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Señor Director
DIRECCIÓN DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

Trámite : 011
Evento : 378
Actuación : 523

Referencia : Expediente No. **11.165.436**
Asunto : Solicitud de patente para: **NUEVAS SALES
FARMACÉUTICAMENTE ACEPTABLES DE 4-(1H-
IMIDAZOL-4-ILMETIL)PIRIDINA**
Solicitante : BIOPROJET
Fecha de solicitud : 01 de Diciembre de 2011

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio No. **13431** notificado por fijación en lista el 30 de Julio de 2013.

2. Mediante oficio No. **13431** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio se refieren a la claridad del pliego reivindicatorio, a la claridad del título, a las reivindicaciones 15 a 20 que definen un método de tratamiento, las reivindicaciones 21 y 22 relativas a usos, a la novedad de las reivindicaciones 1 a 9 y 23 en vista del documento D1: WO 92/14453, y al nivel inventivo de las reivindicaciones 1 a 14 y 23 en vista del documento D2: US6465486, al nivel inventivo de las reivindicaciones 1 a 9 en vista del documento D3: J. Med. Chem. 2004, 47, 2414-2417, y al nivel inventivo de las reivindicaciones 10 a 14 en vista del documento D4: US60124296.

3. Respuesta a las objeciones

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desea presentar las siguientes modificaciones, aclaraciones y argumentos:

"EXCELENCIA LEGAL EN UN MUNDO SIN FRONTERAS"

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3.1 Modificación de una solicitud en trámite

De acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar un nuevo pliego reivindicatorio conformado por 12 reivindicaciones.

El título se ha modificado de la siguiente manera:

“NUEVAS SALES FARMACÉUTICAMENTE ACEPTABLES DE 4-(1H-IMIDAZOL-4-ILMETIL)PIRIDINA”

Se han introducido las siguientes modificaciones al capítulo reivindicatorio:

- La reivindicación 1 ha sido modificada de manera que ahora especifica que los solvatos son formas hidratadas. Además, la reivindicación 1 ahora recita que las sales farmacéuticamente aceptables se seleccionan de la lista específica de las sales anteriormente recitadas en la reivindicación 5. Por lo tanto, la reivindicación 5 ha sido eliminada.
- La reivindicación 9 (nueva 7) ha sido modificada con el fin de omitir la referencia a la Figura 1 y en lugar se incluyen las características de los picos característicos del espectro de difracción de polvo de rayos X, como se muestra en la Figura 1. Por lo tanto, la reivindicación 9 (nueva 7) ahora recita los picos característicos. Los picos se identifican por las flechas en la Figura 1 adjunta y por lo tanto, es claro que dichos valores se encuentran soportados en la descripción tal como fue originalmente presentada.
- La reivindicación 7 ha sido eliminada.
- Las reivindicaciones 10 y 11 han sido modificadas con el fin de recitar los ácidos específicos, el disolvente orgánico, el átomo de halógeno y el grupo protector como se ha indicado en los ejemplos.
- Las reivindicaciones 14 a 22 han sido eliminadas.

Así, las reivindicaciones son ahora muy claras y precisas en el momento de señalar la naturaleza de la invención reivindicada y es evidente que las características técnicas esenciales de la invención están establecidas.

Por lo anterior, las objeciones formuladas por el Examinador en cuanto a la claridad y concisión del pliego reivindicatorio se ven superadas. En consecuencia, se solicita atentamente tener en cuenta este nuevo pliego reivindicatorio para el nuevo análisis de patentabilidad.

Con respecto a las objeciones realizadas por el señor Examinador hacia las reivindicaciones 8, 4 y 6, respetuosamente manifestamos nuestro desacuerdo por las siguientes razones:

- La reivindicación 8 (nueva 6) se dirige específicamente a las sales de oxalato de immetridina. Por lo tanto, la objeción elevada hacia esta reivindicación, en la que “se reclaman de forma general las sales de immetridina” no tiene validez.
- Las reivindicaciones 4 y 6 no repiten la misma materia: la reivindicación 4 se refiere a una composición farmacéutica, mientras que la reivindicación 6 está dirigida a un compuesto *per se*.

En este sentido, teniendo en cuenta las anteriores aclaraciones y las modificaciones realizadas al pliego reivindicatorio, atentamente señalamos que las objeciones anteriormente argumentadas, con respecto a la claridad y concisión del pliego reivindicatorio carecen de validez y por lo tanto, se consideran superadas.

3.2 Argumentos a favor de la falta de Novedad

El artículo 16 de la Decisión 486 señala lo siguiente:

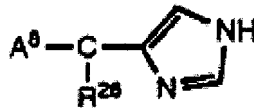
“Artículo 16: Una invención se considerará nueva cuando no esté comprendida en el estado de la técnica.

El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente, o en su caso, de la prioridad reconocida.”

La novedad de una invención se evalúa a través de la comparación uno a uno de sus características esenciales. De este modo, una invención puede ser objetada por novedad cuando sus características esenciales son exactamente iguales a las de un antecedente que represente el estado de la técnica conocido al momento de presentar la solicitud. Por lo tanto, en caso que llegue a existir al menos una diferencia en dicha comparación, se afirma que la invención SI cumple con el requisito de novedad.

Con base en lo anterior, respetuosamente manifestamos nuestro desacuerdo con el análisis llevado a cabo por el Examinador y con las conclusiones del mismo y solicitamos atentamente la reconsideración de las razones por las cuales se objeta la novedad, con base en la siguiente información:

En primer lugar, amablemente señalamos que el documento D1 divulga la fórmula general:



Esta fórmula es genérica y cubre una pluralidad de diferentes compuestos como R8 y R28 que pueden variar.

A pesar de que, posiblemente, puede abarcar immetridina, D1 no describe específicamente immetridina.

Como principio general para la evaluación de la novedad, una divulgación general, no anticipa una especie en particular. Por lo tanto, D1 no afecta la novedad de las presentes reivindicaciones.

De acuerdo con los argumentos anteriormente explicados, la invención de la presente solicitud de patente es novedosa sobre el documento del estado de la técnica D1 citado por el Examinador.

3.3 Argumentos a favor de la falta de Nivel Inventivo

El artículo 18 de la Decisión 486 señala lo siguiente:

“Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica.”

Una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis del nivel inventivo de una solicitud de patente, se considera que una anterioridad o una combinación de dos anterioridades puede afectar dicho requerimiento de patentabilidad, si una persona medianamente versada en la materia encuentra enseñanzas o sugerencias claras que, a partir de un conocimiento medio en dicho campo, le permitan llegar de manera obvia a la invención reivindicada. Adicionalmente, se considera que una mejora sustancial, un efecto inesperado o una ventaja con respecto al arte previo es un indicio de nivel inventivo, por cuanto aporta un salto tecnológico en la regla técnica.

A este respecto, respetuosamente manifestamos nuestro desacuerdo con el análisis llevado a cabo por el Examinador y con las conclusiones del mismo y solicitamos atentamente la reconsideración de las razones por las cuales se objeta el nivel inventivo, con base en la siguiente información:

Como primera medida, el nivel inventivo ha sido objetado en vista de D2 en combinación con D3. Aunque D3 da a conocer immetridina, no da a conocer una composición farmacéutica del mismo.

Las presentes reivindicaciones se encuentran dirigidas a los usos farmacéuticos de sales de immetridina.

En este sentido, los documentos D2 y D3 no afectan el nivel inventivo ya que no hay sugerencia alguna de tal uso farmacéutico de immetridina.

D2 no describe ni sugiere immetridina. Adicionalmente, D3 no describe ni sugiere composiciones farmacéuticas.

Los autores de D3 no hacen referencia o indican alguna conclusión con respecto al uso farmacéutico posible de immetridina, ni siquiera un uso especulativo hipotético.

Aunque la actividad de H3 *in vitro* de immetridina fue reportada por el documento D3, los autores consideran solamente la immetridina como una herramienta y no como un posible candidato a fármaco para el tratamiento humano.

Esto se debe a que era conocido generalmente en la fecha de D3 que immetridina, la cual contiene un anillo de imidazol, exhibe todas las desventajas conocidas de compuestos que contienen imidazol. Estas desventajas de los anillos que contienen imidazol son las siguientes:

La presencia de un anillo de imidazol dentro de las moléculas hace que estos compuestos no sean aptos para usos terapéuticos, según lo establecido en el pliego reivindicatorio tal como fue presentado.

1° En primer lugar, los compuestos que contienen imidazol son buenos sustratos para la histamina N-metil-transferasa, la cual es abundante en el hígado humano y la cual por lo tanto, inactiva sustancialmente estos compuestos cuando se administran en humanos.

Por lo tanto, a pesar de que una actividad *in vivo* puede haber sido detectada en ratas o conejillos de indias, esto no es ilustrativo de la actividad en humanos. Estas especies generalmente muestran una tasa de histamina N-metil-transferasa hepática menor que en humanos. Por lo tanto, los fármacos que contienen imidazol, tales como

immetridina fueron considerados por los autores como poco potentes en humanos debido a la extensiva inactivación del primer paso hepático, y en una medida que no es compatible con los requisitos terapéuticos.

2 ° En segundo lugar, los fármacos que contienen imidazol son a menudo inhibidores potentes y/o inductores de enzimas de citocromo P450 y por lo tanto, no son adecuados para los propósitos médicos. Es conocido que la inhibición o la inducción del citocromo P450 resulta la interacción de fármaco-fármaco potencial y los candidatos a fármacos están diseñados para que carezcan de actividad P450. Por lo tanto, durante las últimas décadas, la química médica ha desarrollado la investigación hacia los ligandos del receptor H3 que no tienen una fracción de imidazol.

Como se conocen todas estas desventajas de ligandos que contienen imidazol, se sabe que la immetridina es inadecuada para un posible candidato a fármaco en humanos. Por lo tanto, la investigación sobre immetridina y, en particular, la enseñanza de D3 se limita solamente al uso de immetridina como una herramienta. Por lo tanto, el objetivo de la enseñanza de D3 no es el uso farmacéutico de immetridina en humanos.

A pesar de este prejuicio conocido, los inventores han demostrado ahora que la immetridina es sorprendentemente carente de los efectos secundarios esperados, como es evidente por los datos dados en la presente descripción tal como fue presentada:

- Sustrato pobre de histamina N-metil-transferasa (HMT): (página 20, línea 10 a la página 21, línea 4 de la solicitud PCT (en HMT humana, *in vivo*);
- Interacción pobre con los citocromos P450 humanos: página 23, líneas 24-32 de la solicitud PCT.

La ausencia de estas desventajas en humanos hace por lo tanto, que el uso farmacéutico de immetridina ahora sea posible de acuerdo con la invención.

Así, el problema técnico a ser resuelto por la presente invención es la provisión de medicamentos para el tratamiento humano de los trastornos relacionados con H3.

Como se discutió anteriormente, la persona del oficio normalmente versada en la materia que busca proporcionar agentes alternativos para el tratamiento de trastornos relacionados con H3, simplemente no habría considerado immetridina como una posible solución incluso en vista de D3. Esto es porque, debido a su fracción de imidazol, immetridina habría sido considerada inmediatamente como no compatible para aplicaciones médicas en humanos debido a la inactivación mediante interacciones de histamina N-metil-transferasa y citocromos de fracciones de imidazol en humanos. Como soporte a este prejuicio conocido, se proporcionan referencias científicas que establecen la interacción reconocida de imidazol que contienen fracciones con citocromo y el efecto perjudicial de esta interacción para el tamisaje de los candidatos (Tang *et al.*; Zlokarnik *et al.* Ambos adjuntos a este documento).

En este sentido, la persona del oficio normalmente versada en la materia que busca nuevos ligandos H3R no habría estado motivada a considerar compuestos que contienen imidazol y no habría sido considerado la immetridina en la búsqueda del candidato a fármaco para el consumo humano.

Contra todas las expectativas, se ha encontrado que immetridina o sus sales son adecuadas para los tratamientos terapéuticos humanos. A pesar de todo perjuicio, estos compuestos fueron probados por los inventores para uso humano. De manera sorprendente, los inventores han encontrado que ellos carecen de los inconvenientes esperados en humanos. Esto no podría haber sido esperado de manera razonable por las personas versadas en la materia.

En efecto, la presente descripción tal como fue presentada muestra que el problema se resuelve en realidad de la siguiente manera:

- Los resultados de la tabla en la página 35 (para immetridina como la forma base y sus diversas sales) muestran que estos derivados están metilados a una tasa extremadamente baja con la histamina N-metil-transferasa humana, lo que conduce a la ausencia de inactivación por la histamina N-metil-transferasa y, por lo tanto, la posibilidad de utilizarlas como medicamento en humanos;

- Los resultados en la páginas 38-39 también muestran que immetridina no interactúa significativamente con el citocromo humano a pesar de todas las expectativas, y
- Los resultados dados en la tabla en la página 37 evidencian el efecto de immetridina en forma de base y sus diversas sales como activador del receptor H3. Cuando se administra por vía oral en ratones a 0,3 mg/kg, provocan una reducción de tele-metil-histamina a nivel cerebral.

Por lo tanto, los datos presentados en la descripción presentan evidencias de que el problema ha sido resuelto por immetridina, así como sus diversas sales.

Por lo tanto, la immetridina y sus sales fueron consideradas como no aptas para usos terapéuticos. Sin embargo, podrían ser utilizados como herramientas útiles para la comprensión de los mecanismos del receptor H3. Este es específicamente el objetivo de D3.

Por lo tanto, en vista de la parcialidad conocida a partir del uso de immetridina en humanos en el momento de la invención, el objetivo de la enseñanza de D3 no puede ser una composición farmacéutica, ni siquiera implícitamente como lo afirma el Examinador.

Así, la persona del oficio normalmente versada en la materia en vista de D3 con su conocimiento general de las desventajas del imidazol que contiene un anillo, no habría ido en contra de tales principios generalmente aceptados para considerar immetridina para uso farmacéutico, sin la participación de sus habilidades inventivas. De haberlo hecho, habría ido en contra de un prejuicio o parcialidad fuerte, que a su vez refleja una actividad inventiva.

Por lo tanto, un técnico con conocimientos medios en la materia no estaba en condiciones de plantearse el problema, tampoco de resolverlo en la forma en que se reivindica y tampoco de prever el resultado, por lo que la presente solicitud posee la altura inventiva para que se le conceda el privilegio solicitado.

En consecuencia de lo anterior, la invención de la presente invención tiene nivel inventivo en vista de los documentos D2, D3 y D4 del estado de la técnica citados por el Examinador.

3.4 Concesión de Solicitudes equivalentes

Si bien entendemos y aceptamos que cada país es autónomo en la concesión o negación de los privilegios de patente, queremos resaltar el hecho que el análisis de los requisitos para el otorgamiento de esos derechos es prácticamente universal. La presente invención fue concedida por la Oficina de Patentes de Marruecos, patente No. MA33284 (B1).

3.5 Conclusión

Por todo lo anterior, el documento D1 no resulta una anterioridad válida capaz de afectar la novedad de la invención de la presente solicitud. De la misma manera, el documento D2, el documento D3, el documento D4, o su combinación, no resultan anterioridades válidas capaces de afectar el nivel inventivo de la invención de la presente solicitud.

Por lo tanto, consideramos que el Examinador está pasando por alto que una sola diferencia con respecto al estado de la técnica, permite concluir que el objeto reivindicado es nuevo. Además, está excluyendo el hecho de que el inventor en el momento de solucionar el problema técnico de la solicitud tuvo que emplear habilidad inventiva, pues la presente invención no resulta de la mera extrapolación de las enseñanzas del arte previo, como se afirma. Se está desconociendo el mérito investigativo empleado y que está aportando una ventaja al arte previo.

4. Puesto que se ha dado respuesta a todas las objeciones formuladas por el Examinador, se solicita atentamente proceder al otorgamiento de la solicitud de patente. En este sentido, respetuosamente recordamos al Examinador que la patente no podrá ser negada con base en objeciones diferentes a las expuestas en la acción oficial No. 13431, toda vez que al no haber sido trasladadas a mi representada, dicha decisión violaría tanto el tenor literal del artículo 45 de la Decisión 486 como el derecho constitucional al debido proceso. En este evento, y con base en el inciso 2 del artículo 45, respetuosamente

solicitamos al Examinador expedir una nueva acción oficial que permita a mi representada contra argumentar las mismas o modificar la solicitud con el fin de cumplir los requisitos de patentabilidad.

De otro lado, respetuosamente manifiesto que a pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud, beneficiándose de la fecha de prioridad inicialmente reivindicada.

5. Anexos

- Nuevo pliego reivindicatorio conformado por 12 reivindicaciones
- Figura 1 de la presente solicitud
- Artículo 1: Zlokarnik, G., Grootenhuis, P. & Watson, J. (2005). High throughput P450 inhibition screens in early drug discovery. *Drug Discovery Today*. Vol. 10(21): 1443-1450
- Artículo 2: Tang, C., Chiba, M., Nishime, J., Hochman, J., Chen, I., Williams, T. & Lin, J. (2000). Comparison of imidazole- and 2-methyl imidazole-containing farnesyl-protein transferase inhibitors: interaction with and metabolism by rat hepatic cytochrome P450S. *Drug Metabolism and Disposition*. Vol. 28(6): 680-686.

Señor Director,


LUZ CLEMENCIA DE PAEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

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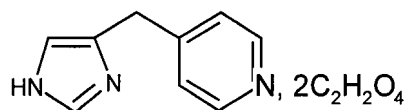
REIVINDICACIONES

1.- Una composición farmacéutica caracterizada porque comprende immetridina o una de sus sales farmacéuticamente aceptables seleccionadas de las sales de diclorhidrato, dihidrogensulfonato, toluensulfonato, ditoluensulfonato, dialcanforsulfonato, dibencensulfonato, fosfato, dimetansulfonato, alfa-cetoglutarato, oxalato, monosulfato de immetridina, así como sus formas hidratadas.

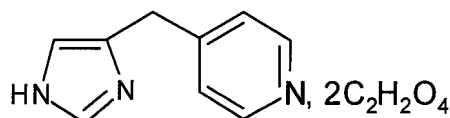
2.- Una composición farmacéutica de conformidad con la reivindicación 1, caracterizada porque la immetridina está en la forma de una sal farmacéuticamente aceptable.

3.- La composición farmacéutica de conformidad con la reivindicación 1 ó 2, caracterizada porque la immetridina está en la forma de una sal de oxalato.

4.- Una composición farmacéutica de conformidad con la reivindicación 1, 2 ó 3, caracterizada porque la immetridina está en la forma de la sal de dioxalato de la fórmula:



5.- El compuesto de conformidad con la reivindicación 4, caracterizado porque es la sal de dioxalato de immetridina, de la fórmula (I):



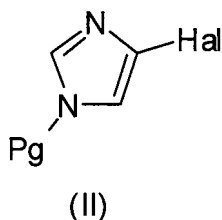
6.- Las sales de immetridina de conformidad con la reivindicación 4, caracterizadas porque son sales de oxalato de immetridina.

7.- El compuesto caracterizado porque tiene el espectro de difracción de polvo de rayos X que comprende los siguientes picos: (2-Θ en °): 16.55, 19.44, 19.63,

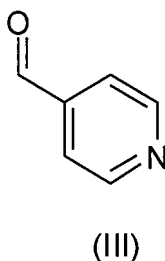
21.04, 21.45, 23.22, 25.04, 25.53, 26.01, 26.67, 27.16, 29.26 referido como la fase III BP1.5375L.

8.- Un procedimiento para la preparación del compuesto de conformidad con cualquiera de las reivindicaciones 4 a 7 caracterizado porque comprende el paso de hacer reaccionar immetridina con el ácido correspondiente seleccionado de ácidos bromhídrico, clorhídrico, sulfúrico, p-toluensulfónico, alcanforsulfónico, bencensulfónico, fosfórico, metansulfónico, α -cetoglutárico, oxálico, en un solvente orgánico propan-2-ol.

9.- El procedimiento de conformidad con la reivindicación 8, caracterizado porque además comprende los pasos de preparar immetridina mediante el acoplamiento de un compuesto de la fórmula (II)



con un compuesto de la fórmula (III)



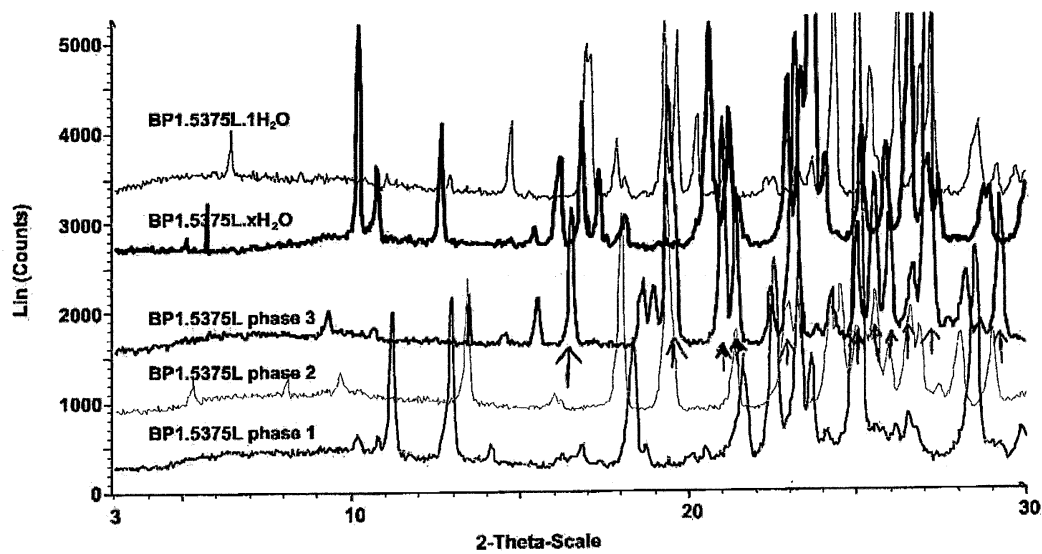
en donde Hal representa un átomo de halógeno seleccionado de I, Pg representa un grupo protector seleccionado de trifenilmetilo seguido por la desoxigenación/desprotección del grupo protector Pg.

10.- El procedimiento de conformidad con la reivindicación 8 o 9, caracterizado porque comprende el paso de hacer reaccionar immetridina con dos equivalentes de ácido oxálico en acetona.

11.- El procedimiento de conformidad con cualquiera de las reivindicaciones 8 a 10, caracterizado porque comprende:

- agregar lentamente acetona a una solución al 10% (p/p) de dioxalato de immetridina en agua a 20°C a una composición final de agua/acetona de 10/90 p/p;
- agitar durante 24 horas a temperatura ambiente;
- filtrar, enjuagar el sólido con acetona;
- secar.

12.- Las combinaciones caracterizadas porque comprenden una sal farmacéuticamente aceptable de immetridina así sus formas solevantadas, incluyendo hidratadas, y formas polimórficas de las sales y/o formas polimórficas de las formas solvatadas (incluyendo hidratadas) con un modulador positivo GABA-A.

FIG.1

High throughput P450 inhibition screens in early drug discovery

Gregor Zlokarnik, Peter D.J. Grootenhuis and John B. Watson

This review of high throughput (HT) P450 inhibition technologies and their impact on early drug discovery finds the field at a mature stage. The relationship between P450 inhibition and drug–drug interactions is well understood. A wide variety of P450 inhibition detection technologies are readily available off-the-shelf, but what seems still to be missing is a general agreement on how much weight one should give to the various types of early discovery HT P450 inhibition data. Method-dependent potency differences are a cause of concern, and to resolve this issue the authors advocate calibration of the HT methods with a large set of marketed drugs.

► Rationale for early compound profiling

Drug discovery is a multi-parameter optimization process in which compounds are optimized for interaction with the desired target and minimal off-target activities, while imparting drug-like properties on the candidate compounds. Project teams often pursue a parallel optimization approach in which multiple scaffolds are explored along multiple axes, including potency, selectivity, physicochemical properties and absorption, distribution, metabolism, excretion and toxicity (ADME–Tox) parameters [1–6]. This is to minimize the risk of making potent and target-class selective compounds in a chemical space that might be entirely incompatible with modifications needed to address other liabilities. Hence, all these molecular activities and properties need to be determined early on many compounds, to progress the overall best leads or scaffolds to the pre-clinical candidate stage. Such an approach requires a high throughput (HT) assay infrastructure, providing all relevant parameters for many, preferably all, members of privileged scaffolds at a reasonable cost and speed. Optimally,

all data should be available concurrently with primary-target affinity information.

Multi-parameter optimizations are complex and project teams need to decide early upon which parameters, in addition to potency and selectivity, to focus on and to devise strategies to tackle the optimization challenges. Ideally, some liabilities can be addressed with targeted scaffold modifications that are tolerated with regard to target potency and class selectivity. Examples relating to CYP3A4 inhibition will be presented below.

Case study

As part of ADME–Tox profiling, compounds are routinely tested *in vitro* for the potential to cause drug–drug interactions [7]. These are adverse events in which a drug influences the levels of a co-administered drug, either increasing or decreasing its levels. The most prominent cases involve the inhibition of the drug's metabolism, which can raise drug levels to toxic concentrations [8]. Prominent contributors to drug metabolism are a family of oxidizing enzymes

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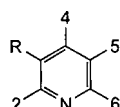
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TABLE 1
CYP3A4 inhibition within a series of pyridine-containing analogues



IC ₅₀ (μM) with substituent at position:				
Substituent	2	4	5	6
H	3	3	3	3
F	71	–	5	>200
OCH ₃	>200	7	1	>200
CH ₂ OH	74	–	–	–
NMe ₂	13	5	1	–
OH	–	–	10	–
Cl	–	15	–	–
CH ₃	–	5	–	–
OCH ₂ Ph	–	2	–	–

Key: R, side chain; –, not tested.
Table reproduced, with permission, from Ref. [16].

called cytochromes P450 (P450s). These monooxygenases are involved in the metabolism of >60% of all marketed drugs. Cimetidine (Tagamet®, GlaxoSmithKline), a drug for the treatment of heartburn and acid reflux, quickly lost sales to competing products as its drug–drug interaction liability emerged in clinical and post-market approval studies [9]. Mibefradil (Posicor®, Roche) was withdrawn from the market in 1998 because it potently inhibits CYP3A4, the predominant drug metabolizing P450, after having led to adverse events in patients concurrently taking a variety of other commonly prescribed medicines [10]. Alerted to the dire consequences of bringing P450 inhibitors to market, the pharmaceutical industry began testing candidate drugs sooner in discovery to be able to act early and move away from unwanted interactions.

Examples of the use of P450 inhibition data in drug discovery are CYP3A4 inhibitions by two pyridine-attached scaffolds. In the first example, which relies on a small dataset generated by traditional low throughput HPLC-based methods [11], the authors investigated CYP3A4 inhibition by changing the steric hindrance of the pyridine nitrogen via 2- or 6-substitution relative to the pyridine nitrogen, as shown in Table 1. Steric bulk next to the pyridine nitrogen weakens the coordination of the nitrogen to the P450 prosthetic heme iron. This approach is very efficient in changing CYP3A4 activity and several small substituents reduced CYP3A4 inhibition by more than one order of magnitude.

The other example is from an in-house project that employed HT screens to monitor the progress of an optimization for target potency while minimizing CYP3A4 inhibition, shown in Figure 1. The medicinal chemistry effort focused on a 4-substituted pyridine series. The initial 2,6-unsubstituted 4R-pyridines showed a wide spread of

target as well as CYP3A4 activities (Figure 1, gray diamonds), with the majority of compounds being sub-micromolar inhibitors of CYP3A4. Although one could argue that a few potent inhibitors of the target had a lower CYP3A4 liability, it was realized that further optimization of these series for other parameters ran the danger of creating compounds with activities closer to the center of the distribution and towards sub-micromolar inhibition of CYP3A4 (Figure 1, region indicated by the arrow). It seemed safer to substitute positions 2 or 6 of the pyridine and indeed, for most compounds, various 2-substituents (Figure 1, blue triangles, yellow circles and green square) reduced CYP3A4 inhibition potency to >1 μM, whereas, for instance, 3-fluoro substitution (Figure 1, red stars) was ineffective in dealing with the CYP3A4 liability. In both examples, the early optimization of the CYP3A4 inhibition parameter in scaffold evolution by targeted substitution removed this factor from the multi-parameter optimization matrix, allowing teams to focus on other issues, probably without the need to revisit CYP3A4 inhibition at a later stage.

In addition to pyridine moieties, terminal imidazoles are notorious for inhibiting multiple P450 isozymes, and azole antifungals as well as the antihistamine cimetidine [9] have been reported to display drug–drug interactions as a result of this activity. To ameliorate the P450–imidazole interactions, substitution patterns on imidazole derivatives have been explored. In an SAR study on imidazoles, 2- and/or 4-substitution led to compounds that are essentially inactive in inhibiting aldrin epoxidation in rat liver microsomes [12]. In humans, however, 2-methylimidazoles are known precursors to reactive metabolites, and potent P450 inhibition has been observed upon metabolism of this moiety [13]. There are several other chemical features that lead to inhibition of P450 enzymes, such as terminal olefins and acetylenes, quinolines, amines, hydrazines, hydrazones and methylenedioxy phenyls, to mention a few [14].

Prediction of potential drug–drug interactions involving lesser-studied P450 isoforms

In a recent publication, Walsky *et al.* [15] demonstrated the use of large P450 inhibition datasets in uncovering the potential of a drug to cause drug–drug interactions as a result of the inhibition of the P450 isoform CYP2C8. They tested the inhibition of CYP2C8 by 209 commonly prescribed drugs, to identify medicines with the potential to interact with other drugs that involve in their metabolism this P450 isozyme, such as paclitaxel, repaglinide, rosiglitazone and cervistatin. Although most tested drugs were not expected to cause clinically relevant drug interactions, montelukast was identified as a potent inhibitor of CYP2C8, with the potential of causing clinically relevant interactions with CYP2C8 drug substrates. Armed with such knowledge, companies should gauge the risk of inhibiting a minor P450 isoform with a new candidate drug. To establish *in vivo* relevance, companies should

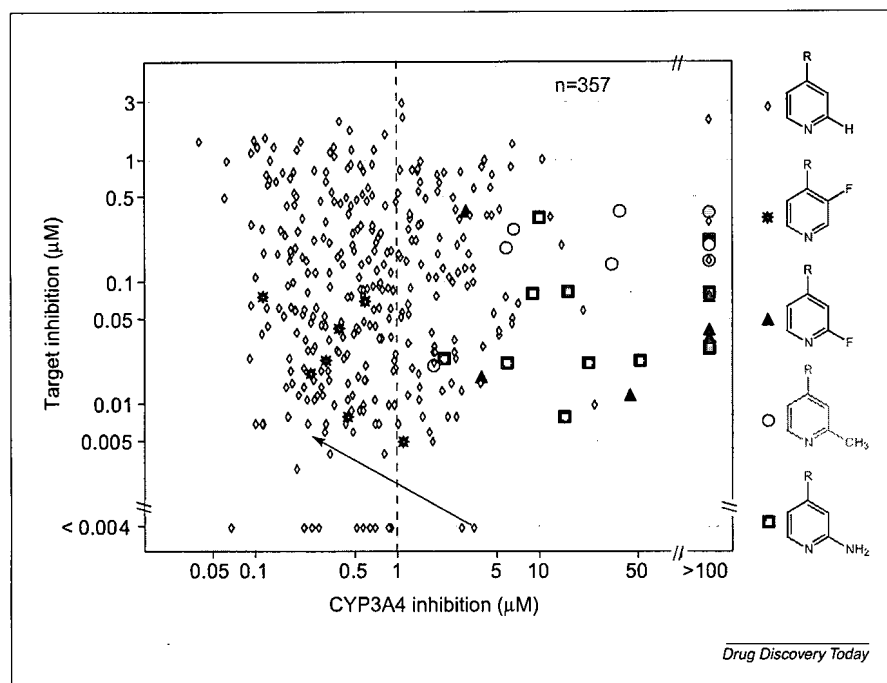


FIGURE 1

Monitoring of a series of substituted pyridines for CYP3A4 inhibition in a fluorescence HT-CYP3A4 inhibition assay and target potency in early discovery. The fluorogenic CYP3A4 probe was Vivid™3A4 Red (aka BOMR) [38,39]. Unsubstituted pyridines (gray diamonds) show a wide spread of target and CYP3A4 inhibition. Further optimization of these series for other parameters could create compounds with activities closer to the center of the distribution and towards sub-micromolar inhibition of CYP3A4 (as indicated by the arrow). Fluoro substitution far (meta) to the pyridine nitrogen (red stars) are ineffective in dealing with CYP3A4 liability, whereas compounds with substitutions adjacent to the pyridine nitrogen (blue triangles, yellow circles and green squares) have reduced CYP3A4 inhibition potency. The preferred activity zone is colored in green.

conduct in the early clinical stage *in vivo* drug–drug interaction studies with drugs that are likely to be co-administered, therefore limiting the risk of late failures.

Rendic [16] has compiled a comprehensive list of human P450 isozymes and their known metabolic reactions and interactions, incorporating >1000 references published through 2001. Of the 57 named human P450s, only 11, including CYP2C8, have >100 references [17]. As the contribution of the lesser-studied P450s to human drug metabolism becomes clear, similar studies on their inhibition and their potential to be involved in clinical drug–drug interactions will be desirable.

Detection technologies for HT P450 inhibition testing

In the 1990s, the efforts of combinatorial and parallel synthesis required improved assay throughput to find the most potent compounds in increasingly larger compound collections. This task was much aided by the advances in HTS. HTS accelerates compound testing by providing automation and instrumentation that supports parallel processes, such as plate-handling robots, multi-channel fluidics, multi-channel analyzers, high-density assay plates and plate-scanning readers [18–20]. For assays requiring sample fractionation, multi-channel parallel separation cartridges [21], parallel mass-spectrometry (MS) analysis

[22] and multiplexed assays as well as analytical sample multiplexing provide increased throughput [23]. Many of these technologies were readily adapted for increased experimentation demands in the ADME–Tox profiling of compounds [23–25].

To detect P450 inhibition, the enzymatic reaction of a P450 (plus cofactors) with the substrate is monitored and the effect of the test compound on the reaction determined. Over the past two decades, P450 inhibition assays evolved from determining substrate metabolism by HPLC-based methods in individual reactions to detecting optical signal changes indicative of substrate metabolism in parallel experiments, in plate-based formats and without the need for sample separation or purification. Traditional HPLC-based methods have also received throughput upgrades. Enhancements include multiplexing of P450 isoform probe substrates and analyses of the probe–metabolite cocktail, affording simultaneous inhibition experiments on several P450 isoforms in the same sample [26–28] and tandem liquid chromatography–mass spectrometry (LC–MS–MS) analysis, which shortens analytical run times significantly. Table 2 provides a comparison of these methods with higher throughput radioactivity and optical methods.

Radioactivity-based methods

Radioactivity-based methods rely on ^3H - or ^{14}C -labeled drug substrates, which are selectively oxidized by the P450 isozyme of interest [29]. The metabolite is separated from the remaining substrate by HPLC and quantified via radiochemical detection. In some methods P450-mediated oxidation causes substrate demethylation with liberation of ^{14}C -formaldehyde, which can be extracted and detected by radioluminescence in a microplate scintillation counter [26,30]. To avoid the separation step, a scintillation proximity assay version of this method has also been developed. Here, the scintillant-containing beads are impregnated with polyethylenimine to bind the ^{14}C -formaldehyde, formed in the metabolic reaction. The proximity of the bound ^{14}C -formaldehyde results in efficient excitation of the scintillant with light emission [31]. An advantage of these radioactive assays is the ability to use known drug substrates with good solubility and well-characterized kinetics, although they generate radioactive waste, which requires subsequent decontamination and disposal.

Fluorescence-based methods

Fluorogenic P450 substrates were first studied in the early 1970s. These, and more recently developed substrates,

TABLE 2
P450 inhibition screening methods

Method	HPLC Methods	Radioactivity	Fluorescence	Luminescence
Substrates	Selective drugs	³ H- or ¹⁴ C-labeled drugs	Fluorogenic probes	Luciferin derivatives
Product	Drug metabolites	Extractable radiolabel	Fluorescent dye	Luciferin (light)
Detector	Mostly UV or MS	Scintillation counter	Fluorescence reader	Luminescence reader
P450 source	Liver microsomes or recombinant	Liver microsomes or recombinant	Mostly recombinant	Recombinant
Addition only assay	No	Mostly no	Yes	Yes
Non-coupled assay	Yes	Yes	Yes	No
Throughput	Medium-high	High	Very high	Very high
Market penetration	Full	Partial	Substantial	Nascent
FDA accepted	Yes	No	No	No

combined with recombinantly expressed P450 isoforms [32], formed the basis for facile P450 inhibition testing. An early provider and vendor of this technology was Gentest Corporation (now BD-Biosciences). The assays employ low-fluorescence substrates, which are converted to more-fluorescent metabolites. The progression of the reaction is measured by an increase in fluorescence intensity during P450 metabolism of the substrate. Inhibitors of a particular P450 reduce the rate of metabolism of the substrate and this results in a reduced fluorescence signal. Advantages of these assays are the ability to use 96-well and higher-density formats, addition-only protocols, the availability of a large number of substrates and the lack of radioactive waste [33–37]. These low-cost methods mostly rely on recombinant sources of P450 enzymes, as most fluorescent probes are not sufficiently P450 isoform-selective to be used with liver microsomal preparations. For compounds that are very fluorescent at the wavelength employed in the assay, P450 inhibition measurements cannot be obtained. More recently, Aurora Biosciences (now Vertex) [38] has developed more-sensitive substrates that produce larger fluorescence signal changes and are, therefore, less sensitive to optical interference [39,40] (commercial source of Vivid™ P450 substrates: Invitrogen, Carlsbad, CA, <http://gcms.invitrogen.com/downloads/vivid.pdf>).

Bioluminescence-based methods

The most recent technology developed for P450 inhibition is based on substrates that release luciferin as the metabolite (commercialized by Promega, Madison WI, www.promega.com/cnotes/cn007/cn007_02.htm, www.promega.com/cnotes/cn010/cn010_06.htm) [41]. This is a coupled assay system in which addition of luciferase and ATP converts the freed luciferin to des-carboxyluciferin with light emission. The format is similar to fluorescence methods, requiring addition-only manipulations and employing a luminescence plate reader instead of a fluorescence plate reader. This assay also requires use of recombinant sources of P450 enzymes, as the probes are not sufficiently P450 isoform-selective to be used with human liver microsomes. A potential source for false positives in these assays can

be compounds that interfere with light generation directly, such as compounds that interfere with luciferase enzymatic activity.

Any given organization's choice of P450 inhibition technology is driven by the work flow in discovery, the existing in-house expertise and instrumentation and the confidence in the validity and accuracy of any of the new technologies. Undoubtedly the latter is a major driver in instrument development to provide increased throughput for conventional methods, which are well established and have received FDA approval (www.fda.gov/cder/guidance/clin3.pdf) [7]. P450 screens developed for HT rely on high-density plate formats, low assay cost, little compound consumption and assay robustness. The probe substrates employed give large signal changes but typically have low P450 isoform selectivity and therefore require a preparation with individual expressed P450 isoforms. The methods readily allow routine broad P450 inhibition profiling including, if so desired, screening of entire compound libraries for P450 liabilities.

Finally, recent progress in computational methods for prediction of P450 compound interactions needs to be mentioned [42–44]. *In silico* screening has been very appealing as, with the increasing power of desktop computing screening, cost could become negligible. Virtual compounds could be screened for P450 liabilities, reducing the numbers of compounds with P450 liabilities a team would otherwise synthesize, providing additional savings [45–48]. In a recent publication [49], scientists working on a κ -opioid receptor agonist series demonstrated how molecular docking of candidate compounds into a homology model of CYP2D6 in conjunction with compound testing in a HT-CYP2D6 fluorescence inhibition assay efficiently guided medicinal chemistry efforts away from the CYP2D6 liability. As a major advance, the crystal structures of several human P450 isozymes have been solved [50–52]. These structures provide more-accurate geometries of the enzymes' active sites and should lead to better representations than previous homology models that were based on crystal structures of soluble bacterial enzymes [43,53].

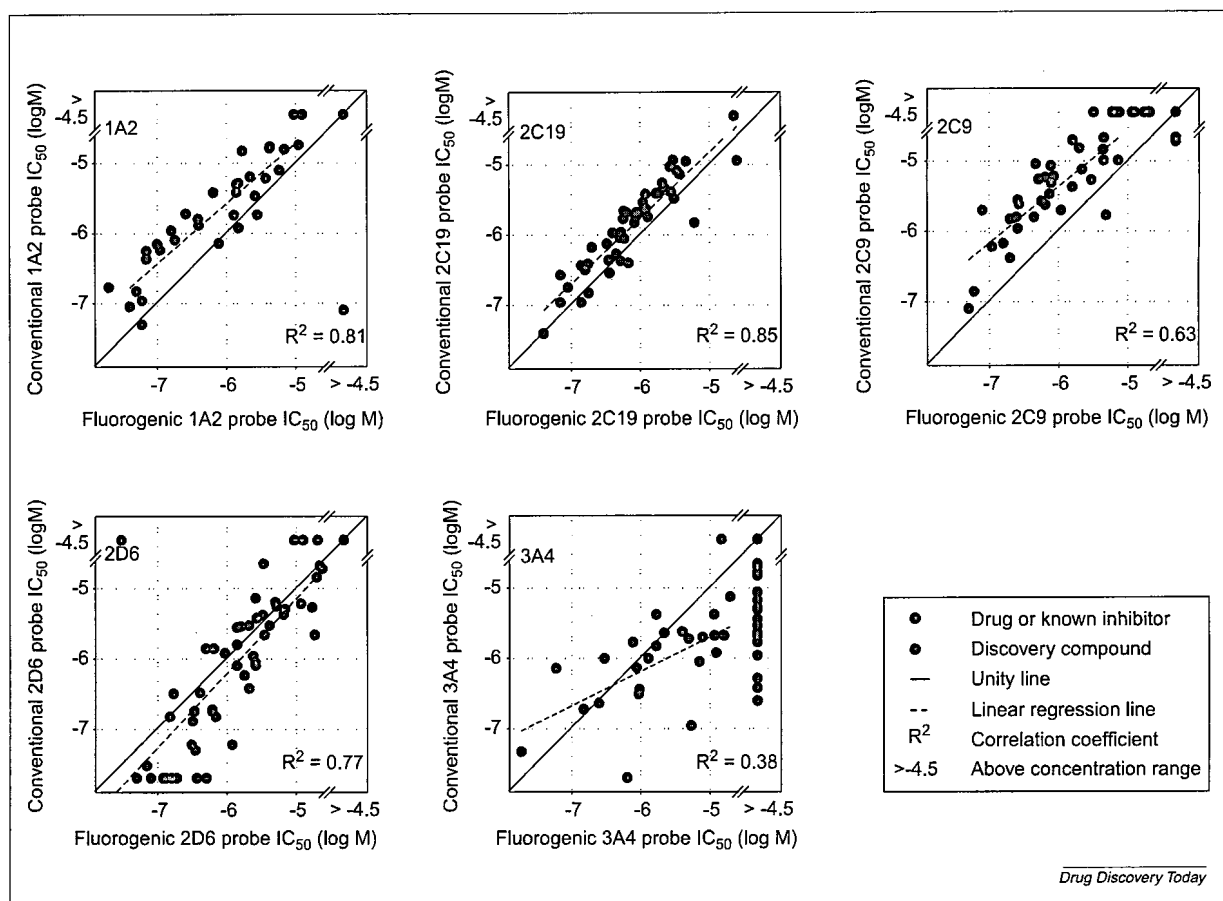


FIGURE 2

Fluorescence HT P450 inhibition methods compared with HPLC-based methods. The Figure is based on data from Ref. [40], converted from linear to logarithmic values. It compares the log IC₅₀ values for compounds determined with traditional drug (ordinate) and fluorogenic substrates (abscissa) for five of the human P450 enzymes. The solid diagonal line represents unity and the log linear regression (represented by the dotted line) was performed with omission of data outside the measurement range (data at >30 μ M were omitted from the fit).

A staged implementation of drug–drug interaction screening methods has been proposed, in which computational models for P450 *K_i* prediction are deployed at the stage of virtual, *in silico* screening. During the ‘hit-to-lead’ stage, HT P450 inhibition screens provide guidance and teams would rely on the gold-standard HPLC-based approaches during the lead optimization phase of discovery [54].

Calibrating HT P450 inhibition screens

For several reasons, HT methods relying on fluorescence and luminescence substrates with recombinant P450 enzymes do not provide the same answers as assays based on selective drug substrates and liver microsomal preparations [55]. For P450 isoforms 1A2, 2C19 and 2D6, the differences might be attributable to enzyme preparations, whereas CYP2C9 and CYP3A4 assays also display substrate-dependent inhibition differences [36,56,57]. But differing results are an issue not only for screens using recombinant preparations. Results obtained with traditional methods, which use the same probe substrate under similar conditions, can vary substantially between laboratories [58], as a result of P450 isozyme variations in liver microsomal

preparations [59,60] and differing microsomal concentrations in the assays [61].

A publication that questioned the quality of data obtainable in fluorescence P450 inhibition assays [40] compared data from several of these assays with traditional methods (Figure 2) on a series of drugs and xenobiotics (red circles) and in-house discovery compounds (blue circles). Figure 2 displays the data from this publication plotted on a log scale to show the correlation of the data, with the solid diagonals indicating unity and the dotted lines the linear fit of the log data (data at >30 μ M are omitted from fit). Potency trends between methods are quite similar for CYP1A2, 2C9, 2C19 and 2D6 data, whereas CYP3A4 inhibition data are less well correlated. Importantly, for the well-correlated assays, there is an average shift in potency. For CYP1A2, 2C9 and 2C19, the enzyme seems more readily inhibited in the recombinant assay using the fluorescence probe than in the liver microsomal preparations, similar to trends that have been seen with traditional probe substrates in differing enzyme preparations [6,30,55]. Hence, for calibration purposes, it is recommended to perform such an analysis on HT screens that employ

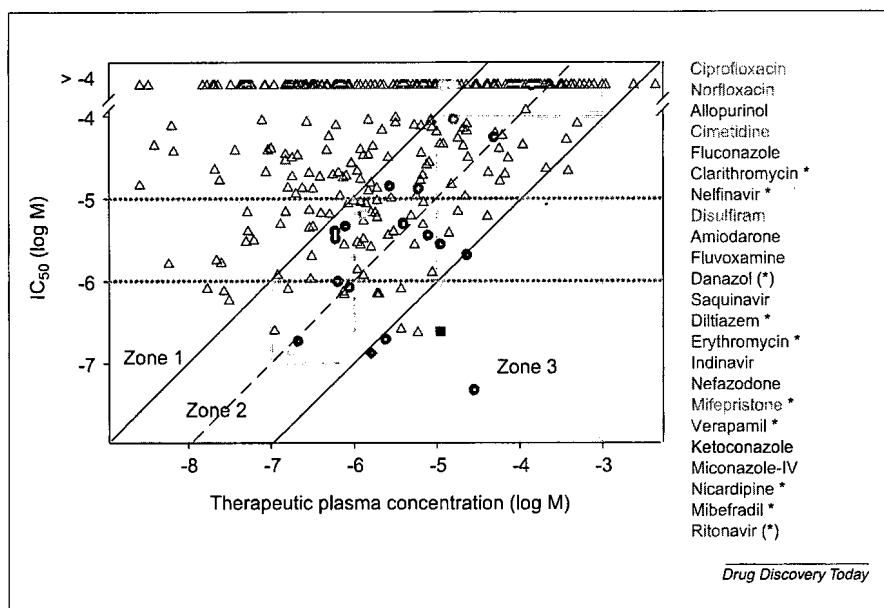


FIGURE 3
CYP3A4 inhibition potencies of 337 drugs plotted against their highest human therapeutic plasma concentrations. The values for therapeutic plasma concentrations were taken from Ref. [65]. Inhibition of CYP3A4 was assessed with a fluorescence HT P450 inhibition screen (Vivid™3A4 Red, aka BOMR, [38,39]). Drugs without any known CYP3A4 inhibition-related drug–drug interactions are depicted with open green triangles. The remaining drugs, denoted in other symbols and colors, do display such interactions. The identities of these drugs are listed in increasing order of potency of CYP3A4 inhibition (from Ciprofloxacin >100 μ M to Ritonavir at 50 nM). An asterisk next to the drug name indicates a reported contribution of a non-competitive mechanism to CYP3A4 inhibition *in vivo*. The blue dashed line represents unity, which means that drugs on the line have the same CYP3A4 IC_{50} as their maximum therapeutic plasma concentration. Zones 1–3 indicate areas in which drugs are below (1), within (2) or above tenfold (3) in potency for CYP3A4 inhibition, compared with their therapeutic concentrations.

recombinant assay preparations. The situation for the CYP3A4 inhibition assays is more complex, as it has been demonstrated that the choice of the enzyme as well as the substrate can lead to different results [62–64].

Relevance of HT CYP data to the clinical setting

An additional study was performed to demonstrate the relevance of HT P450 inhibition data as it relates to *in vivo* results in human patients. In this analysis, the assay that Vertex scientists used to determine CYP3A4 inhibition is about tenfold more sensitive to inhibitors than traditional human liver microsome-based methods with testosterone as probe substrate, similar to the findings for the fluorescence CYP1A2 and CYP2C9 assays mentioned above. The CYP3A4 inhibition potencies for 337 systemic drugs were determined and the results graphed in relation to their highest therapeutic plasma levels (C_{max} or highest therapeutic concentration reported for each drug [65]; Figure 3). Of these, 314 medications do not have any known CYP3A4 inhibition-related drug–drug interactions (Figure 3, green triangles). Of the remaining 23, one is Mibefradil (Posicor®, Roche, Figure 3, purple diamond) [15], which was withdrawn from the market. An additional 17 drugs are known to increase cyclosporine A and/or simvastatin levels due to CYP3A4 inhibition (Figure 3, red

filled circles) [66,67]. The remaining 5 drugs show other CYP3A4-related drug–drug interactions (Figure 3, yellow filled circles, <http://medicine.iupui.edu/flockhart/>).

Figure 3 indicates that, as would be expected for a predictive screen, few drugs are administered at higher plasma concentrations than the CYP3A4 inhibition IC_{50} concentration determined. Drug candidates that have tenfold or higher plasma concentrations than the determined HT CYP3A4 IC_{50} are very likely to receive a black box warning label or could fail to reach the market (Figure 3, zone 3). Drug candidates that have therapeutic plasma levels within tenfold of the HT CYP3A4 IC_{50} have a moderate chance of interacting with other marketed drugs (Figure 3, zone 2), but drug candidates with therapeutic plasma levels of less than 10% of the HT CYP3A4 IC_{50} s are likely to be safe with respect to these types of drug interactions (Figure 3, zone 1). This shows that the HT CYP3A4 inhibition data can provide guidance in the selection of drug candidates with lower development risk.

One might wonder why in the assay drugs that display an apparent K_i ten times below their total plasma concentrations would display clinically significant drug–drug interactions. This might seem

particularly puzzling for drugs that are highly bound to plasma proteins. Following are some possible explanations for the finding. For drugs administered orally, one would expect a decreasing gradient of total concentrations from the gut to the pre-hepatic circulation, the liver, the lungs, and to the systemic circulation, at which location drug plasma levels are typically monitored [68,69]. Metabolism by CYP3A4 in the gut contributes significantly to the first-pass clearance of many drugs. The concentration of a co-administered CYP3A4 inhibitor in the gut would likely be underestimated by systemic plasma concentrations. It is also known that many drugs accumulate in the liver at much higher levels than found in plasma [59,70,71]. Some drugs are metabolized to inhibitory species in the liver, which concentrations and potencies are not related to K_i values and plasma concentrations of the parent drug. Also, some drugs act as mechanism-based inhibitors of CYP3A4 [72]. Turnover of these drugs with CYP3A4 inactivates progressively and irreversibly the enzyme and the liver has to synthesize new enzyme to recover CYP3A4 activity. Such drugs can appear more potent *in vivo* than in the HT inhibition assay P450, because *in vivo* exposure to a drug lasts in the order of hours to days, whereas the enzyme in the HT assay is exposed to compound on the timescale of minutes [71]. In Figure 3, drugs that display

a mechanism-based component to CYP3A4 inhibition are indicated in the list with an asterisk.

In early discovery, the likely upper limit of the therapeutic human plasma concentration of the drug candidate will not be known. Yet, to avoid late failures due to drug-drug interactions, a risk assessment needs to be performed early. Some companies suggest guidelines for CYP3A4 inhibition, which are used to assign risk [73]. Compounds with potent CYP3A4 block in traditional inhibition assays, for instance with K_i values below 1 μM , are classified as high risk, compounds between 1 and 10 μM are assigned to moderate risk, whereas compounds with K_i values above 10 μM are classified as lower risk. With calibration of the screens, HT data can be assigned to analogous risk brackets. In the analysis presented in Figure 3, 95% of drugs have CYP3A4 inhibition values of $>1 \mu\text{M}$ in the recombinant HT CYP3A4 inhibition screen (above lower dotted gray line), whereas

~80% are less potent than 10 μM (above upper dotted gray line).

Conclusions

Many HT technologies are now available to detect P450 inhibitors, which should decrease the number of withdrawals of novel drugs from the market due to inhibition of the major P450 isozymes. HT P450 data can be used to guide medicinal chemistry away from these interactions in an early stage and in certain cases might entirely solve the issue by targeted modification of the P450 interacting functionality, leading to a reduced dimensionality of the drug optimization process. To be generally useful, P450 inhibition screens need to be calibrated against standard methods and preferably also tested with a large set of drugs, for which the human drug-drug interaction outcome is known.

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COMPARISON OF IMIDAZOLE- AND 2-METHYL IMIDAZOLE-CONTAINING FARNESYL-PROTEIN TRANSFERASE INHIBITORS: INTERACTION WITH AND METABOLISM BY RAT HEPATIC CYTOCHROME P450S

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ABSTRACT:

Methylation at the 2-position of the imidazole ring of IBN (I), a 1, 5-substituted imidazole-containing compound, was carried out to minimize its inhibition of rat cytochrome P450 (CYP)3A activity. The resulting analog 2-MIBN (II) exhibited an inhibitory potency 70-fold weaker ($K_i = 25 \mu\text{M}$) than that of I ($K_i = 0.3 \mu\text{M}$) toward CYP3A, the major rat liver microsomal P450 isoform(s) for the metabolism of I and II by rat liver microsomes in the presence of NADPH. The structural modification did not switch the major metabolic pathways for I and II, but significantly decreased the affinity of II to the metabolizing enzyme(s) as reflected by the difference in their K_i values for CYP3A. Enzyme kinetic studies also demonstrated that I had a lower apparent K_m ($0.3 \mu\text{M}$) than II ($18 \mu\text{M}$), but an apparent $V_{\max}$ 14 times lower than II. This finding indicates that methylation

at the imidazole ring reduced the affinity of the compound to CYP3A, but increased the catalytic capacity, turning I as a substrate of low K_m value but low capacity into a compound of high K_m but high capacity for the metabolism. Our results suggest the significance of substrate concentration in comparing the metabolic stability of compounds with different kinetic parameters. Although higher intrinsic clearance is implied for I when the substrate concentration is below or close to its K_m value, higher metabolic rate was constantly seen with II over micromolar range. The different kinetic parameters of I and II may also explain the observation that no significant difference in pharmacokinetic behavior was seen after an i.v. administration of I and II to the rat.

Oncogenically activated Ras product has been implicated in many forms of human cancers, including 30 to 50% of colon and 90% of pancreatic cancers (Rodenhuis, 1992). The *S*-farnesylation of the terminal cysteine residue of Ras is required for its ability to relay extracellular growth signals to nuclear proteins by enabling association with the cell membrane (Casey et al., 1989; Kato et al., 1992). Thus, efforts to regulate Ras function with the goal of developing new anticancer therapy have focused mainly on inhibition of the zinc metalloenzyme farnesyl-protein transferase (FTase)¹. Studies have shown that FTase inhibitors (FTIs) can prevent protein farnesylation in cell culture and selectively inhibit *ras*-transformed cell growth, as well as suppress the growth of *ras*-dependent tumors in mice (Gibbs, 1991; Moores et al., 1991; Gibbs et al., 1993, 1994; Kohl et al., 1993).

Avenues toward improving the biological properties of FTIs revealed that nitrogen heterocycles are alternatives to the cysteine thiol included in a wide array of FTIs that mimic the tetrapeptide C-terminus of Ras (Reiss et al., 1990), probably due to their ability to bind a zinc atom at the enzyme active site (Hunt et al., 1996; Leonard et al., 1997). Dinsmore et al. (1999) reported a class of imidazole-containing diarylether and diarylsulfone FTIs that showed dramatically improved potency and gave high cell activity. Ever since, a variety of differently substituted imidazole-containing FTIs have been identified and characterized in Merck (Anthony et al., 1999; Williams et al., 1999). IBN (4-{5-[4-(3-trifluoromethoxyphenyl)-3-oxopiperazin-6-butyl-1-ylmethyl]-imidazol-1-ylmethyl}-benzonitrile) (Fig. 1) is one of those compounds that yield high inhibitory potency ($\text{IC}_{50} < 0.15 \text{ nM}$) toward FTase (Williams et al., 1999).

It has been established that imidazole ring-containing drugs, such as ketoconazole, itraconazole, and clotrimazole, are known to be potent cytochrome P450 (CYP)3A inhibitors (Wilkinson et al., 1974; Ahmed et al., 1995; Halpert, 1995; Miranda et al., 1998). The lone pair electrons with sp^2 orbital on the nitrogen atom could chelate with the ferric heme in the enzyme to elicit inhibition. The CYP3A family comprises the most abundant CYP forms found in human liver and catalyzes the metabolism of a host of medications and steroids (Guengerich, 1995; Li et al., 1995). Therefore, inhibition of the activity of CYP3A forms may contribute to potential toxicity associated with drug-drug interactions (Hooper, 1999). Bearing an imidazole ring in its structure, IBN (I) was found to be a CYP3A4 inhibitor

¹ Abbreviations used are: FTase, farnesyl-protein transferase; FTIs, farnesyl-protein transferase inhibitors; CYP, cytochrome P450; IBN, 4-{5-[4-(3-trifluoromethoxyphenyl)-3-oxopiperazin-6-butyl-1-ylmethyl]-imidazol-1-ylmethyl}-benzonitrile; 2-MIBN, 4-{5-[4-(3-trifluoromethoxyphenyl)-3-oxopiperazin-6-butyl-1-ylmethyl]-2-methyl-imidazol-1-ylmethyl}-benzonitrile; LC-MS, liquid chromatography-mass spectroscopy; MS/MS, tandem mass spectroscopy; ES, enzyme-substrate complex; TFA, trifluoroacetic acid.

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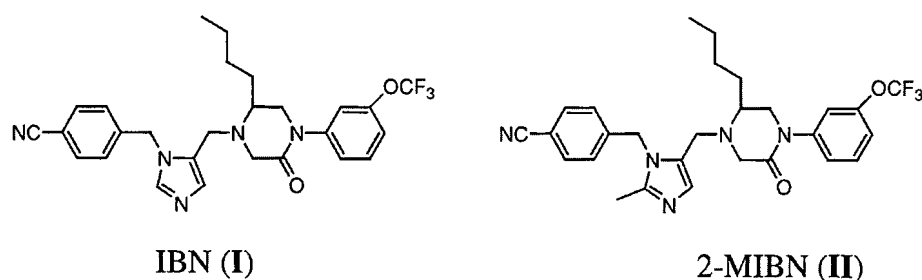


FIG. 1. Structures of IBN (I) and 2-MIBN (II).

TABLE 1

IBN (I) and 2-MIBN (II) remaining in the incubation with rat liver microsomal preparations for 20 min in the presence of NADPH

The data are expressed as percentage of remaining parent amount compared with the control ($t = 0$ min) and represent mean values from duplicate determinations.

Concentration	IBN		2-MIBN	
	0.5 ^a	1.0	0.5	1.0
μM	mg/ml		mg/ml	
5	61.4	49.0	17.6	6.4
10	78.5	55.1	31.0	12.1
20	85.6	91.7	48.6	30.2

^a Protein content of rat liver microsomes.

in human liver (M. Chiba, personal communication). To minimize its inhibitory activity, the analog of I, 2-MIBN (II; 4-{5-[4-(3-trifluoromethoxyphenyl)-3-oxopiperazin-6-butyl-1-ylmethyl]-2-methyl-imidazol-1-ylmethyl}-benzonitrile) has been developed via substitution of the hydrogen atom at the 2-position with a methyl group, a common approach to attenuate the interaction with CYP by introducing steric hindrance (Rogerson et al., 1977). The purpose of this study was to assess how the structure modification affects the metabolism along with the expected alteration of the inhibitory potency against drug-metabolizing enzyme(s). Using the rat as a model, we examined the inhibition of various CYP isoforms by I and II, determined their metabolic pathways, identified the important CYP isozymes involved in their metabolism, and compared the in vitro kinetic parameters with the in vivo pharmacokinetic profile.

Materials and Methods

Chemicals. I, II, and L-775,842 (4-{5-[4-(5,6,7,8-tetrahydronaphthalenyl)-3-oxopiperazin-6-butyl-1-ylmethyl]-imidazole-1-ylmethyl}-benzonitrile) were synthesized at Merck Research Laboratories (West Point, PA). Testosterone, bufuralol, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Anti-Rat CYP Sera. Polyclonal goat anti-rat CYP3A2, anti-rat CYP2C11, anti-rat CYP2B1 sera, and control goat sera were purchased from Gentest Corp. (Woburn, MA). These antibodies gave 90% inhibition of 6 β -hydroxylation of testosterone, 95% inhibition of 16 α -hydroxylation of testosterone, and 60% inhibition of lidocaine methyl-hydroxylation at 25 μl sera/100 μg microsomes.

Rat Liver Microsomes and Incubation Conditions. Liver microsomes from mature (3-month-old) and immature (3-week-old) male rats were prepared by standard procedures (Fujita et al., 1990), and aliquots were stored at -70°C until use. Protein concentrations were determined according to the method previously published (Lowry et al., 1951) using BSA as the standard. The animals were treated in accordance with the Merck Institutional Animal Care and Use Committee guidelines.

Effects on the metabolism of testosterone and bufuralol. I and II (0.1–100 μM) were preincubated with 20 μM testosterone and bufuralol, 10 mM

MgCl₂, 1 mM EDTA, 20 mM glucose 6-phosphate, 4 I.U./ml of glucose 6-phosphate dehydrogenase, 150 mM Tris-HCl (pH 7.4), and 0.2 mg microsomal protein/ml (in a final volume of 0.25 ml) for 5 min in a shaking bath at 37°C . The reaction was initiated by the addition of NADPH (1 mM) and carried out for 10 min.

Substrate consumption. The time course consumption of I and II (5–20 μM) was determined in the same incubation mixture described above except for the liver microsomal protein concentration (0.5 and 1.0 mg/ml). Aliquots (0.5-ml) were taken over a 60-min period.

Metabolite identification. Samples used for metabolite identification were prepared by incubating I and II (20 μM) with rat liver microsomes (1 mg protein/ml) for 60 min in the same incubation mixture as described above.

Enzyme kinetic studies. Various substrate concentrations (0.1–20 μM) were incubated with the microsomal preparations at a protein content of 0.4 mg/ml for 3 to 10 min at 37°C . The incubation duration was adjusted to ensure the substrate consumption $\leq 10\%$.

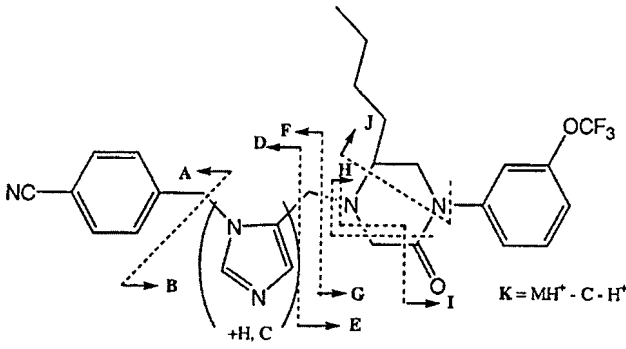
Immunoinhibition studies. The effect of anti-rat CYP sera on the metabolism of I and II was examined by preincubation of microsomes with antisera (5–25 μl /100 μg of microsomes) for 30 min at room temperature before the addition of the compounds and NADPH-generating system. The incubation was carried out for 30 min at 37°C with the final concentrations of the substrates and microsomal protein of 5 μM and 0.4 mg/ml, respectively. Parallel incubations were performed with control goat sera.

All reactions described above were terminated by either heat inactivation (100°C for 3 min) or 2 volumes of acetonitrile. After centrifugation, the supernatants were analyzed directly by HPLC, or concentrated for liquid chromatography-mass spectroscopy (LC-MS) analysis. L-775,842 and cortisone were used as the internal standard for the quantitation of the test compounds and the hydroxylated metabolites of testosterone, respectively.

Animals and Treatment. Male Sprague-Dawley rats (Taconic Farms, Germantown, NY), weighing 280 to 400 g, were used for the in vivo pharmacokinetic studies. The animal studies were reviewed and approved by the Merck Research Laboratories (West Point) Institutional Animal Care and Use Committee. All rats had an indwelling cannula (silicone rubber/polyethylene) implanted in the right jugular vein for blood sampling and drug administration. During the study, all animals were housed individually in plastic metabolism cages and were unrestrained throughout the experiment. Two rats received a single i.v. dose of I or II at 4 mg/kg. The drugs were administered as a solution in dimethyl sulfoxide (0.5 ml/kg) via the cannula in the jugular vein. Blood samples were collected periodically at appropriate time intervals, and plasma samples were kept frozen (-20°C). Before analysis by HPLC, the samples were thawed and precipitated with acetonitrile. After centrifugation, the supernatant was evaporated to dryness under a nitrogen stream. The residues were reconstituted with HPLC mobile phase. L-775,842 was used as the internal standard.

HPLC Analysis. The HPLC system consisted of two Shimadzu-LC-10A pumps (Kyoto, Japan). For the analysis of I and II, chromatography was conducted on a MAC-MOD (Chadds Ford, PA) Zorbax C18 column (4.6×150 mm, 5 μm). The mobile phase consisted of 0.05% aqueous trifluoroacetic acid (TFA) solution (solvent A) and 0.05% TFA in acetonitrile (solvent B), delivered at a flow rate of 1 ml/min with linear increase of solvent B from 10 to 55% over 35 min. The analysis of testosterone and its metabolites was carried out on a Supelco (Bellefonte, PA) C18 column (4.6×150 mm, 5 μm). The mobile phase, consisting of 30% methanol aqueous solution (solvent A)

TABLE 2
Summary of key MS fragmentation for IBN (I) and its metabolites

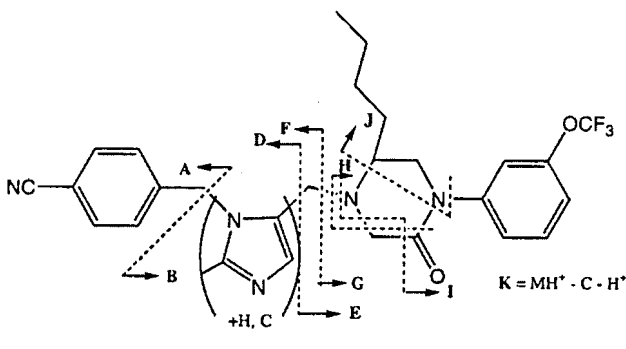


$K = MH^+ - C - H^+$

Compounds	Retention Time	MH ⁺	Fragments										
			A	B	C	D	E	F	G	H	I	J	K
	min		m/z										
IBN	36.0	512	116 ^a	395	81 ^a	184 ^a	329	196	315	273	301	100 ^a	432 ^b
M1	13.3	214	116 ^a		81 ^a								
M2	15.2	429							349				
M3	19.3	333							315			116 ^a	
M4	22.5	413			81 ^a				333			116 ^a	
M5	28.4	397			81 ^a				317			100 ^a	
M6	26.3	528	116 ^{a,c}		81 ^a		345	196	331			116 ^{a,c}	448 ^b

^a The fragments only seen on TSQ7000.
^b The fragments predominant on LCQ ion mass spectrometer.
^c Fragments A and J for M6 were tentatively differentiated by analogy with the mass spectra of parent drug and metabolites along with the absence of fragment at m/z 100 in the metabolites.

TABLE 3
Summary of key MS fragmentation for 2-MIBN (II) and its metabolites



$K = MH^+ - C - H^+$

Compounds	Retention Time	MH ⁺	Fragments										
			A	B	C	D	E	F	G	H	I	J	K
	min		m/z										
2-MIBN	36.2	526	116 ^a	409	95 ^a	198	329	210	315	273	301	100 ^a	432 ^b
M1'	12.2	244	116 ^a		111 ^a	214							
M2'	14.0	228	116 ^a		95 ^a	198							
M3'	15.5	443							349				
M4'	19.9	427			95 ^a				333				
M5'	22.8	427			111 ^a				317				
M6'	26.4	542	116 ^{a,c}	425	95 ^a	198	345	210	331		317	116 ^{a,c}	448 ^b
M7'	28.9	411			95 ^a				317			100 ^a	
M8'	34.1	542	116 ^a		111 ^a		329	224		273	301		
M9'	24.4	558						226	333				

^a The fragments only seen on TSQ7000.
^b The fragments predominant on LCQ.
^c Fragments A and J for M6 were tentatively differentiated by analogy with the mass spectra of parent drug and metabolites along with the absence of fragment at m/z 100 in the metabolites.

and 90% methanol/10% acetonitrile (solvent B), was delivered at a flow rate of 1 ml/min with linear increase of solvent B from 20 to 45% over 15 min. The analysis of bufuralol and its metabolite was carried out on a Keystone (Bellefonte, PA) C18 column (4.6 × 150 mm, 5 μm). The mobile phase, consisting of 0.05% TFA aqueous solution (solvent A) and 0.05% TFA acetonitrile (solvent B), was delivered at a flow rate of 1 ml/min with linear increase of solvent B from 20 to 70% over 10 min. The UV detection was performed on a Shimadzu (Kyoto, Japan) SPD-10A detector with wavelengths set at 235 and

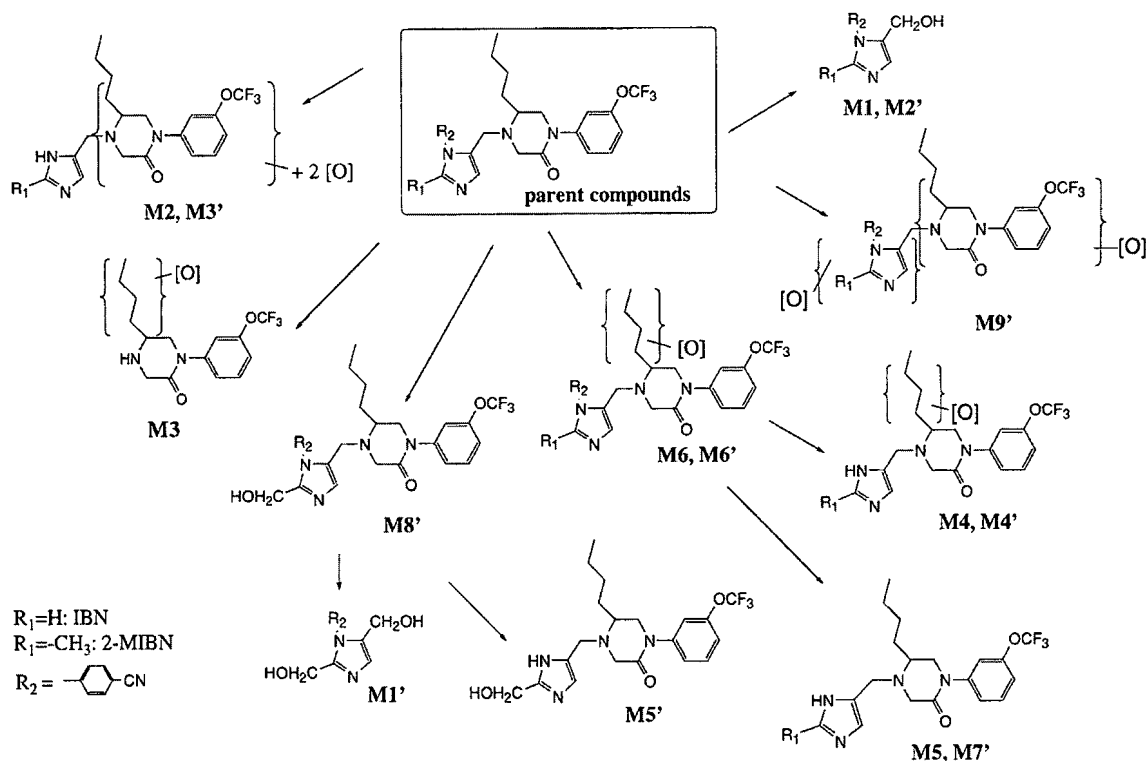


FIG. 2. Metabolites of IBN (I) and 2-MIBN (II) after incubation with rat liver microsomes in the presence of NADPH.

The letters without an apostrophe indicate the metabolites derived from IBN; those with an apostrophe indicate the metabolites derived from 2-MIBN.

245 nm for the test compounds and testosterone, respectively. Bufuralol and its metabolite were monitored on a Shimadzu RF-10A fluorescence detector with excitation wavelength set at 252 nm and emission wavelength at 302 nm.

LC-MS Analysis. HPLC separation was carried out on a HP-1050 gradient system (Hewlett-Packard, Palo Alto, CA) using a Keystone Betasil C18 column (2×150 mm, $5 \mu\text{m}$). The mobile phase consisted of 0.2% aqueous acetic acid with pH adjusted to 7.6 with NH_4OH (solvent A) and acetonitrile (solvent B), and was delivered at a constant flow rate of 0.2 ml/min. The initial mobile phase consisted of 10% of solvent B, which was held for 3 min and then linearly increased to 80% over 35 min.

The column eluent was coupled directly to a Finnigan MAT LCQ ion trap mass spectrometer. Mass spectral analyses were performed using electrospray ionization in the positive mode. The electrospray ionization voltage was set at 4.5 kV, and the heated capillary temperature was maintained at 230°C for all analyses. Collision energy value was set at 25% for the MS/MS or MS^3 (MS/MS/MS) experiments.

Kinetic Analysis. The apparent Michaelis-Menten parameters (K_m and V_{max}) for the overall metabolism of I and II were estimated from Lineweaver-Burk plots using the reduced substrate amount to reflect total turnover. The K_i values for I and II were estimated from a Dixon plot of the data of testosterone metabolite formation in the presence of the inhibitors. In all cases, the parameters were obtained using linear regression.

Results

Metabolic Stability and Profile. To examine their metabolic stability and pathways, I and II were incubated with rat liver microsomal preparations in the presence of NADPH. The metabolic stability of drugs was estimated by measuring the remaining unchanged drugs. As shown in Table 1, the metabolic stability was highly dependent on drug and protein concentration. A faster turnover was observed with a lower drug concentration, whereas a lower protein concentration yielded a slower turnover. Over the concentration range ($5\text{--}20 \mu\text{M}$), at the end of the 20-min incubation, the remaining amount of I was consistently greater than that of II.

A full-scan LC-MS analysis of microsomal incubations of I and II in the presence of NADPH detected several peaks that did not appear in control samples. The parent drugs and their metabolites were well resolved under the conditions used. The structural information of metabolites was obtained through mass spectral analysis. The metabolites were first analyzed using an ion trap mass spectrometer (Finnigan MAT LCQ) and confirmed on a triple quadrupole mass spectrometer (Finnigan TSQ7000). On the basis of the protonated molecular weight (MH^+) and the interpretation of the MS/MS spectra (Tables 2 and 3), the metabolites were tentatively identified as the products derived from *N*-dealkylation, side chain hydroxylation, and the combination of both pathways (Fig. 2).

Because of the limitation of scan range for MS/MS ($1/3$ mass of parent for lowest scan range), some diagnostic fragment ions (fragments A, C, and J) could not be detected by the LCQ. Therefore, the structure of metabolites was also confirmed by using a triple quadrupole mass spectrometer (Finnigan TSQ7000). Generally, similar frag-

TABLE 4

Effect of IBN (I) and 2-MIBN (II) on the activities of testosterone hydroxylases in rat liver microsomes

I and II ($0.1\text{--}100 \mu\text{M}$) were incubated with rat liver microsomes (0.2 mg/ml) at 37°C for 5 min in the presence of testosterone ($20 \mu\text{M}$).

Metabolites (Hydroxylated Testosterone)	IC_{50}	
	IBN (I)	2-MIBN (II)
	μM	
6 β -OH (CYP3A)	<0.2	20
7 α -OH (CYP2A)	>100	>100
16 α -OH (CYP2C)	22	55
16 β -OH (CYP2B)	3	4
2 α -OH (CYP2C)	22	50
2 β -OH (CYP3A)	<0.2	10

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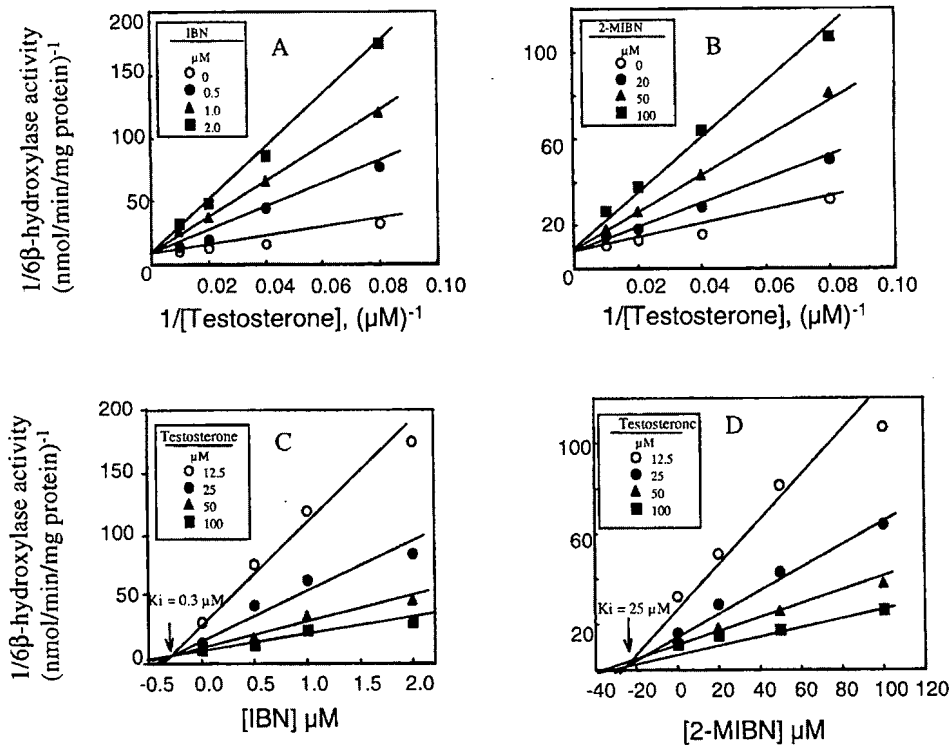


Fig. 3. Inhibition of 6β-hydroxylated testosterone formation in rat liver microsomes by IBN (I) and 2-MIBN (II).

A, double reciprocal plot for the inhibition by I; B, double reciprocal plot for the inhibition by II; C, Dixon plot for the inhibition by I; D, Dixon plot for the inhibition by II.

mentation patterns were seen on both types of mass spectrometers, but the triple quadrupole mass spectrometer gave rise to fragments with low *m/z* values, which were not seen on the ion trap mass spectrometer. However, a significant fragment ion (fragment K) was generated upon a MS/MS experiment carried out on the ion trap mass spectrometer, which helped to identify the potential modification on the imidazole moiety. This ion may be derived from rearrangement occurring in the ion trap by virtue of loss of imidazole moiety and re-linking of the cyanobenzyl group and trifluoromethoxyphenyl piperazinone moiety. Schaefer (1997) reported a similar rearrangement for other compounds. Nevertheless, the combined information obtained from the ion trap and triple quadrupole mass spectrometers on the MS/MS spectra facilitated a better elucidation of metabolite structures in this study.

Inhibitory Effect on Rat Liver CYP Activities. The inhibitory effects of I and II on CYP-mediated reactions were evaluated based on their effect on the hydroxylation of testosterone (Waxman et al.,

1985; Ryan and Levin, 1990) and bufuralol (Orishiki et al., 1994), two CYP marker compounds. As shown in Table 4 and Fig. 3, both I and II competitively inhibited CYP3A activity, as indicated by a decrease in the formation of 6β-OH testosterone; but I was greater than 100 times more potent than its analog. To a lesser extent, the activity of CYP2C11 was also suppressed, as shown by the attenuated formation of 16α- and 2α-hydroxylated testosterone, with I showing an inhibitory potency 2- to 3-fold greater than II. The activity of CYP2D was not affected according to the fact that no effect of the compounds on 1'-OH bufuralol formation was observed (data not shown).

Effect of Anti-Rat CYP Antibodies. To substantiate the results from inhibition of P450 isoform activities, polyclonal antibodies against rat CYP3A2, CYP2C11, and CYP2B1 were used to identify the CYP isoforms involved in the metabolism. I and II were incubated with rat liver microsomes that had been pretreated with respective anti-rat CYP450 sera at three different concentrations. The treatment gave rise to an antibody concentration-dependent inhibition of metab-

TABLE 5

Effects of three anti-rat CYP sera on the formation of major metabolites of IBN (I) and 2-MIBN (II) in rat liver microsomes

Values are expressed as percent formation compared with control (antisera = 0 μl). IBN and 2-MIBN (5 μM) were incubated at 37°C for 30 min with rat liver microsomes (0.4 mg/ml) preincubated with antisera (5–25 μl/100 μg of microsomes).

Antisera/RLM ^a	IBN (I)						2-MIBN (II)					
	Anti-rat CYP2C11		Anti-rat CYP3A2		Anti-rat CYP2B1		Anti-rat CYP2C11		Anti-rat CYP3A2		Anti-rat CYP2B1	
	M1	M6	M1	M6	M1	M6	M2'	M6'	M2'	M6'	M2'	M6'
	μl/100 μg	%	%	%	%	%	%	%	%	%	%	%
0	100	100	100	100	100	100	100	100	100	100	100	100
5	118	66	17	151	98	89	111	58	8.9	193	88	88
15	123	46	10	148	47	109	132	34	5.5	188	76	96
25	137	34	7.9	144	55	86	177	34	4.6	179	78	74

^a RLM, rat liver microsome.



TABLE 6

Enzyme kinetic parameters for IBN (I) and 2-MIBN (II) metabolism obtained using immature male rat liver microsomes

The parameters are expressed as mean value obtained from two separate experiments that were carried out in duplicate.

Parameters	IBN (I)	2-MIBN (II)
K_m (μM)	0.34	18.29
V_{max} (nmol/min/mg protein)	0.055	0.782
V_{max}/K_m (ml/min/mg protein)	0.162	0.043
V^a ([S] = 0.1 μM)	0.012	0.004
V^a ([S] = 0.3 μM)	0.026	0.013
V^a ([S] = 1.0 μM)	0.041	0.040
V^a ([S] = 5.0 μM)	0.051	0.168

^a Calculated velocity (nanomoles per minute per milligram of protein) using the parameters listed in the table.

TABLE 7

Pharmacokinetic parameters of IBN (I) and 2-MIBN (II) for the rat after an i.v. administration of 4 mg/kg

Data are presented as the average value from two rats.

Compounds	CL _{total} ^a ml/min/kg	Vd _{ss} l/kg	t _{1/2} min	AUC ^b $\mu M \cdot h$
IBN	46.1	2.51	67.4	3.21
2-MIBN	37.9	1.79	38.1	4.33

^a CL_{total}, total clearance.

^b AUC, area under the curve.

olite formation summarized in Table 5. Anti-rat CYP2C11 sera significantly decreased the formation of side chain hydroxylate metabolite (M6 of I; M6' of II), whereas anti-rat 2B1 sera only slightly suppressed the formation of the metabolite derived from *N*-dealkylation, but did not affect the metabolite derived from side chain hydroxylation and parent depletion. However, anti-rat CYP3A2 sera strikingly decreased the formation of metabolites associated with *N*-dealkylation (M1 of I and M2' of II). These results indicated that the metabolism of both I and II was primarily catalyzed by CYP3A2 and CYP2C11, and that CYP3A played a more important role than CYP2C11. Thus, I and II were characterized by a similar CYP reaction phenotype.

Enzyme Kinetic Studies. Because both I and II were mainly catalyzed by CYP3A enzymes and because I showed much lower IC₅₀ and *K_i* values for CYP3A than II (Table 4 and Fig. 3), it was speculated that I would have higher affinity to the enzyme. Therefore, enzyme kinetic studies were carried out to determine apparent *K_m* and *V_{max}* values for the metabolism of these two compounds.

Because the metabolism involved multiple pathways, the velocity was determined on the basis of the disappearance of parent drug over a given substrate concentration (0.1–5 μM for I; 1–20 μM for II). Immature male rat liver microsomal preparations, which contained a negligible amount of CYP2C11 (Waxman, 1984), were used as the enzyme source to minimize the influence of CYP2C11. The apparent kinetic parameters derived from this experiment are listed in Table 6, along with the calculated velocities at different substrate concentrations using these parameters. The *K_m* values are 0.34 μM for I and 18 μM for II, comparable with their *K_i* values for the inhibition of 6 β -hydroxylation of testosterone. However, the *V_{max}* value for II is 14 times greater than that for I. The finding clearly shows that methylation at the imidazole ring turned I, a substrate of high affinity but low capacity, into a compound of low affinity but high capacity for metabolism. Under first order conditions ([S]/*K_m* $\leq$ 0.1 for I), the clearance of I would be approximately 4-fold greater than that of II,

whereas the clearance of II would be equal to or exceed that of I when the concentrations are greater than 1 μM (Table 6).

Pharmacokinetics after i.v. Administration. An i.v. administration of I and II at a dose of 4 mg/kg to the rat gave rise to a plasma concentration range of ca. 5 to 0.1 μM over 3 h and revealed similar pharmacokinetic profiles. Comparable pharmacokinetic parameters were obtained for both compounds (Table 7). It appeared that the difference in their intrinsic clearance did not result in different in vivo disposition.

Discussion

Like some potent CYP inhibitors containing a 1, 4(5)-substituted imidazole ring (Wilkinson et al., 1974; Ahmed et al., 1995; Halpert, 1995; Miranda et al., 1998), I strongly inhibited the activity of rat CYP3A (*K_i* = 0.34 μM). The mechanism of the inhibition is thought to relate to the coordination of the lone pair electrons with sp² orbital on the nitrogen atom with the prosthetic heme iron. The process shifts the P450 from a high- to low-spin state, making its reduction by P450 oxidoreductase more difficult and, consequently, inhibits enzyme activity (Schenkman et al., 1982; Ortiz de Montellano and Correia, 1995). This undesirable feature of I as a drug candidate led to the development of its analog II by means of substitution of the hydrogen atom at 2-position with a methyl group, a common approach to attenuate the interaction by introduction of steric hindrance (Rogerson et al., 1977). As expected, II showed an inhibitory potency (*K_i* = 25 μM) 70-fold weaker than I.

Methylation of the imidazole ring did not alter the major metabolic pathway. Although the presence of the methyl group on the imidazole ring in II provided an additional site for enzyme attack, resulting in some minor additional metabolites, the major metabolic pathways for these two compounds are qualitatively similar. The modification of the structure also did not switch the metabolizing enzymes. The CYP3A family appeared to be the major CYP isoform responsible for the oxidation of I and II in the rat, while CYP2C11 was also involved, although to a lesser extent. Antibody study indicates that CYP3A and CYP2C11 may attack different sites of the compounds. Namely, CYP3A mediates *N*-dealkylation and CYP2C11 side chain hydroxylation. These two metabolic pathways may be affected to a different extent by methylation of imidazole ring, because over 70 times decrease in the inhibitory potency against CYP3A4 was seen with II, but the modification gave rise to only 2- to 3-fold changes in the effect on CYP2C11.

The change in their kinetic parameters (*K_m* and *V_{max}*) resulting from this minor structural modification reflects a different interaction of I and II with the metabolizing enzyme(s). I has a greater affinity to the enzyme to form enzyme-substrate complex (ES) that, however, may not be favored for the product formation. On the other hand, the disadvantageous step to form ES for II due to its lower affinity may get compensated by the faster turnover of its ES to the products.

It is of importance to note that for compounds differing in the kinetic properties as such, the net consequence of their reaction velocity will depend on the substrate concentration even though their intrinsic clearance (*V_{max}*/*K_m*) may be considerably different. The in vitro intrinsic clearance has been widely used to predict in vivo metabolic clearance (Houston, 1994; Houston and Carlile, 1997). However, in spite of the *V_{max}*/*K_m* for I being 4-fold higher than that for II, no significant difference in pharmacokinetic profiles was observed for these two compounds after i.v. administration to the rat. Because I and II both extensively bound to rat plasma in a similar extent (data not shown), their comparable pharmacokinetics could be attributed to the impact of actual drug concentration in the plasma (or at metabolic sites). As illustrated in Table 6, the favorable metabolic

rate varies with substrate concentration for compounds like **I** and **II**. A greater rate would be seen for **I** when the concentration of the compounds is below or close to its K_m , but **II** would surpass its analog when the concentration reaches micromolar level. This situation would hold true for any drug that is metabolized by two enzymes with distinct K_m and V_{max} values, as in the case of meloxicam (Chesne et al., 1998). The pathway mediated by the low K_m enzyme would be predominant at low substrate concentrations. At higher concentrations, however, the reaction catalyzed by the high K_m enzyme would dominate. Therefore, the importance of each enzyme in the overall metabolism depends on the actual drug concentration. With a high affinity and low capacity, **I** is more likely subject to nonlinear kinetics than its analog. At the maximal plasma concentration of ca. 5 μM , **I** would follow a zero order (saturated) metabolism, but **II** would assume a first order kinetics. Because of its low catalytic capacity, **I** would not be favored. In the in vivo situation, the actual concentration of the compounds varied with time. As a result, the overall pharmacokinetics of **I** and **II** may fail to show a significant difference at that given dose (4 mg/kg). The difference of their K_m and V_{max} values may also explain the higher turnover of **II** at the concentration range of 5 to 20 μM in the in vitro experiment (Table 1). If the assumption is valid that the compounds would be distributed and excreted to comparable extents, when given a lower dose, a significantly shorter $t_{1/2}$ and smaller AUC for **I** might be observed. This situation would be consistent with the predication made on the basis of the difference in their intrinsic clearance. Our results demonstrate that concentration is an important factor when comparing the pharmacokinetics of two compounds having different enzyme affinities and catalytic capacities.

In conclusion, we have shown that methylation of **I** at its imidazole ring decreased its inhibitory potency against metabolizing enzymes. Accordingly, the affinity to the metabolizing enzyme decreased and the catalytic capacity enhanced. The structural modification did not switch the major metabolic pathways and the metabolizing enzymes involved. In addition, the overall effect of this structure modification on the metabolic rate depends on the substrate concentration.

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