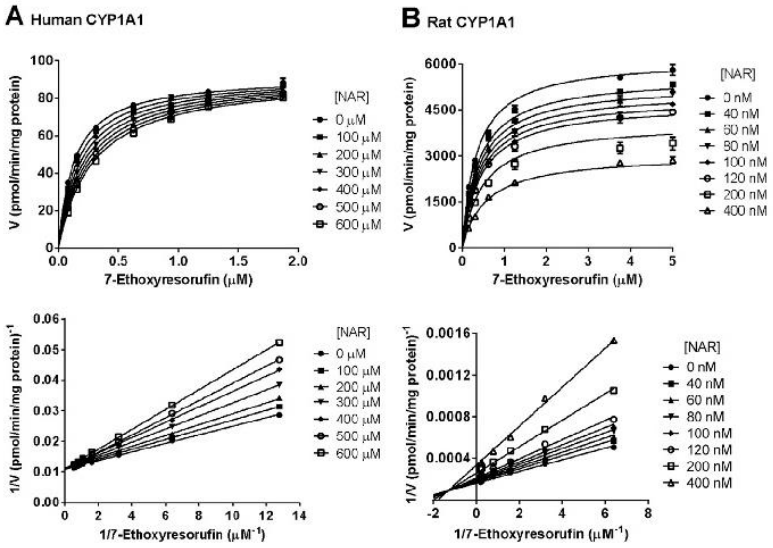

Fig. 1. Inhibition kinetics of NAR for human and rat CYP1A1 activity. Human (A) and rat CYP1A1 Supersomes® (B) containing 10 pmol/mL P450 were used in the assays. Top panels: the plots of velocity (V) versus substrate concentration. Bottom panels: the Lineweaver–Burk plots. The solid lines show the best fit determined by software of GraphPad Prism. Each point represents the mean ± SE obtained from three independent experiments.

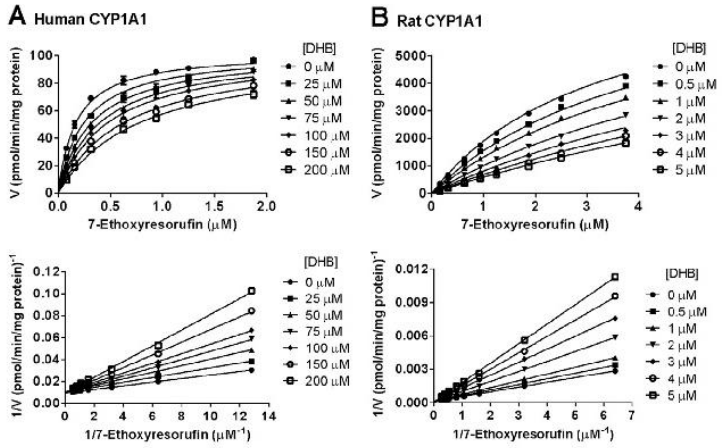

Fig. 2. Inhibition kinetics of DHB for human and rat CYP1A1 activity. Human (A) and rat CYP1A1 (B) containing 10 pmol/mL P450 were used in the assays. Top panels: the plots of velocity (V) versus substrate concentration. Bottom panels: the Lineweaver–Burk plots. The solid lines show the best fit determined by software of GraphPad Prism. Each point represents the mean ± SE obtained from three independent experiments.

docking results, two potential binding sites for both inhibitors were found relatively distant from the human CYP1A1 heme moiety, corresponding to the main binding sites identified in the conformations from de MD simulations (Table 3 and Fig. 4). The primary binding pocket, identified as the catalytic site (pink in Fig. 4), is overlapped with the binding site of the selective inhibitor α-naphthoflavone experimentally defined by crystal structure of the complex between human CYP1A1 and this compound (PDB: 4I8V). Most conformations obtained by docking simulations that were bound in the primary binding pocket are compatible with the reported orientation between the heme group and the oxidizable atom in the human CYP1A1-α-naphthoflavone crystal structure (Walsh et al., 2013) and with that suggested by Lewis et al. (1999) for rat CYP1A1.

From the docking simulations, ligand interaction diagrams were calculated and are shown in Fig. 5. In human CYP1A1, the number of interactions with the active site amino acid residues

Table 3
Volume of the main binding sites.

Binding site	Human CYP1A1	Rat CYP1A1
	Volume (Å³)	Volume (Å³)
1 (catalytic site)	374.2 ± 43.5	282 ± 139.2
2	250.6 ± 64.7	459.9 ± 172.7
3	175.3 ± 28.1	247.2 ± 34.4

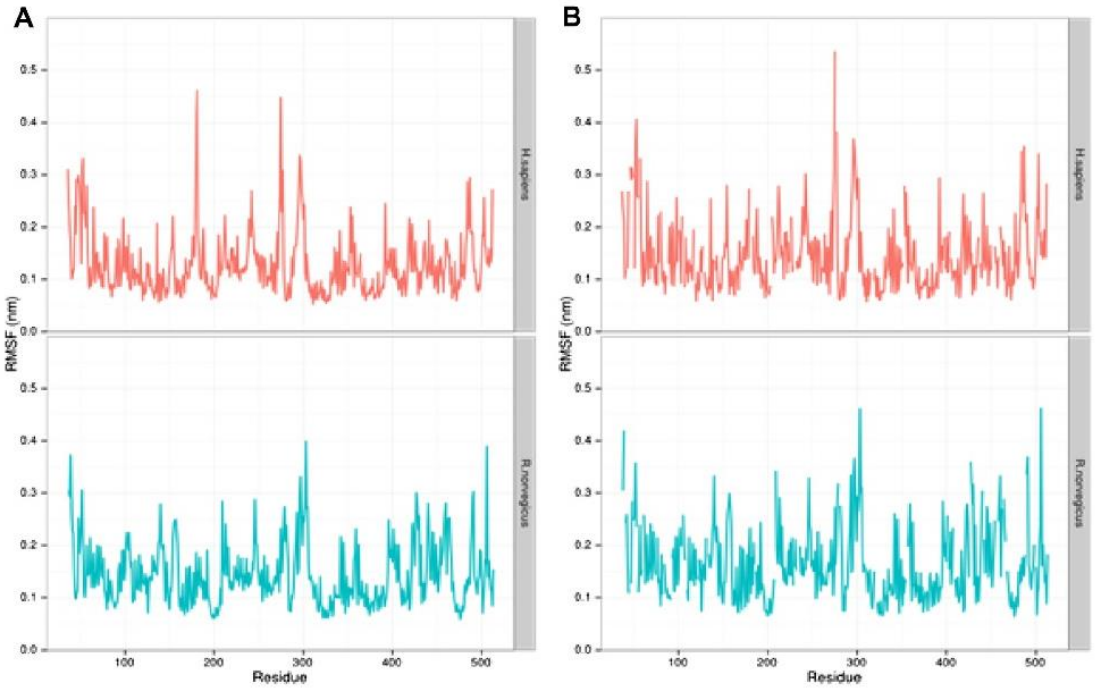

Fig. 3. Root-mean-square fluctuations (RMSF) of atomic positions for human and rat CYP1A1 obtained by MD simulations. (A) Backbone atoms' positions. (B) Sidechain atoms' positions.

Table 4
Main conformational clusters found in the MD simulations.

Human CYP1A1			Rat CYP1A1		
Cluster	Size	Representative conformation at time (ps)	Cluster	Size	Representative conformation at time (ps)
1	2664	5652	1	1436	8006
2	1125	332	2	1430	4310
3	516	9732	3	692	1814
4	293	1390	4	383	6660
5	285	3328	5	332	408

(Fig. 5A) was consistent with previous findings of a dominant π–π interaction between the inhibitor α-naphthoflavone and Phe224 in the active site of CYP1A1 (Walsh et al., 2013), and aromatic rings of inhibitors were involved in a similar interaction with the same residue. Additional stabilization was provided by further interactions with a number of amino acid residues in the binding pocket (Table 5), including the hydrophobic residues (Ile115, Ala317, and Leu496), the aromatic residues (Phe123, Phe258, and Phe319), and the polar residues (Ser122, Asn222, and Asp320). On the other hand, interactions of inhibitors with rat CYP1A1 occur

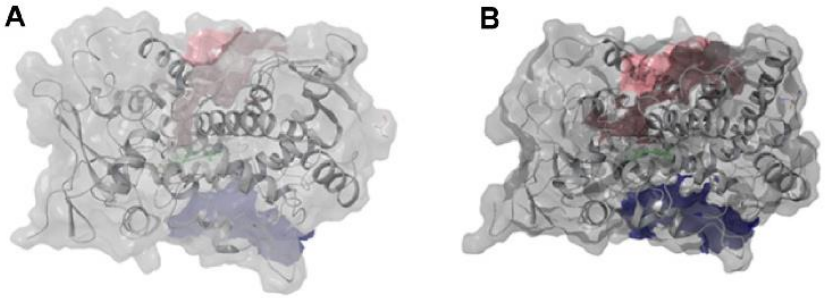

Fig. 4. Main binding sites obtained by MD simulations. (A) Human CYP1A1 and (B) rat CYP1A1. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

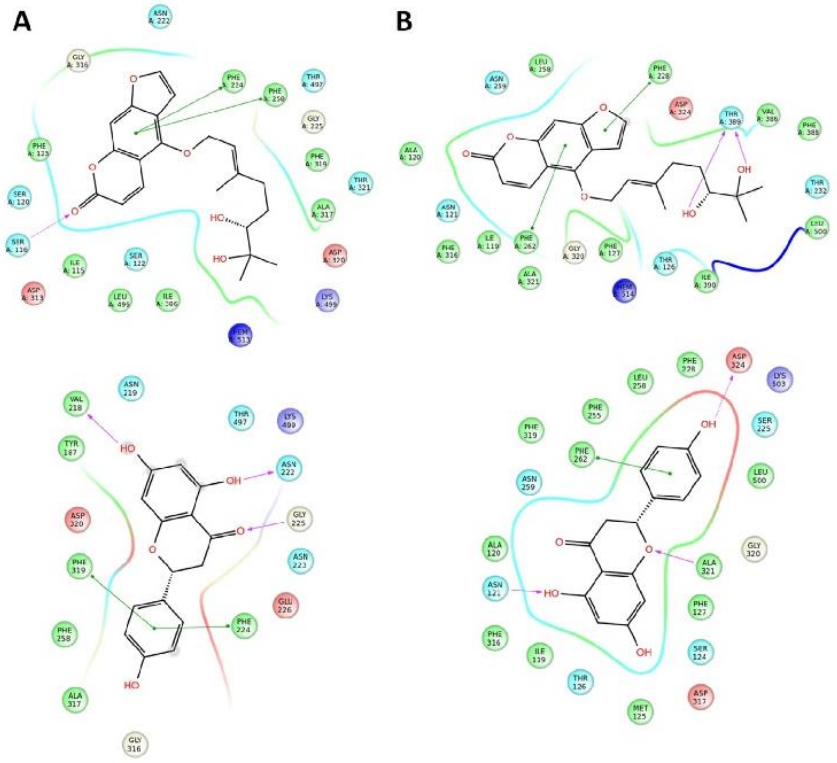

Fig. 5. Representative ligand interaction diagrams for DHB and NAB. (A) Human CYP1A1 and (B) rat CYP1A1.

Table 5
Docking scores of NAR and DHB in the catalytic site and neighboring residues at 4 Å.

Compound	Human CYP1A1		
	Conformation	Docking score (kcal/mol)	Residues at 4 Å[a]
NAR	1390 ps	NA	NA
	3328 ps	NA	NA
	5652 ps	−6.96	I115 S116 S120 S122 N222 F224 G225 L312 D313 G316 F319 D320 L496 T497 K499
	9732 ps	−6.34	Y 187 V218 N219 N222 N223 F224 G225 E226 F258 G316 A317 F319 D320 K499
	Mean	−6.65 ± 0.44	
DHB	1390 ps	NA	NA
	3328 ps	−8.87	I115 S120 S122 F123 Y187 V191 V218 N219 N222 F224 G225 V228 D313 G316 A317 F319 D320 T323 T324 L496 T497 K499
	5652 ps	−9.31	I115 S116 S120 S122 F123 N222 F224 F258 D313 G316 A317 F319 D320 T321 I386 L496 T497 HEM513
	9732 ps	−8.36	S122 F123 N222 F224 G225 F258 L312 D313 G316 A317 F319 D320 T321 V382 I386 L496 T497 HEM513
	Mean	−8.85 ± 0.48	
	Rat CYP1A1		
	Conformation	Docking score (kcal/mol)	Residues at 4 Å[a]
NAR	1814 ps	NA	NA
	4310 ps	−4.63	I119 A120 N121 S124 T126 F127 S225 F228 L258 N259 F262 F316 D317 G320 A321 F323 D324 L500 K503
	6660 ps	−7.19	I119 A120 N121 S124 N125 T126 F127 S225 F228 L258 N259 F262 F316 D317 G320 A321 D324 L500 K503
	8006 ps	−5.48	I119 A120 N121 S124 I221 V222 L224 S225 F228 L258 N259 F262 F319 G320 A321 F323 D324 L500 K503
	Mean	−5.77 ± 1.30	
DHB	1814 ps	−4.59	S115 F116 I119 F127 T232 G233 S234 G320 A321 D324 T325 V386 F388 T389 I390 P391 F412 L500 T501 HEM514
	4310 ps	−10.82	I119 A120 N121 S124 T126 F127 T232 S234 N259 F262 N266 F316 D317 F319 G320 A321 T325 V386 F388 T389 I390 L500 HEM514
	6660 ps	−8.70	I119 A120 N121 T126 F127 F228 T232 L258 N259 F262 F316 G320 A321 V386 F388 T389 I390 L500 HEM514
	8006 ps	−8.09	I119 A120 N121 S124 T126 I221 V222 L224 S225 F228 F255 L258 N259 F262 F316 D317 F319 G320 A321 F323 D324 L500
	Mean	−8.05 ± 2.59	

[a] Amino acids in one letter code.

mainly with hydrophobic residues (Ala120, Ala321, and Leu500), the aromatic residues (Phe127 and Phe228), and the polar residues (Asn121, Thr126, Thr232, and Asp324) located in the active site (Fig. 5B).

4. Discussion

Differences in CYP activity and inhibition between species have been reported before (Eagling et al., 1998; Guengerich, 1997; Hodek et al., 2002; Martignoni et al., 2006; Moon et al., 2006; Zhai et al., 1998). As reported by Eagling et al. (1998), the effects of inhibitors are not the same when using rat and human liver preparations. These differences in the effects of inhibitors on *in vitro* drug metabolism may be attributable to different reasons (Boobis et al., 1990), including the following: (i) the metabolism of a given substance is mediated by different isoforms depending on the specie involved; (ii) the inhibitory site is different between species when inhibition is not via direct competition at the active site, and (iii) the active site is different amongst different species.

Human CYP1A1 (512 amino acid residues) is smaller by 12 amino acids than its rat counterpart (524 residues) with which it exhibits 79% identity, and differences in amino acid composition include the active sites in both species (Ioannides, 2005). To the best of our knowledge, the comparison of the efficiency of human and rat CYP1A1 metabolism of the same compounds, or its sensitivity to be inhibited, has not been explored.

In order to detect possible differences in sensitivity between human and rat CYP1A to NAR and DHB, two important drug inhibitors present in grapefruit juice were tested. First, we used liver microsomes and found that NAR and DHB are capable of inhibiting the EROD activity in both rat and human microsomes (Table 1). Results obtained showed that rat liver microsomes were three orders of magnitude more sensitive than human microsomes to the inhibitory effects of NAR and DHB. Although EROD activity has been widely used as a marker for CYP1-specific activity (Ono et al., 1996), it is not a specific substrate for the uninduced hepatic CYP1A1 isoform. CYP1A2 may also participate in its metabolism (Burke et al., 1994); for this reason and to better understand the mechanism of inhibition, kinetic analysis was performed using recombinant CYP1A1 enzyme.

We hypothesize that differences in amino acid sequences between human and rat could affect its three-dimensional structure or catalytic site size, and thus, ligand recognition and affinity. Our studies on the metabolism of ethoxyresorufin by human and rat CYP1A1 demonstrated that rat enzyme is more active under identical conditions, showing a higher Vmax value (Table 2). Studying the kinetic parameters of EROD activity in human liver microsome samples from two nonsmokers, Williams et al. (1986) reported a Vmax of 60 and 185 pmol/min/mg protein. On the other hand, a Vmax of 6282 pmol/min/mg protein was reported for rat liver microsomes induced with benzo[a]pyrene (Floreani et al., 2013). These data are similar to those in Table 2 and support the results obtained with CYP1A1-enriched Supersomes® in this work.

The above-mentioned results may be explained by the nature of the CYP1A1 human and rat catalytic sites. The catalytic site in the rat enzyme is rich in threonine (Lewis et al., 1999) and the human in phenylalanine (Walsh et al., 2013), supporting different kinds of interactions between the catalytic site and xenobiotic compounds, as observed in this study with the analysis of molecular modeling.

Yamazaki et al. (2011) reported four residues in the active site for the metabolism of 3,3′,4,4′,5-pentachlorobiphenyl that are not conserved between human and rat CYP1A1 (Ser116/Ala 120, Ser 122/Thr126, Asn221/Ser225, Leu312/Phe316). These amino acids are present in all ligand-protein conformations found in this work (Table 5) and might play an important role in the differences in mechanism and potency of inhibition between species. Additionally, the above-mentioned amino acids for human CYP1A1 are involved in substrate recognition sequences reported by Kesharwani et al. (2014). We cannot rule out that the inhibitory effect observed is due to the metabolites of the compounds tested, which need to be evaluated in future research.

Docking assays suggested two potential binding sites for grapefruit compounds (Fig. 4). A secondary binding pocket (blue in Fig. 4) was found "below" the catalytic site and relatively exposed to the solvent; remarkably, this binding pocket in the rat CYP1A1 was larger than the catalytic site (Table 3), which could be related to the different ability to metabolize ligands between species. Because of the size of this binding site, some docking simulations with the best scores were posed in this pocket.

However, biochemical assays indicated the presence of one binding site for the grapefruit compounds on CYP1A1, because the secondary re-plot of slope or Y-intercept vs. [inhibitor] was linear (Supplementary Fig. S1). Nevertheless, we cannot rule out the possibility that other inhibitors can interact with the second binding site suggested by the computational analysis.

Substrate specificity of P450 enzymes in a single species varies from one isoenzyme to another. To understand the specificity of CYP1 isozymes to several ligands, several directed mutagenesis assays (Lewis et al., 2007; Liu et al., 2003; Liu et al., 2004) and *in silico* studies have been carried out (Don et al., 2003; Prasad et al., 2007; Rosales-Hernández et al., 2010; Sangamwar et al., 2008; Sridhar et al., 2010; Szklarz and Paulsen, 2002). These investigations have highlighted the importance of hydrophobic interactions, mostly due to π-stacking between CYP phenylalanine residues and aromatic rings of the studied compounds. Our data in Fig. 5 showing the participation of Phe224 and Phe262 in human and rat CYP1A1, respectively, agreed with these findings.

CYP1A1 is one of the major P450 enzyme involved in the metabolic activation of promutagenic compounds. Experimental data suggest that inhibition of this enzyme may lead to a decrease in cancer incidence in animals simultaneously exposed to promutagens, such as polycyclic aromatic hydrocarbons (Firdous et al., 2013; Gum et al., 2008; Zhao et al., 2008). Understanding the biochemical basis of CYP inhibition would provide crucial clues to develop potent and selective inhibitors that may be employed in cancer prevention (Cui et al., 2013; El Massry et al., 2012; Liu et al., 2012; Shimada et al., 2011; Sridhar et al., 2012).

Chemical inhibitors of CYP1 have been used in bioassays involving specific species; nevertheless, the selectivity of the inhibitors has not been investigated in detail (Bozcaarmutlu and Arinç, 2004; Cui et al., 2013; Shertzer et al., 1999; Takahashi et al., 1995). While the interpretation of these results might be accurate, in other instances the inferences might be incorrect. Therefore, the use of inhibitors in different species, without further characterization, must be interpreted with caution.

For instance, naringenin has been evaluated in several *in vivo* models of chemical carcinogenesis (Arul and Subramanian, 2013; Ekambaram et al., 2008; Krishnakumar et al., 2013). Treatment with naringenin effectively suppressed N-nitrosodiethylamine-initiated hepatocarcinoma and the associated preneoplastic lesions by modulating xenobiotic metabolizing enzymes, alleviating lipid peroxidation, and decreasing levels of liver marker enzymes (Arul and Subramanian, 2013). This compound acts as a chemopreventive agent against 7,12-dimethyl benz(a)anthracene-induced oral carcinogenesis (Krishnakumar et al., 2013) and gastric carcinogenesis induced by N-methyl N-nitro-N-nitrosoguanidine, where administration of naringenin regulated the redox status to decrease the risk of cancer (Ekambaram et al., 2008). Given the high sensitivity of rat (lower Ki) over human CYP1A1 inhibition by naringenin shown in this work, results from chemoprevention

studies in animal models may overestimate the protective potential in humans.

5. Conclusions

The present study represents a step forward in understanding the structural basis behind human and rat CYP1A1 interaction with its ligands and highlights the importance of considering the CYP species-specific differences when extrapolating results from animal models to human. Finally, it provides insight for the study and optimization of new CYP1A1 inhibitors. We cannot rule out that the inhibitory effect observed is due to the metabolites of the compounds tested, which need to be evaluated in future research.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2016.07.023.

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Research Article

Gas Chromatography-Mass Spectrometry Analysis of *Ulva fasciata* (Green Seaweed) Extract and Evaluation of Its Cytoprotective and Antigenotoxic Effects

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The chemical composition and biological properties of *Ulva fasciata* aqueous-ethanolic extract were examined. Five components were identified in one fraction prepared from the extract by gas chromatography-mass spectrometry, and palmitic acid and its ethyl ester accounted for 76% of the total identified components. Furthermore, we assessed the extract's antioxidant properties by using the DPPH, ABTS, and lipid peroxidation assays and found that the extract had a moderate scavenging effect. In an experiment involving preexposition and coexposition of the extract (1–500 μg/mL) and benzo[a]pyrene (BP), the extract was found to be nontoxic to C9 cells in culture and to inhibit the cytotoxicity induced by BP. As BP is biotransformed by CYP1A and CYP2B subfamilies, we explored the possible interaction of the extract with these enzymes. The extract (25–50 μg/mL) inhibited CYP1A1 activity in rat liver microsomes. Analysis of the inhibition kinetics revealed a mixed-type inhibitory effect on CYP1A1 supersome. The effects of the extract on BP-induced DNA damage and hepatic CYP activity in mice were also investigated. Micronuclei induction by BP and liver CYP1A1/2 activities significantly decreased in animals treated with the extract. The results suggest that *Ulva fasciata* aqueous-ethanolic extract inhibits BP bioactivation and it may be a potential chemopreventive agent.

1. Introduction

Marine seaweeds have been harvested for several years in the Far East and Asia Pacific countries, where they are consumed as food. In the last decade, this practice has also extended to North America and Europe [1]. At present, the economic potential of the seaweed industry is widely recognized [2]. In addition, seaweeds are considered an attractive avenue for the screening of biologically active compounds, due to their biodiversity and safety [2, 3].

Until now, the seaweed species studied have shown variations in their chemical composition (proteins, carbohydrates, lipids, minerals, and vitamins) associated with the influence of environmental factors such as seasonal periods, temperature, light, salinity, location, and storage conditions [4]. Seaweeds are able to produce secondary metabolites with interesting bioactive properties, including antibacterial, antifungal, antiviral, and antioxidant effects [5–9]. *Ulva fasciata* Delile, also known as sea lettuce, grows abundantly along coastal seashores. The antioxidant and antibacterial

properties of this seaweed have been previously reported [10, 11].

Many extracts or partially purified polysaccharides from various algae have shown antitumor activity against experimental tumors in animal models [2, 12, 13]. The mechanism underlying this effect could be related to their antioxidant properties or control of cell proliferation. Additionally, Ryu et al. [14] showed that the ethanolic extract of *U. fasciata* has anticancer activity associated with the modulation of apoptotic signals, including mitochondria- and caspase-dependent processes, in human colon cancer HCT116 cells.

Cancer is a serious global health problem and the primary cause of morbidity and mortality in Cuba [15]. Thus, the search for novel nutraceuticals with potential benefits for the prevention or therapy of cancer is well justified. An aqueous-ethanolic extract of *U. fasciata* collected from the north coast of Cuba was obtained and analyzed. Phytochemical study of this extract shows a high content of chlorophyll b, carotenoids, protein, carbohydrates, fiber, Ca, Mg, K, Fe, Zn, Cr, and Mn [16].

In order to contribute to the chemical characterization of the *U. fasciata* extract, here we report some nonpolar constituents of the extract. A chloroform-diluted fraction of the extract was prepared and its composition was determined by using gas chromatography-mass spectrometry (GC-MS). We also studied the protective effects of the whole extract by assessing its ability to protect against benzo[a]pyrene-(BP-) induced cytotoxicity in C9 hepatic cells in mice. The antioxidant capacity and inhibitory effects of *U. fasciata* on CYP1A1/2 and CYP2B1/2 activities involved in the metabolism of several human mutagens/carcinogens were also investigated.

2. Materials and Methods

2.1. Chemicals. Analytical-grade reagents and reference substances were obtained from Aldrich (Milwaukee, MN, USA). Phenobarbital (PB) was purchased from Abbott Laboratories (Mexico City, Mexico). Beta-naphthoflavone (β-NF), resorufin, 7-ethoxyresorufin (ER), methoxyresorufin (MR), benzyloxiresorufin (BR), pentoxyresorufin (BR), NADPH, BP, 2,2-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), thiobarbituric acid (TBA), corn oil, and Giemsa stain were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Microsomes of *Baculovirus* expression systems from rat CYP1A1-expressing insect cells (Supersomes) were purchased from BD-Gentest (Woburn, MA, USA).

2.2. Material. Ulva fasciata Delile (Chlorophyta) was collected from the estuary of Quibu River in Cuba (82°27′48″W and 23°53′04″N). The seaweeds were collected by hand from the intertidal zone in October 2013. After collection, the materials were immediately washed to remove epiphytes and sand and transported to the laboratory. After washing with distilled water, the samples were dried at 60 ± 1°C to constant weight, milled, and stored desiccated in plastic receptacles. Fifty grams of dried *U. fasciata* powder was continuously macerated with 500 mL of ethanol : H_2O (1 : 1 vol/vol) for 24 h at room temperature. The extract obtained was filtered and concentrated to dryness under reduced pressure at 45°C.

2.3. Gas Chromatographic/Mass Spectrometric Analysis. One hundred milligrams of the dried extract was partitioned within $CHCl_3/H_2O$ (1 : 1 v/v). The resulting crude organic phase was filtered and concentrated to dryness under reduced pressure at 45°C by using a rotary vacuum evaporator. Then, the fraction obtained was analyzed by gas chromatography-mass spectrometry (GC-MS).

The analyses were performed using a GC-MS system (Model QP 2010 series, Shimadzu, Tokyo, Japan) equipped with an autosampler model AOC-20i and an RTX-1 fused silica capillary column of 30 m in length, 0.25 mm in diameter, and 0.1 μm of film thickness. The column oven temperature was programmed from 50 to 300°C for 2°C min^{-1}. Ionization of the sample components was performed in electron impact mode (EI, 70 eV). The temperature of the injector was fixed at 300°C and that of the detector at 310°C. Helium (purity, 99.9995%) was the carrier gas; its flow rate was fixed at 1 mL min^{-1}. The mass range from 40 to 1000 m/z was scanned at a rate of 3.0 scans/s. One microliter of the organic extract of *U. fasciata* was manually injected into the GC-MS system by using a Hamilton syringe, for total ion chromatographic analysis by split injection (1 : 40). The total running time of the GC-MS system was 15 min. The relative percentage of each extract constituent was expressed as percentage with respect to peak area normalization. The conversion of analog data to digital data was performed using the GC Solution software.

2.4. Antioxidant Study

2.4.1. Assay of 2,2-Diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) Scavenging Activity. The antioxidant capacity of the extract was measured as DPPH radical scavenging ability according to the method described by Tabart [17] with minor modifications. DPPH (1500 μL) in ethanol (0.075 mg/mL) was mixed with 750 μL of the extract at five different concentrations (10–1500 μg/mL). A control sample (ethanol) and a reference sample (ethanol plus DPPH) were used. The antioxidant ascorbic acid was used as a positive control. The decrease in the absorbance (Abs) at 515 nm was determined using a UV-1201 spectrophotometer (Shimadzu, Japan), until the reaction plateau step was reached. The IC_{50} values were determined, where they represent the concentration of extract that caused 50% inhibition of the maximum effects, and the scavenging effect was calculated as a percentage of DPPH scavenged, where % DPPH inhibition = (control Abs − sample Abs)/(control Abs) × 100, control Abs = ethanol + DPPH Abs and sample Abs = sample + DPPH Abs.

2.4.2. Assay of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic Acid) ($ABTS^{\bullet+}$) Radical Scavenging Activity. The scavenging activity of *U. fasciata* extract was also tested as previously described [18]. Briefly, $ABTS^{\bullet+}$ solution (7 mM) was mixed with potassium persulfate (2.45 mM) and stored during 16 h in the dark to generate the $ABTS^{\bullet+}$ radical cations. Then, the $ABTS^{\bullet+}$ absorbance was adjusted to 0.70 ± 0.02 at 734 nm. Free radical scavenging activity of *U. fasciata* was assessed by

mixing 300 μL of test sample with 3.0 mL of $ABTS^{\bullet+}$ radical solution. The decrease in absorbance at 734 nm was measured after six minutes. The percentage inhibition was calculated as follows: Scavenging activity (%) = (control Abs − sample Abs)/(control Abs) × 100. The antioxidant capacity of *U. fasciata* was expressed as IC_{50} values (μg/mL).

2.4.3. Assessment of Lipid Peroxidation. The capacity of the inhibition of liver lipid peroxidation by *U. fasciata* extract was assessed as described elsewhere [19] with slight modifications. The livers of male mice were removed and placed on ice. One gram of tissue was homogenized in cold 0.1 M Tris buffer at pH 7.4 (1 : 10 w/v) in a tissue homogenizer (Sakura, Japan). The homogenates were centrifuged at 12000 rpm for 5 min at 4°C. Then, the extract was incubated at 37°C for 1 h with 1 mL of the homogenate solution. Afterward, 8.1% sodium dodecyl sulfate (SDS), 1.33 M acetic acid (pH 3.4), and 0.6% thiobarbituric acid (TBA) were added to the medium. The reaction mixture was incubated at 97°C for 1 h and later the absorbance was measured at 532 nm. TBARS concentrations were estimated from a standard curve of malondialdehyde bis-(dimethyl acetal) and reported as nmol MDA/mg protein. Data was expressed as percentage of inhibition. It was calculated from the absorbance values of the control and experimental tubes and IC_{50} value was calculated. Experiment was repeated three times and values were represented as mean ± S.E. of the experiments. Protein concentration was also determined [20].

2.5. Effects of U. fasciata Extract on BP-Induced Cytotoxicity in Hepatic C9 Cells. Rat hepatocytes clone 9 culture (gift from Dr. M. Marina-Silva, IFC, UNAM, Mexico) was grown in DMEM supplemented with 10% newborn calf serum, 50 U penicillin/mL, and 50 μg streptomycin/mL. For subcultures, 5×10^5 cells at 1 : 10 dilution were plated in a 100 mm petri dish. The medium was changed every three days and the cells were harvested at ~100% confluence with 0.25% trypsin-EDTA. For treatments, C9 cells were seeded at a density of 5,000 cells/well and allowed to grow and equilibrate for 24 h.

To explore the potential toxicity of *U. fasciata*, the cells were exposed to a range of extract concentrations (10–1000 μg/mL) for 24, 48, and 72 h. For experiments conducted to evaluate cytoprotection, the concentrations of the extract tested were 1, 5, 10, 100, and 500 μg/mL at a BP concentration of 10 μM.

In the first experimental series, the cells were pretreated with increasing concentrations of the extract for 12 h. Then, they were treated with the extract and BP for an additional period of 6 h. In the second experimental series, the cells were treated with the extract for 12 h; they were then washed and exposed to BP or to the extract plus BP for an additional 24 h or 48 h period. A third experimental series was performed in which the cells were exposed to both the extract and BP for 24 or 48 h. Finally, cell viability was evaluated by conducting the [3-4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [21]. The percentage of cell viability was calculated relative to that of the nontreated cells, which were assumed to be 100% viable.

2.6. Effects on CYP1A and CYP2B Isoforms in Rat Liver Microsomes

2.6.1. Preparation of Liver Microsomes. For the *in vitro* assays, liver microsomes were obtained from the phenobarbital and 5,6-β-naphthoflavone-induced S_9 fraction [22]. For the *in vivo* assay, microsomes were prepared from the liver of animals exposed to *Ulva fasciata* extract or controls. Livers were excised, washed, and homogenized in 0.15 M KCl solution. The homogenate was centrifuged for 10 min at 9000 ×g and the supernatant was collected (S_9 fraction). The S_9 fraction was further centrifuged at 100,000 ×g for 60 min and the pellet was resuspended in 0.1 M phosphate buffer (pH 7.4) and 0.25 M sucrose and centrifuged again at the same conditions. The microsomal fraction was resuspended in 0.1 M phosphate buffer (pH 7.4), 1 mM EDTA, 0.1 mM dithiothreitol (DTT), and 20% v/v glycerol. Protein concentration was determined [20] and the microsomal fraction was kept at −80°C until use.

2.6.2. CYP1A and CYP2B Activities. The activities of CYP1A1-related ethoxyresorufin-*O*-deethylase (EROD), CYP1A2-related methoxyresorufin-*O*-demethylase (MROD), CYP2B1-related pentoxyresorufin-*O*-dealkylase (PROD), and CYP2B2-related benzyloxy-resorufin-*O*-dealkylase (BROD) were measured as described elsewhere [23] with minor modifications. The extract was added to the incubation mixtures as an aqueous solution. The incubation mixture containing different concentrations of the extract (or water as control), rat liver microsomes (0.01–0.1 mg), substrate (1 μM ER, 5 μM MR, 5 nM PR, or 20 nM BR), and buffer with pH 7.6 (50 mM Tris-HCl, 25 mM $MgCl_2$) was incubated for 3 min at 37°C. The reaction was started by the addition of NADPH (0.5 mM) and was monitored for 3 min, with the fluorescence signal being recorded every 15 s. The activities were calculated from a standard curve of resorufin (5–50 pmol/mL).

2.7. Biochemical Characterization of the Extract's CYP1A1 Inhibitory Effect. The final reaction mixture contained 1 pmol CYP1A1 Supersome, 0.5 mM NADPH, different ER concentrations, and 0, 10, 20, 30, 40, and 50 μg/mL of *U. fasciata* extract. Appropriate controls without the tested extract were established. The reaction was started with the addition of NADPH. V_{max} and K_m values were obtained from the $1/y$- and $1/x$-intercepts of a Lineweaver-Burk plot after incubation at 37°C for 10 min. Furthermore, a kinetic analysis was performed by using the Dixon plot and replot of the Y-intercept of the Linewaver-Burk plot.

2.8. Antigenotoxic Effects of the Extract on the DNA Damage Induced by BP. Male BALB/C mice (18–20 g) were obtained from Biomedical Research Institute, National University of Mexico (UNAM, Mexico, DF, México). The animals were adapted to standard conditions (temperature: 20 ± 2°C, humidity: 40–60%, and 12 h light/dark cycle) for one week. They were fed with a commercial standard rat diet and water *ad libitum*. The experiment was conducted in accordance with the ethical guidelines for investigations with laboratory animals of the Institute of Biomedical Research, UNAM.

The study was conducted using 4 experimental groups (5 animals per group). The animals were orally administered 10, 100, and 250 mg/kg *U. fasciata* extract for 5 days. The control group received only distilled water (vehicle). One hour after administration of the last dose of the extract, the animals received 250 mg/kg BP by the oral route. After 24 h, the animals were sacrificed and their livers were removed, weighed, washed (0.15 M KCl), and conserved at −70°C until the microsomal fraction was obtained.

2.8.1. Liver CYP1A1/1A2 Activities in Mouse Liver Microsomes. The formation of resorufin after *O*-dealkylation of 7-ethoxyresorufin and 7-methoxyresorufin were measured in microsomes from treated and control animals is as described above.

2.8.2. Determination of Micronuclei (MN) Induction. Once the animals were sacrificed, both femurs were removed and freed from skin and muscle by traction. The proximal end of the femur was carefully shortened until the marrow canal became visible. One milliliter of serum was introduced into the bone canal and the femur was submerged in a centrifuge tube filled with fetal calf serum. The marrow was aspirated and flushed several times. Cells were centrifuged at 1000 rev/min for 5 min. Two drops of the cell fraction were placed onto clean, dry slides and smeared, fixed in methanol, and stained with Giemsa 5% (v/v) for 12 min. The percentage of micronucleated cells was determined using a sample of 2000 polychromatic erythrocytes (PCE). Normochromatic erythrocytes (NCE) were also scored in 2000 erythrocyte samples to determine the PCE/NCE ratio [24].

2.9. Statistical Analysis. Results were expressed as the mean ± SD from three independent experiments. The statistical analysis was performed by one-way ANOVA, followed by a Dunnett's test for multiple comparisons using the GraphPad Prism 5 statistical software package. Statistical significance (p) was set at 0.05.

3. Results

3.1. Chemical Characterization of U. fasciata Extract. The chromatogram obtained from the GC-MS analysis of the fraction obtained after elution with $CHCl_3/H_2O$ (1 : 1 v/v) from the *U. fasciata* extract showed 8 chromatographic peaks from 6 to 10 min and 5 new components were identified (Figure 1, Table 1). Most of the compounds and their derivatives were acidic in nature. The main component was palmitic acid (51.3%) and its ethyl ester (24.7%). These results increase the knowledge on the phytochemical composition of *U. fasciata* extract and it should add to the understanding of the product pharmacological properties.

3.2. Antioxidant Capacity of U. fasciata Extract. As the antioxidant activity is usually system-dependent, three methods were used to test the antioxidant effectiveness of this product, including two based on the evaluation of the free-radical scavenging capacity of the extract in a cell-free system and one based on its effects on lipid peroxidation in liver homogenates. Results concerning the antioxidant capacity of the extract are shown in Figure 2. As it can be seen in cell-free systems, the extract showed a moderate scavenging effect, showing IC_{50} values of 155.3 μg/mL and a maximum effect of 58.26% at the concentration of 500 μg/mL in the DPPH assay. Meanwhile, for the $ABTS^{\bullet+}$ radical the IC_{50} value was in the order of 240.4 μg/mL and the maximum effect was observed at 500 μg/mL. In accordance with these results, *U. fasciata* inhibited lipid peroxidation when added to liver homogenates, showing IC_{50} values of 259.4 μg/mL. In all the cases, the positive control (ascorbic acid) showed the expected effect (Figure 2).

Table 1: Compounds isolated from *Ulva fasciata* extract.

Compounds	RT (min)	Area (%)
Azelaic acid	6.205	3.08
NI	6.886	2.79
n-Pentadecanoic acid	6.922	1.21
Hexahydrofarnesyl acetone	7.344	11.84
Palmitic acid	7.787	51.31
Palmitic acid ethyl ester	7.928	24.67

RT: retention time %; relative percentage of the each extract constituent expressed as percentage with respect to peak area normalization.

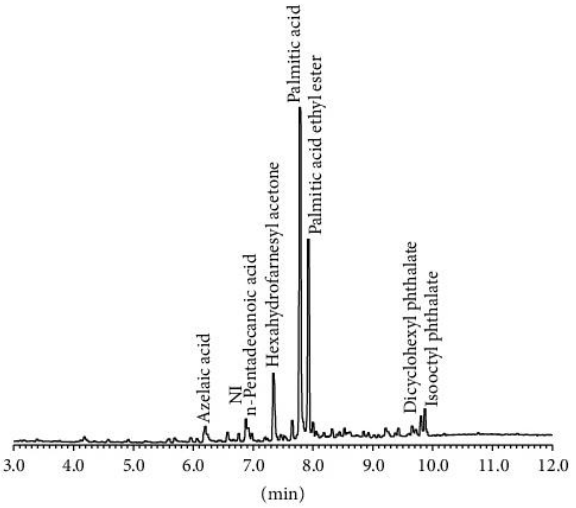

Figure 1: Chromatogram obtained by GC/MS analysis of the extract of *Ulva fasciata*.

3.3. Effects of U. fasciata Extract on the BP-Induced Cytotoxicity in Hepatic C9 Cells. *In vitro* toxicity effects of *Ulva fasciata* extract were evaluated in the C9 cell line. No significant cytotoxic effects were observed after exposing cells to *Ulva fasciata* for 24–72 h at any of the concentrations tested (10–1000 μg/mL) (data not shown). In order to evaluate the potential cytoprotection of *Ulva fasciata* against the toxicity induced by the BP, the viability of C9 cells was scored after different treatment protocols. Exposure of C9 cells to BP induced the expected reduction in cell viability; however,

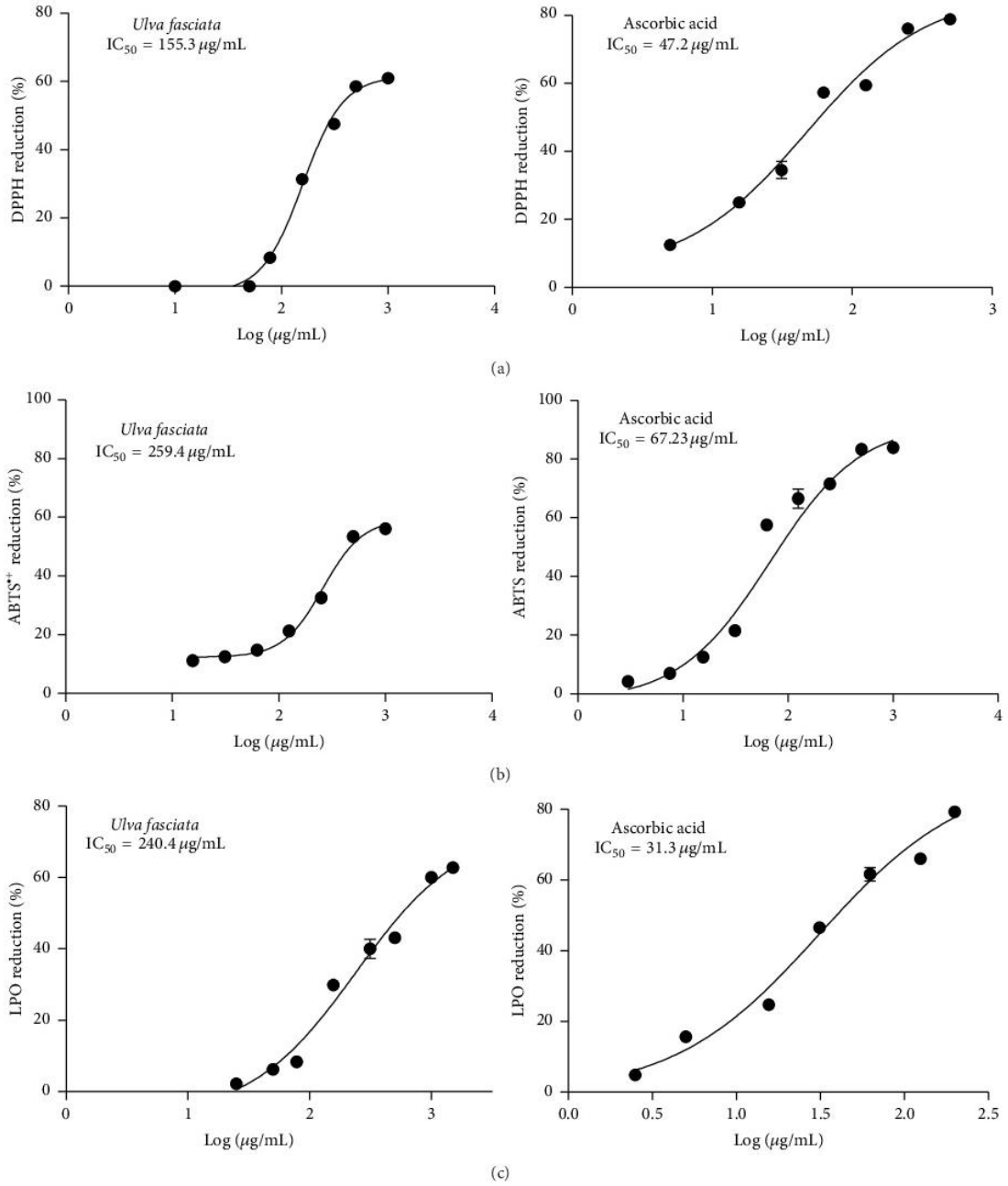

FIGURE 2: Antioxidant effects of *Ulva fasciata* extract. (a) DPPH radical, (b) $ABTS^{\bullet+}$ radical, and (c) lipid peroxidation assay. Results represent the mean value ± SD of three experiments carried out in triplicate. Antioxidant effectiveness was expressed as IC_{50}. Ascorbic acid was used as standard. Coefficients of covariance were minor of 15 percent in all the cases.

a noticeable recovery was observed when the cells were pre-exposed to the extract (Figure 3). A similar level of recovery was noted in the cells coexposed to the extract plus BP, whereas the protective effect was more evident at the lower concentrations tested (1 and 5 μg/mL), being significantly different than control cells. In addition, the exposure time where the effects of the extract were more consistent was 48 h (Figure 3).

3.4. In Vitro Effect of U. fasciata Extract on Hepatic P450 Activities. The activities of CYP1A1, CYP1A2, CYP2B1, and CYP2B2 were measured in induced rat liver microsomes

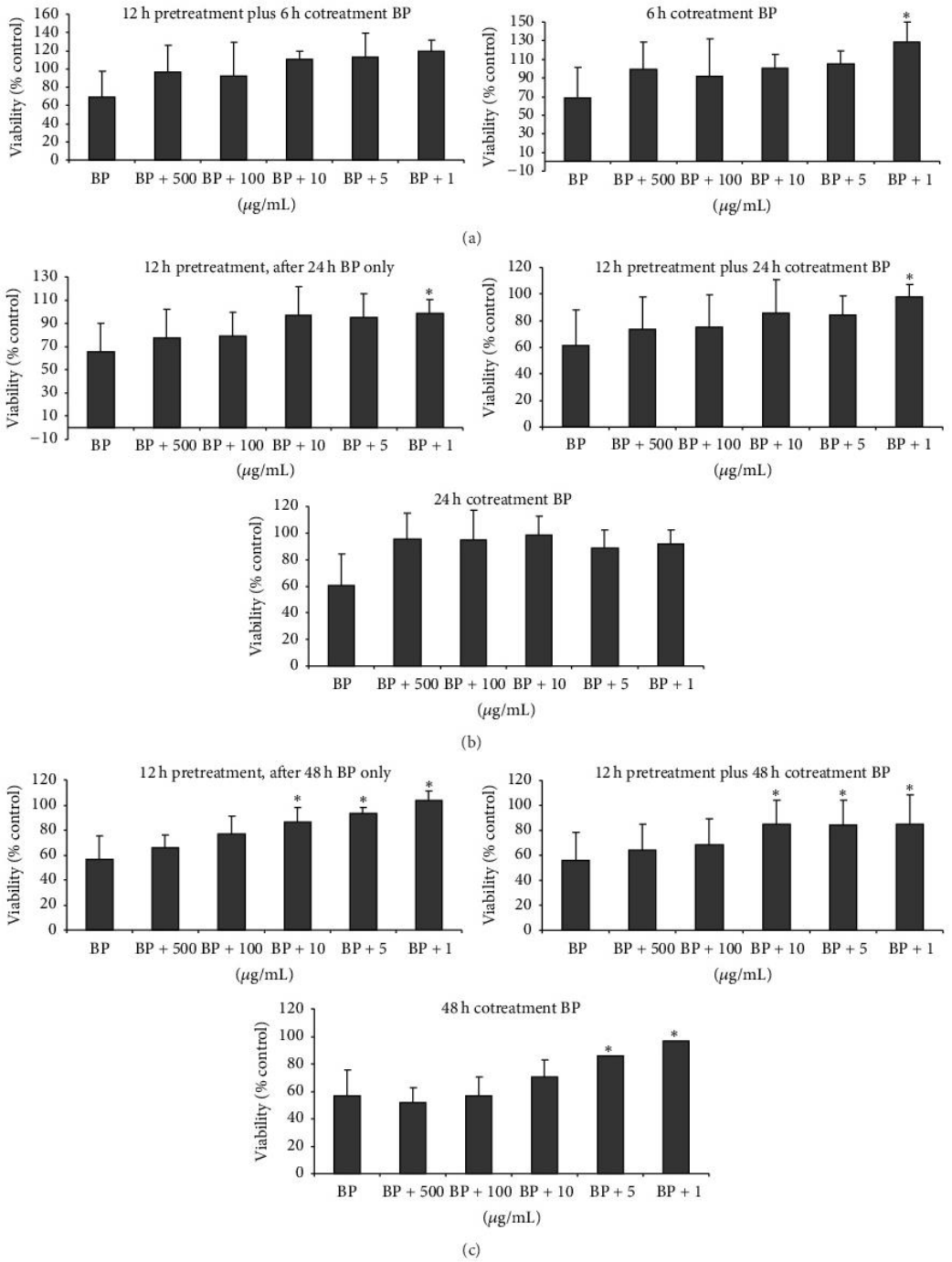

FIGURE 3: Effects of the *Ulva fasciata* extract on benzo[a]pyrene- (BP-) induced toxicity to rat C9 cells. (a) Cells were exposed for 12 h to increasing concentrations of *Ulva fasciata* extract and for an additional 6 h period in the presence of the extract and BP, or they were exposed for a 6 h period to both products. (b) Cells were exposed for 12 h to increasing concentrations of the extract; then, they were washed and exposed to the extract plus BP or BP only for an additional 24 h period. In the other condition, cells were exposed to both products for 24 h. (c) Cells were treated in conditions similar to those described in (b); however, in this case, the additional period was 48 h long. In all series, the BP concentration used was 10 μM. Finally, cell viability was determined by the MTT assay. Results are expressed as the percentage of control (untreated) cells. Each point represents the mean ± SD of three experiments with three replicates. $^*p < 0.05$ in relation to cells treated to the toxin (Dunnett's test).

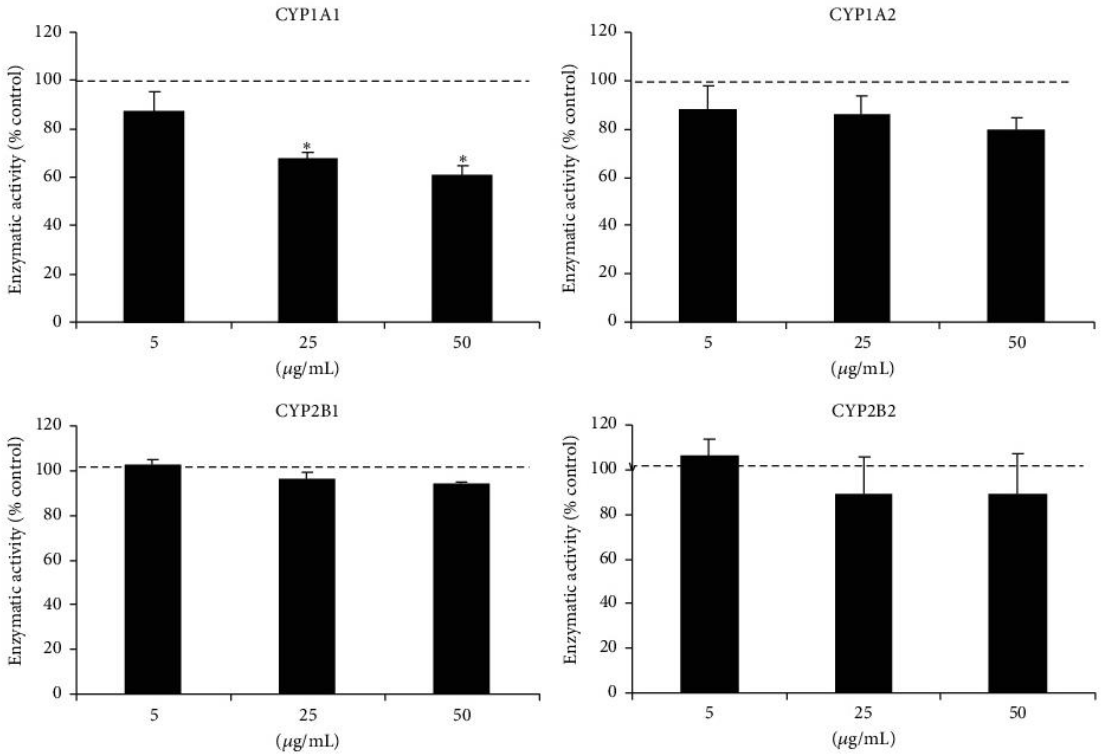

FIGURE 4: Effects of *Ulva fasciata* extract on microsomal P450 activities in rat PB-induced microsomes. P450 isoform activities were assayed in rat PB-induced microsomes incubated with appropriated substrates and *Ulva fasciata* concentrations. The values represent mean ± SEM of three independent experiments, $^{*}p < 0.05$ (Dunnett's test).

alone, or in the presence of the extract at concentrations of 5, 25, and 50 μg/mL. As seen in Figure 4, the extract produced a significant reduction of 35–40% in CYP1A1-associated EROD activity with respect to the activity recorded in the controls. A slight nonsignificant inhibition was observed for CYP1A2-associated MROD activity. No appreciable changes in CYP2B activity were found. Taking into account the above-mentioned results, we decided to investigate the type of inhibition produced by the extract on CYP1A1 catalytic function. The kinetic parameters of rat recombinant CYP1A1 (Supersomes), using ethoxyresorufin as the substrate, were V_{max} = 2677 ± 51 pmol/min/mg protein and K_m = 1.44 ± 0.07 μM (Table 2). CYP1A1 activity was inhibited in the presence of the extract at concentrations of 10–50 μg/mL (Figure 5), with a Ki of 67.9 ± 9.3 μg/mL (Table 2). Results in Figure 6 show the mixed-type inhibitory mechanism of the *U. fasciata* extract.

3.5. Effects of U. fasciata Extract on BP-Induced Genotoxicity in Male BALB/C Mice. The results in Table 3 shows that pretreatment of animals with different doses of the *U. fasciata* extract prevented an increase in the frequency of micronucleated bone marrow polychromatic erythrocytes (MNPCEs) observed in bone marrow cells of BP-exposed animals. The reductions ranged from 47 to 65%. Compared to the controls, the treated animals groups showed no significant changes in the cytotoxicity index. Additionally, no significant differences in body weight and water consumption were observed between the groups (data not shown).

TABLE 2: Effect of *Ulva fasciata* extract on kinetics parameters of CYP1A1 Supersome.

Kinetic parameters	*Ulva fasciata*
V_{max} (pmol/min/mg protein)	2677 ± 51
K_m (μM)	1.44 ± 0.07
Type of inhibition	Mixed type
Ki	67.9 ± 9.3 μg/mL
αKi	130.8 ± 15.5 μg/mL

On the other hand, the effects of the *U. fasciata* extract on the enzymatic activity of CYP1A1 and CYP1A2 in liver microsomes from the treated animals and controls are shown in Figure 7. Mice orally exposed to the extract for 5 days before BP exposure showed a decrease in hepatic CYP1A1 activity compared to the activity recorded in the BP-positive control group.

4. Discussion

The use of chemoprotective agents in everyday life has been suggested to be effective in preventing the increase of cancer

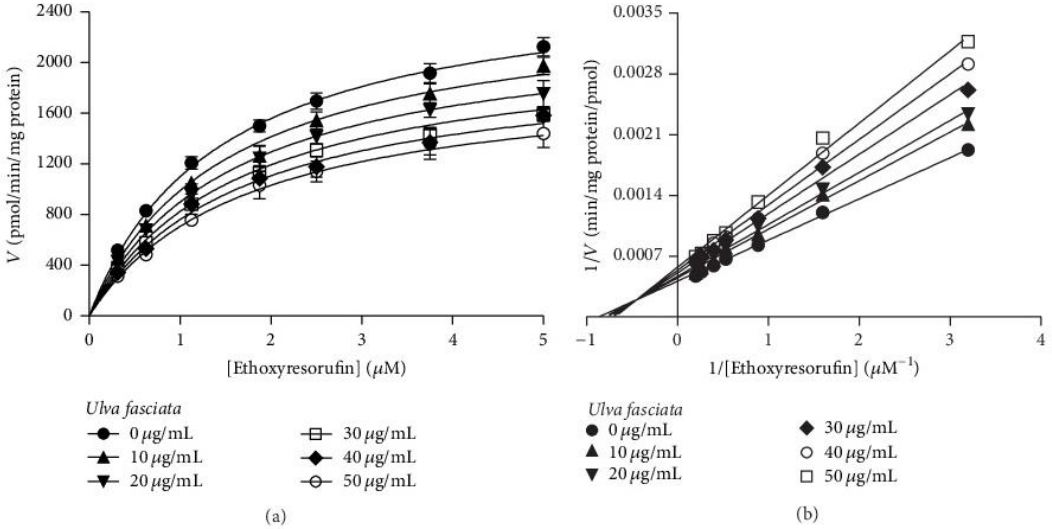

FIGURE 5: CYP1A1 associated ethoxyresorufin-*O*-deethylase activity in the absence and presence of different concentrations of *Ulva fasciata*. (a) Reaction in a final volume of 200 μL was monitored for 10 min recording the fluorescence signal each 15 s. The reactions consisted of 1 pmol Supersome protein, 50 mM NADPH, and ethoxyresorufin at different concentrations. For the inhibition experiments, the extract was added at different concentrations to the reaction mixture. (b) Lineweaver-Burk plot analysis was performed to obtain the kinetic parameters. Each point in (a) represents the mean ± SD obtained from three independent experiments.

TABLE 3: Effects of *Ulva fasciata* extract on micronucleus assay in Balb/C male mice.

Group (mg/kg)	IC	MN/PCE
Negative control	10.8 ± 0.28	4.3 ± 0.8
Vehicle control	2.1 ± 0.47	6.6 ± 2.6
Benzo(a)pyrene only	2.23 ± 0.75	15.3 ± 2.6
Benzo(a)pyrene + *Ulva* (50 mg/kg)	2.48 ± 0.50	7.9 ± 1.9**
Benzo(a)pyrene + *Ulva* (100 mg/kg)	1.88 ± 0.25	5.3 ± 1.4**
Benzo(a)pyrene + *Ulva* (250 mg/kg)	2.63 ± 0.47	5.15 ± 0.65**

MN: micronucleus; PCE: polychromatic erythrocytes; 2000 cells/animal were examined; IC: cytotoxicity index: polychromatic erythrocytes/normochromatic erythrocytes; ** $p < 0.01$; comparisons between treated groups with the extract plus PB and the control group treated with BP only (Dunnett's test).

frequency in human populations. For instance, many dietary antioxidants were shown to be potentially beneficial agents by reducing oxidative stress involved in the development of different chronic diseases, including cancer [25].

Natural products contain bioactive constituents that potentially block or reverse the carcinogenesis process at early stages. Therefore, modification in lifestyle habits, including diet, may lead to a reduction in the incidence of these diseases [25]. The central point of this strategy is that dietary constituents may inhibit carcinogenesis through different mechanisms. Blocking biotransformation of procarcinogens through inhibition of the CYP system is one of them. During phase I of xenobiotic metabolism, polar metabolites are formed which are substrates for phase II enzymes in order to render easily excreted products. Nevertheless, metabolites resulting from phase I may be highly electrophilic and carcinogenic, capable of interacting with DNA, and causing mutations [26, 27].

In the present study, we demonstrated the *in vitro* scavenging properties and protective effects of *U. fasciata* extract on BP-induced damage, a recognized human carcinogen. The extract itself was not cytotoxic to hepatic C9 cells but it showed a cytoprotective effect against BP-induced cytotoxicity (Figure 3). Surprisingly, lower concentrations of the extract (1 μg/mL) completely abolished the cytotoxic effect of BP. Since BP exposure should give rise to electrophilic metabolites and the extract showed moderate scavenging activity measured as DPPH, ABTS, and lipid peroxidation assays (Figure 2), the cytoprotection observed may be due to a decrease of the oxidative stress in C9 cells induced by *U. faciata* extract. On the other hand, the significant *in vitro* and *in vivo* inhibitory effects of the extract on CYP1A1 activity (Figures 3 and 7) may play an important role in the protection from BP-induced damage. The extract showed mixed-type inhibition kinetics with increasing K_m and decreasing V_{max} as the extract concentration increased (Figures 5 and 6, Table 2). These results suggest that the active compound(s) in the extract might bind to both the active site of the enzyme and an allosteric site. To our knowledge, this is the first report concerning the potential chemopreventive effects of this green seaweed on DNA-induced damage.

It is known that BP induces genetic lesions such as DNA single-strand breaks, DNA–protein cross-links, and chromosomal aberrations [28]. In an attempt to demonstrate the *in vivo* antigenotoxic potential of the extract, we explored its capacity to reduce the frequency of micronuclei induced by BP. The levels of BP-induced micronuclei were found to

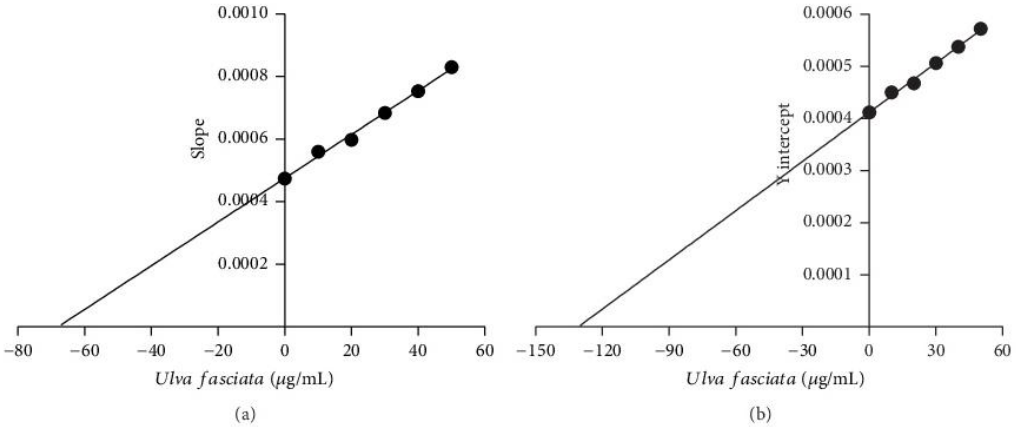

FIGURE 6: Confirmation of the CYP1A1 inhibitory properties of *Ulva fasciata* extract by (a) Dixon plot and (b) Y-intercept of the Linewaver-Burk plot versus inhibitor's concentration. Each plot was obtained from independent reactions containing the desired concentration of ethoxyresorufin (0.31–5.00 μM), 1 pM Supersome protein, 50 mM NADPH, and different concentrations of the extract in a final volume of 200 μL. Each reaction was followed for 10 min, with the fluorescence signal being recorded every 15 s. Each point in (a) represents the mean ± SD obtained from three independent experiments. Slope of each plot in (a) was obtained and plotted versus the inverse of the concentration of ethoxyresorufin (b).

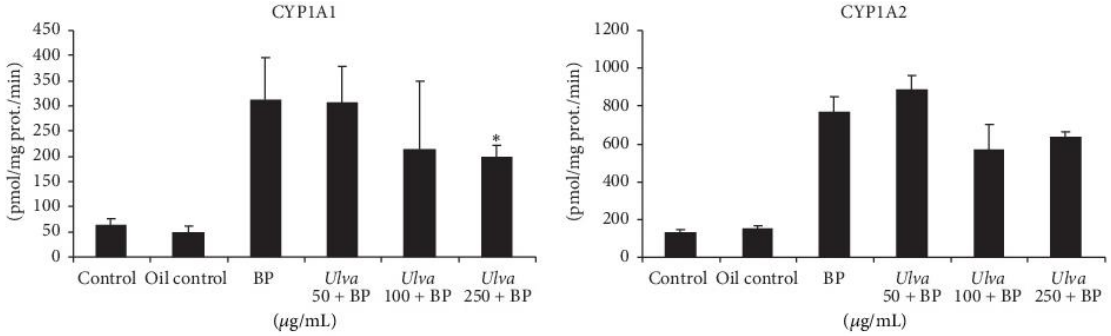

FIGURE 7: Effects of the pretreatment of *Ulva fasciata* extract on CYP1A activities in liver microsomes from male BALB/C mice exposed to benzo(a)pyrene (BP). CYP1A1 and CYP1A2 activities were assayed in liver microsomes from mice pretreated with different doses of *Ulva fasciata* or controls during 5 days and after administration of an oral dose of BP (250 mg/kg). The values represent mean ± SD; $^*P < 0.05$ (Dunnett's test).

be significantly lower in the extract pretreated animals than in those exposed only to BP (Table 3), suggesting that *U. fasciata* protects against DNA damage resulted from endogenously reactive species produced during the intermediary metabolism of BP.

Interestingly, a dose-related decrease in hepatic CYP1A activity was detected in the three extract treated groups compared to the BP-treated group (Figure 7). This finding confirmed the results of the *in vitro* inhibition study described above (Figure 3), and it reinforces the hypothesis that inhibition of metabolizing enzymes may play an important role in *U. fasciata* antigenotoxic properties.

Thus, our results indicate that prevention of BP-induced genotoxic damage by the tested extract may involve the modulation of different molecular targets, where the interaction with Phase I enzymes associated with carcinogen activation (CYP1A subfamily) could play an important role. However, the radical scavenging properties of the extract and the modulation of other antitumor mediators at the cellular level could not be discarded. In addition, our present results as well as others [16] concerning the phytochemical characterization of this extract (Figure 1) indicated that *U. fasciata* is a rich source of many micro- and macronutrients, some of which are associated with the modulation of different biomarkers involved in cancer progression.

Fatty acids and carotenoid pigments are present at high concentrations in seaweeds, including the specie of *U. fasciata* studied here. Carbohydrates, proteins, saponins, alkaloids, and flavonoids are also found to be present [29, 30]. Here, we broaden the phytochemical study of this extract by using GC-MS. The analysis performed in this study (Figure 1) showed palmitic acid to be one of the main components in the fraction analyzed, and the biological importance of this molecule has been highlighted [31]. Ryu et al. [14] reported

the presence of carotenoids in the extract. Nadathur et al. [32] have reported the antimutagenic properties of palmitic acid against the direct mutagen methylnitronitrosoguanidine (MNNG). Its mechanism of action has been suggested to involve trapping of the mutagen inside the micelles formed by the fatty acid. Furthermore, the same group reported that isopalmitic acid also interferes with the mutagenicity of 7,12-dimethylbenz[a]anthracene (DMBA) by inhibiting the activity of CYP1A1. Whether or not the same mechanism of action could be considered for the antigenotoxic effect of *U. fasciata* extract reported here needs to be investigated further.

5. Conclusion

In summary, this study demonstrated that *U. fasciata* has protective effects against *in vitro* and *in vivo* damage induced by BP, and different pathways could be modulated by the extract. Meanwhile, its capacity for inhibiting CYP1A function seems to be the main mechanism involved. Thus, this marine green alga might be an abundant source of potential complementary and alternative functional food for the prevention of cancer and other degenerative diseases associated with xenobiotics bioactivation in the organism. The mechanisms underlying the antigenotoxic effects of *U. fasciata* deserve further investigation.

Conflict of Interests

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

Acknowledgments

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