

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

Abogada
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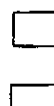
ASUNTO

Radicación 10-036551
Trámite 011
Evento 378
Actuación 519
Oficio **22 - 196 4 2**
Folios 27

Patente de Invención
Vía Nacional
Vía PCT (fase nacional)



Patente de Modelo de Utilidad
Cap. I ☒ Cap. II ☐



No. Solicitud Internacional PCT/FR2008/001384
Fecha de Presentación Internacional 03/octubre/2008

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 146 a 268	26/marzo/2010
Reivindicaciones:	Folios 269 a 275	26/marzo/2010
Prioridad:	FR 0706931	03/octubre/2007

2. Análisis de la Prioridad

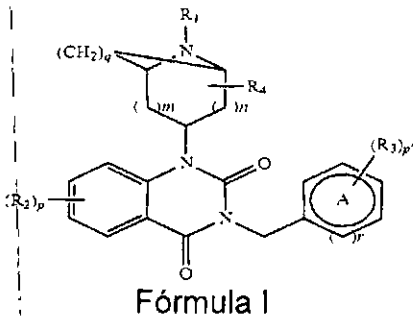
Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Derivados de quinazolinadiona su preparación y sus aplicaciones terapéuticas"

El problema planteado en esta solicitud consiste en la necesidad de suministrar derivados de quinazolinadiona como inhibidores de la fosfodiesterasa para el tratamiento de diversas enfermedades.

Para resolver este problema técnico la solicitud en estudio proporciona compuestos de la fórmula (I).



El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07D403/04, A61K31/517.

4. Reivindicaciones relativas a un uso (Art 14 D 486)

El uso de las reivindicaciones 17 y 18 no es patentable, de acuerdo con el Art 14.

5. De la Solicitud de Patente

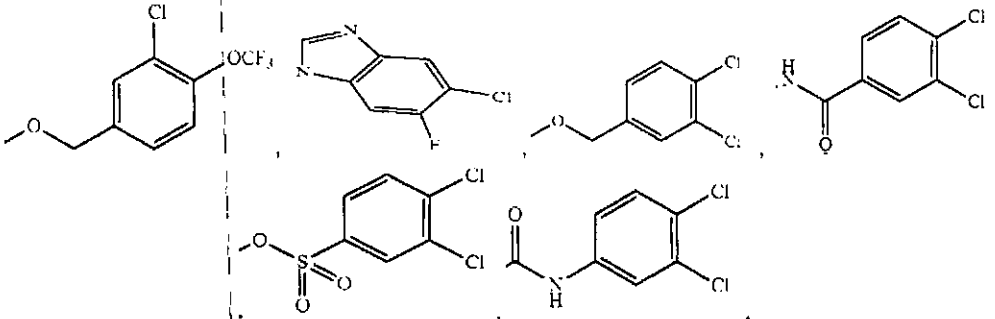
Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 y 6 no son claras porque tienen el término “opcionalmente”, que es impreciso porque hace ocasional la sustitución de los grupos funcionales, y no permite una comparación con el estado de la técnica. Se sugiere incluir las características opcionales, sin ambigüedad.

La reivindicación 1 no es concisa porque los radicales R1, R3 y R4 son cicloalquilo y heterocicloalquilo, los cuales corresponden a grupos funcionales ambiguos y no definen de manera inequívoca los sustituyentes de los compuestos reclamados. Por lo tanto se le sugiere al solicitante, en lo posible, restringir el capítulo reivindicatorio de la solicitud a una generalización razonable de las realizaciones preferidas. Adicionalmente se debe establecer el número de carbonos que los conforman, el número y tipo de heteroátomos.

En la definición de los radicales más representativos de la fórmula (I), de acuerdo con lo que se encuentra debidamente sustentado en el capítulo descriptivo, se encuentra que siempre varían entre los siguientes sustituyentes y átomos:

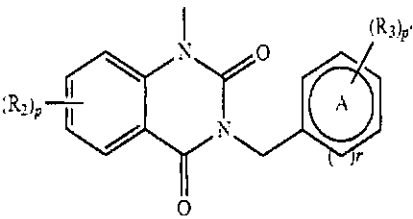
Radical	Soporte en la descripción	
R ¹	COOH	
R ²		

R ³	
R ⁴	H
A	Fenilo, piridina

Por lo tanto se sugiere al solicitante presentar una generalización razonable y eliminar aquellas definiciones que exceden el sustento descriptivo.

6. Unidad de invención (Art 25 D 486)

El concepto común definido en la solicitud consiste en:



y la propiedad en común corresponde a inhibidores de fosfodiesterasas.

El análisis de la unidad de invención *a posteriori*, se realiza considerando el documento D2 el estado de la técnica más cercano porque anticipa los elementos técnicos que definen el concepto común. En el documento D2, A=fenilo y X= C (Pág. 53 y 54), por eso, es equivalente a la solicitud y por lo tanto el concepto común no es inventivo.

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 4 grupos inventivos.

Los grupos inventivos están definidos por las siguientes características esenciales:

Grupo Inventivo 1º: Compuestos de acuerdo a la fórmula (I), de la presente aplicación donde q=0 y la combinación de m y n forman un anillo piperidinilo.

Grupo Inventivo 2º: Compuestos de acuerdo a la fórmula (I), de la presente aplicación donde q=0 y la combinación de m y n forman un anillo pirrolidino.

Grupo Inventivo 3º: Compuestos de acuerdo a la fórmula (I), de la presente aplicación donde q=0 y la combinación de m y n forman un anillo azetidina.

Grupo Inventivo 4º: Compuestos de acuerdo a la fórmula (I), de la presente aplicación donde q=0 y la combinación de m y n forman un anillo homopiperidina.

El primer grupo inventivo es elegido para el análisis de nivel inventivo.

Se sugiere presentar las Solicitudes Divisionales que considere necesarias, ó
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limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de prioridad (03/octubre/2007) y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Item	Documento	Título	Fecha de Publicación
D1	WO9319068	pyrido[2,3-d]pyrimidine derivatives as phosphodiesterase inhibitors	30/septiembre/1993
D2	JOURNAL OF MEDICINAL CHEMISTRY. LOWE J.A. ET AL. vol. 34	Structure-Activity Relationship of Quinazolinone Inhibitors of Calcium-Independent Phosphodiesterase	09/julio/1991

D1. http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=9319068A1&KC=A1&FT=D&ND=&date=19930930&DB=&locale=en_EP
Divulga la fórmula estructural de derivados pirido[2,3-d] pirimidina-2(1H,4H)ona como inhibidores de fosfodiesterasas para el tratamiento del dolor neuropático y la inflamación (Pág. 53 y 54).

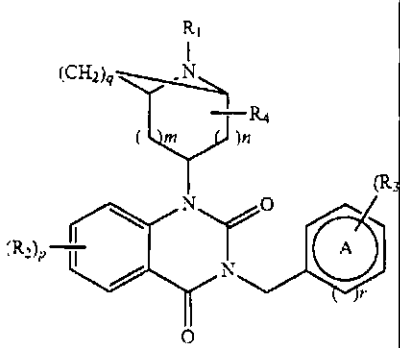
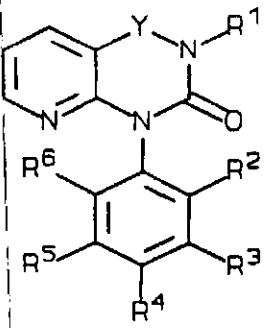
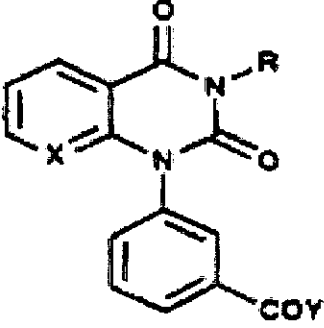
D2. <http://pubs.acs.org/doi/abs/10.1021/jm00106a024>
Divulga la fórmula estructural de derivados quinazolinadonas y azaquinazolinadonas como inhibidores de fosfodiesterasas para el tratamiento del dolor neuropático y la inflamación (Pág. 624 y 625).

8. Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en un compuesto de fórmula (I) donde: (Fl. 269).

Comparación de las **características técnicas esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
<u>Compuesto de fórmula I</u> 	<u>Compuestos de fórmula I</u>  Y=CO Pág. 53 y 54	<u>Compuestos de fórmula I</u>  X=C Pág. 624 y 625
R ¹ = H, -COR donde R=H	R4= COOH	Y=OH
R ² = H, O-Ra donde Ra=H	R2	H
A= arilo o heteroarilo	R1≠ CH2-R7 donde	R=CH ₂ fenil
R ³ = H, O-alquilo, O-N-CR,	R7=arilo o heteroarilo	

NOCR		
$R^4 = H$	H	H
$p = 1,2$	1	1
$q = 0 \text{ o } 2$	0	0
$m \text{ y } n = 0, 1 \text{ o } 2 \text{ m}+n<3$	1	1

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1.

El documento D1 divulga la fórmula estructural de derivados de pirido[2,3-d] pirimidina-2(1H,4H)ona como inhibidores de fosfodiesterasas para el tratamiento del dolor neuropático y la inflamación (Pág. 53 y 54).

El documento D2 divulga la fórmula estructural de derivados de quinazolinadionas y azaquinazolinadionas como inhibidores de fosfodiesterasas para el tratamiento del dolor neuropático y la inflamación (Pág. 624 y 625).

La diferencia entre la invención, definida en la reivindicación 1 y la fórmula del documento D1 consiste en que la invención tiene un anillo fenilo fusionado al anillo pirimidina-2,4-diona y un anillo heterociclo nitrogenado unido al nitrógeno de la posición 1 del anillo pirimidina-2,4-diona.

La diferencia entre la invención, definida en la reivindicación 1 y el compuesto del documento D2 consiste en que la invención define como anillos heterociclos nitrogenados los anillos piperidinilo, pirrolidino, azetidina y homopiperidina.

No se evidencia que por el hecho de tener como anillos heterociclos nitrogenados los anillos piperidinilo, pirrolidino, azetidina y homopiperidina, se tenga un efecto técnico inesperado.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar los compuestos de fórmula I conocidos en el documento D1 para conseguir compuestos derivados de anillos fusionados de pirimidina-2,4-diona como inhibidores de fosfodiesterasas”.

Sin embargo, es conocido en el documento D2, el anillo heterociclo nitrogenado unido al nitrógeno de la posición 1 del anillo pirimidina-2,4-diona, para facilitar la unión al sitio de unión con la enzima fosfodiesterasa y teniendo en cuenta que la distancia con el anillo central es la misma (Pág. 625, columna 2). Adicionalmente es conocido en el diseño de fármacos las numerosas modificaciones alrededor del anillo pirimidina, con el fin de obtener una mayor selectividad por las isoformas de las enzimas fosfodiesterasas (1-10), por lo tanto, se han conocido las diferentes modificaciones a partir de la fórmula estructural del sildenafil para evaluar la inhibición de fosfodiesterasas y es conocido que la sustitución heterocíclica del anillo de pirimidina tiene un efecto marcado en la actividad de inhibición de algunas isoformas de la enzima fosfodiesterasa.¹ Por otra parte respecto a los resultados de inhibición de fosfodiesterasa del documento D1 no se observa un efecto técnico sorprendente. Además es conocido que la selectividad e incremento de la potencia en la fosfodiesterasa 7 fue observada al sustituir el fenilo.²

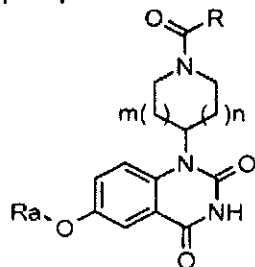
1 J. Perry. Chemotherapeutic potential of phosphodiesterase inhibitors. *Current Biology Publications*. vol. 2, no. 4, 01 de enero de 1998, Pág. 475 a 477.
<http://www.sinab.unal.edu.co/?q=node/46>
2 J. Pitts. Identification of purine inhibitors of phosphodiesterase 7 (PDE7). *Bioorganic & Medicinal Chemistry Letters*. 14. 03 de marzo de 2004. Pág. 2958. <http://www.sinab.unal.edu.co/?q=node/40>
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En consecuencia, la persona del oficio normalmente versada en la materia, estaría motivada a emplear un anillo heterociclo nitrogenado unido al nitrógeno de la posición 1 del anillo pirimidina-2,4-diona. Por lo cual se considera obvia.

Conclusión:
La reivindicación 1 no cumple con el requisito de nivel inventivo (Art. 18).

Puesto que las reivindicaciones dependientes 2 a 11 y 13 a 16 no mencionan características inventivas adicionales y tampoco se pueden considerar inventivas.

La reivindicación 12 consiste en un procedimiento de preparación de un compuesto según una cualquiera de las reivindicaciones 1 a 11, caracterizado porque se hace reaccionar un compuesto de fórmula

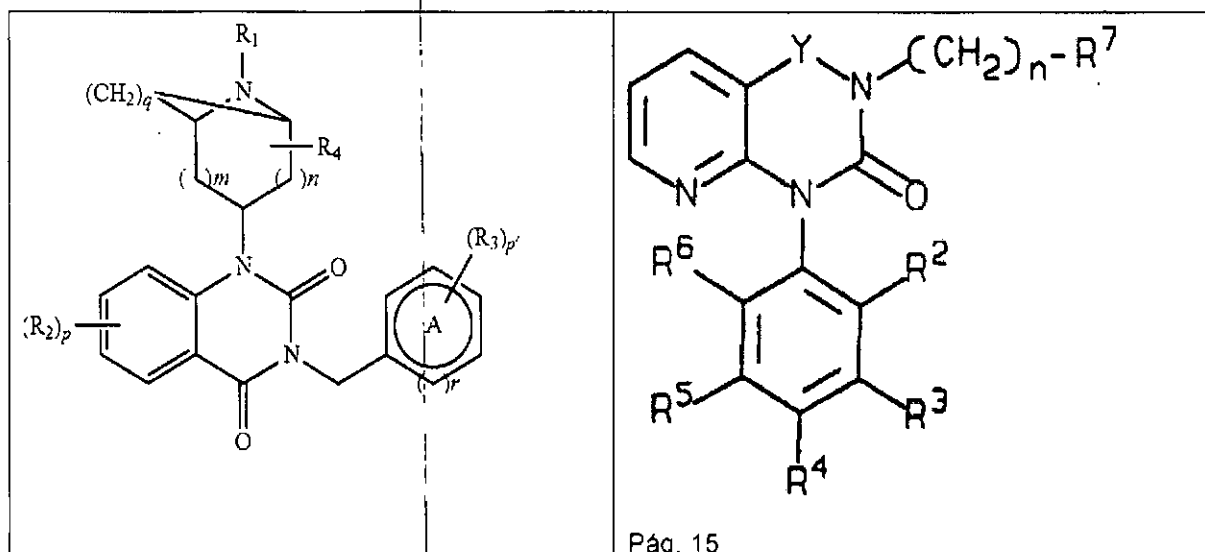


En la que R, Ra, m y n son tal como se definen en la reivindicación 1, y en la que puede además haber un grupo R2, tal como se define para los compuestos de fórmula (I), en posición 7 de la estructura quinazolindiona, con un compuesto de

fórmula $X-CH_2-C_6H_4-R^3$ (XIX) o $HO-CH_2-C_6H_4-R^3$ (XX) por una reacción de alquilación o de Mitsunobu, respectivamente.

Comparación de las **características técnicas esenciales**, con las del Estado de la Técnica:

Características esenciales	D1
Procedimiento de preparación caracterizado por Se hace reaccionar un compuesto de fórmula En la que R, Ra, m y n son tal como se definen en la reivindicación 1, y en la que puede además haber un grupo R2, tal como se define para los compuestos de fórmula (I), en posición 7 de la estructura quinazolindiona, con un compuesto de fórmula $X-CH_2-C_6H_4-R^3$ (XIX) o $HO-CH_2-C_6H_4-R^3$ (XX) por una reacción de alquilación para dar un compuesto según una cualquiera de las reivindicaciones 1 a 11	Procedimiento de preparación caracterizado por Se hace reaccionar un compuesto de fórmula En la que R1 a R6 y Y son tal como se definen en el cuadro de comparación de la reivindicación 1; con un compuesto de fórmula $HO-(CH_2)_n-R^7$ por una reacción de alquilación para dar un compuesto de fórmula I



El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 12.

El documento D1 divulga el procedimiento de preparación de compuestos de la fórmula estructural (I) (Pág. 15).

La diferencia entre la invención, definida en la reivindicación 1 y el procedimiento de preparación del documento D1 consiste en que la invención tiene el intermediario (XVIII) y el producto final de fórmula estructural (I) con un anillo fenilo fusionado al anillo pirimidina-2,4-diona y un anillo heterociclo nitrogenado unido al nitrógeno de la posición 1 del anillo pirimidina-2,4-diona.

No se evidencia que por el hecho de tener como anillos heterociclos nitrogenados los anillos piperidinilo, pirrolidino, azetidina y homopiperidina, se tenga un efecto técnico inesperado.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el intermediario y producto final del procedimiento de preparación para los compuestos de fórmula I conocidos en el documento D1 para conseguir un procedimiento de preparación alternativo".

Sin embargo, es conocido el anillo heterociclo nitrogenado unido al nitrógeno de la posición 1 del anillo pirimidina-2,4-diona, para facilitar la unión al sitio de unión con la enzima fosfodiesterasa y teniendo en cuenta que la distancia con el anillo central es la misma (Pág. 625, columna 2) ³.

En consecuencia, la persona del oficio normalmente versada en la materia, estaría motivada a emplear un procedimiento de preparación en el que el intermediario y por lo tanto en el producto final tengan un anillo heterociclo nitrogenado unido al nitrógeno de la posición 1 del anillo pirimidina-2,4-diona. Por lo cual se considera obvia.

Conclusión:

La reivindicación 12 no cumple con el requisito de nivel inventivo (Art. 18).

9. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO9319068	1 a 16	Nivel inventivo
D2	JOURNAL OF MEDICINAL CHEMISTRY. LOWE J.A. ET AL. vol. 34	1 a 11 y 13 a 16	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 30 OCT. 2012

Elaborado por: Ruth Angélica Lizarazo Pedraza 
Revisado por: Consuelo Leguizamón Leguizamón 

vigorously with 10 mL of toluene scintillator, and the radioactivity in the filters was counted with a Packard Tris-Carb scintillation counter (B-2450). IC_{50} values of the test compounds (the concentrations causing 50% inhibition of ^3H -labeled ligand specific binding) were determined by probit analysis.

Acknowledgment. We are grateful to Dr. M. Hashimoto, the Director of the Laboratories. Thanks are also due to the staff of the Physical & Analytical Chemistry Division of our laboratories for elemental analyses and spectral measurements.

Registry No. 1a, 112885-23-1; 1b, 112885-33-3; 2, 112913-96-9; 3-AcOH, 131322-23-1; 4 ($R_2 = 3-CF_3C_6H_4CH_2$), 112887-04-4; 5 ($R_2 = 3-CF_3C_6H_4CH_2$), 131322-24-2; 6, 4093-28-1; 7, 59-06-3; 8, 4235-43-2; 9, 112914-09-7; 10a, 7206-70-4; 10b, 108282-38-8; 11, 112914-03-1; 12, 131322-25-3; 13, 131322-26-4; 14, 110859-48-8; 15, 112887-44-2; 16, 112914-11-1; 17, 112885-36-6; 18, 112914-02-0; 19a, 131322-27-5; 19a- $C_2H_5O_4$, 131322-36-6; 19b, 112886-52-9; 20b, 131322-28-6; 20b-HCl, 112886-53-0; 21b, 112886-55-2; 21b-HCl, 112886-54-1; 22a, 112885-14-0; 22a- $C_2H_5O_4$, 112885-15-1;

23a, 112885-17-3; 23b, 112885-41-3; 23b-1.25HCl, 131322-37-7; 24a, 112885-18-4; 24a- $2C_2H_5O_4$, 112885-19-5; 24b, 112886-56-3; 24b- $C_2H_5O_4$, 112886-57-4; 25a, 112885-20-8; 25a-0.5 $C_2H_5O_4$, 112885-21-9; 25b, 112886-58-5; 25b- $2C_2H_5O_4$, 112886-59-6; 26a, 112885-05-9; 26a-1.5 $C_2H_5O_4$, 112885-06-0; 27a, 112885-07-1; 27a-1.5 $C_2H_5O_4$, 112885-08-2; 27b, 112886-49-4; 28a, 112885-02-6; 29a, 112885-03-7; 20a- $C_2H_5O_4$, 112885-04-8; 30a, 112885-11-7; 31a, 112885-09-3; 31a-1.5 $C_2H_5O_4$, 112885-10-6; 32a, 112884-98-7; 33a, 112884-97-6; 33a-0.5 $C_2H_5O_4$, 131322-38-8; 34a, 131322-29-7; 34a-HCl, 131322-39-9; 34b, 131322-35-5; 35b, 131322-30-0; 35b, 131322-30-0; 35b-HCl, 131322-40-2; 36a, 112885-32-2; 36a- $C_2H_5O_4$, 112885-35-5; 36b, 112886-47-2; 37a, 112884-99-8; 37a- $C_2H_5O_4$, 112885-00-4; 37b, 112886-45-0; 37b- $C_2H_5O_4$, 112886-46-1; 38a, 112885-01-5; 38b, 112886-48-3; 39a, 112885-13-9; 40b, 112886-44-9; 41b, 131322-31-1; 42b, 131322-32-2; 43b, 131322-33-3; 44a, 112885-12-8; 44b, 112886-50-7; 45a, 112884-94-3; 45a- $C_2H_5O_4$, 112884-95-4; 45b, 112886-38-1; 45b- $C_2H_5O_4$, 112886-39-2; 46a, 112884-92-1; 46a- $C_2H_5O_4$, 112884-93-2; 47b, 112886-37-0; 48a, 112913-72-1; 49a, 112884-96-5; 50b, 112886-42-7; 50b- $C_2H_5O_4$, 112886-43-8; 51b, 112886-40-5; 51b- $C_2H_5O_4$, 112886-41-6; 52b, 112885-34-4; 53b, 131322-34-4; 53b-1.75 $C_2H_5O_4$, 131322-41-3; 54b, 112914-14-4; 55b, 112914-15-5.

Structure-Activity Relationship of Quinazolinodione Inhibitors of Calcium-Independent Phosphodiesterase

John A. Lowe, III,* Robert L. Archer, Douglas S. Chapin, John B. Cheng, David Helweg, Jonathan L. Johnson, B. Kenneth Koe, Lorraine A. Lebel, Peter F. Moore, Jann A. Nielsen, Lorena L. Russo, and John T. Shirley

Central Research Division Pfizer, Inc., Eastern Point Road, Groton, Connecticut 06340. Received July 9, 1990

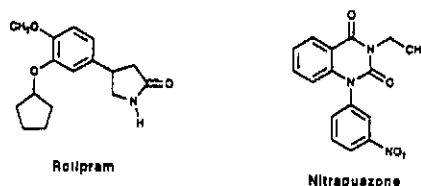
A series of quinazolinodiones and azaquinazolinodiones is described which possess potent inhibitory activity toward the calcium-independent phosphodiesterase enzyme (CaIPDE). In vivo testing showed that this in vitro activity translates to animal models predictive of chronic diseases such as depression and inflammation. These results support the hypothesis that inhibition of CaIPDE may lead to useful activity in such chronic diseases.

The phosphodiesterase (PDE) enzymes have been classified into four groups based on selective inhibition by a number of structurally diverse compounds.¹ We have studied the calcium-independent, low K_m , cAMP-selective PDE (CaIPDE) which is selectively inhibited by the antidepressant drug rolipram² (Chart I) as a target for novel antidepressant agents,³ and in this context, we sought new structures to complement our work. Our attention was thus attracted to a report⁴ describing the drug niraquazone, coded TVX 2706, which was characterized as a phosphodiesterase (PDE) inhibitor with antiinflammatory activity in vivo. Despite its marked structural dissimilarity to rolipram, niraquazone proved to be both a selective CaIPDE inhibitor and a potent binder to the [^3H]rolipram binding site.⁵ Our attempts to understand the structural basis for the inhibition of CaIPDE by both niraquazone and rolipram led us to synthesize an exceptionally potent CaIPDE inhibitor, CP-77,059 (3e). The discovery of 3e and its biological characterization are chronicled in this paper.

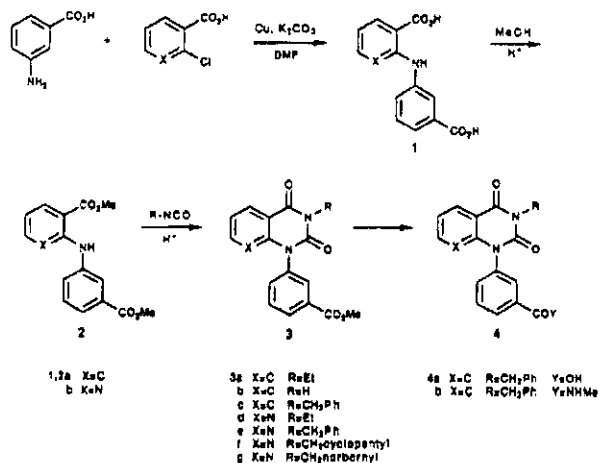
Chemistry

The synthetic methodology developed for the analogues of niraquazone (Table III) is shown in Scheme I; it is

Chart I



Scheme I. Preparation of Quinazolinodiones and Pyridopyrimidinediones



based on literature precedent for the preparation of the quinazolinodione ring system by the reaction of an anthranilate derivative with isocyanate under acid catalysis.⁶

- (1) Reeves, M. L.; Leigh, B. K.; England, P. J. *Biochem. J.* 1987, 241, 535.
- (2) Eckmann, F.; Fichte, K.; Meya, U.; Sastre-y-Hernandez, M. *Curr. Ther. Res.* 1988, 43, 291.
- (3) Koe, B. K.; Lebel, L. A.; Nielsen, J. A.; Russo, L. L.; Saccamano, N. A.; Vinick, F. J.; Williams, I. H. *Drug. Dev. Res.*, in press.
- (4) Glaser, T.; Traber, J. *Agents Actions* 1984, 15, 341.
- (5) Russo, L. L.; Lebel, L. A.; Koe, B. K. *Soc. Neurosci. Abstr.* 1987, 13, 903.

Table I. In Vitro Characterization of PDE Inhibitors 3 and 4

compd	CaIPDE-A ^a	CaIPDE-B ^b	rolipram binding ^c
3a		3.8	
3b	>10	>100	
3c	1.0 ± 0.6 (2)	0.23	180
3d		25.9	
3e	0.040 ± 0.016 (6)	0.13	1.3 ± 0.3 (5)
3f	0.070	1.52	2.5 ± 1.3 (2)
3g	0.10	0.89	3.2 ± 0.1 (2)
4a	>>10		>>1000
4b		21.4	
rolipram	0.49 ± 0.22 (38)	0.59	2.6 ± 0.1 (12)
nitraquazone	1.9 ± 1.0 (4)	11.4	14 ± 1 (5)

^a Inhibition of calcium-independent phosphodiesterase in rat brain cortex, IC₅₀, μM units. IC₅₀ values were determined from dose-response curves of 3 log concentrations of the test compounds; each concentration was run in triplicate. Mean ± standard deviation for (N) separate assays was determined for 3c, 3e, rolipram, and nitraquazone. ^b Inhibition of calcium-independent phosphodiesterase in guinea pig lung, IC₅₀, μM units. IC₅₀ values were determined from dose-response curves of 4 log concentrations of the test compounds; each concentration was run in triplicate. ^c Binding to the [³H]rolipram binding site in rat brain, IC₅₀, nM units. IC₅₀ values were determined from dose-response curves of 3 log concentrations of the test compounds; each concentration was run in triplicate. Mean ± standard deviation for (N) separate assays was determined for 3e-g, rolipram, and nitraquazone.

The N-phenylanthranilate derivatives were prepared by coupling of *m*-aminobenzoic acid with 2-chlorobenzoic or 2-chloronicotinic acid catalyzed by cuprous ion,⁷ followed by esterification. Ring formation catalyzed by camphor-sulfonic acid in refluxing xylene proceeded in modest yield. Those isocyanates which were not commercially available were prepared in situ by reaction of the appropriate carboxylic acid with diphenyl phosphorazidate.⁸

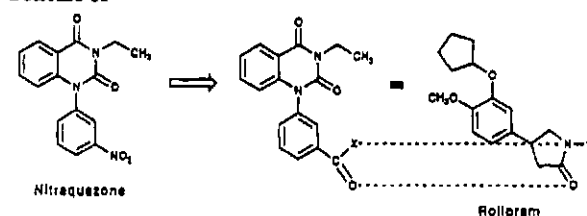
Biology

The procedures for testing compounds for activity against CaIPDE and for binding at the [³H]rolipram binding site are those described previously.⁹ Standard animal models of depression and inflammation were used to evaluate in vivo activity. Thus, depression may be at least partially related to dysfunction of noradrenergic neurotransmission; by causing depletion of noradrenaline, reserpine-induced hypothermia may thus serve as a model of depression. Rolipram, and other inhibitors of CaIPDE, are active in this test, presumably by virtue of their elevation of levels of the second messenger, cyclic adenosine monophosphate (cAMP), in noradrenergic neurons. The forced swim test produces behavior in mice similar to depression in that rolipram and other antidepressants reverse the behavioral deficit. The rat foot edema test in rats reliably predicts the antiinflammatory activity of the nonsteroidal antiinflammatory drugs (NSAIDs) developed thus far and allows an estimate of the effective clinical dose in man.

Results and Discussion

Our finding, reported previously,⁵ that nitraquazone binds with high affinity to the [³H]rolipram binding site is surprising because of the structural dissimilarity of the two compounds. In searching for ways to overlap their basic structural elements, we developed the hypothesis that the nitro group of nitraquazone and the lactam group of rolipram might occupy the same site on the [³H]rolipram

Scheme II



binding site. In contrast to the carbonyl groups of nitraquazone which are structurally rigid and inflexible, the nitro group of nitraquazone provided better overlap with the lactam carbonyl moiety of rolipram because both groups can rotate freely. As shown in Scheme II, we examined replacement of the nitro group by carbonyl functions that would mimic its charge distribution in order to test our hypothesis.

The results of in vitro testing of the compounds resulting from this effort are summarized in Table I, where it can be seen from compound 3a that a carboxylate ester does indeed fulfill much of the function of the nitro group, both in binding at the [³H]rolipram binding site and in inhibition of CaIPDE. Variation of the side chain group at N-3, compounds 3b and 3c, showed that increased size and lipophilicity improve CaIPDE activity. Transformation of the carboxylate ester of 3c to an acid or amide function, compounds 4a and 4b, respectively afforded substantial or complete loss of activity. Although the acid group in 4a would seem a suitable bioisosteric replacement for a nitro group, its negative charge may well exclude it from binding to CaIPDE or the [³H]rolipram binding site. The aromatic amide group in 4b is apparently not an effective bioisostere for the nitro group in this system due to its weaker electron-withdrawing properties. Finally, the pyridopyrimidinone nucleus also afforded potent CaIPDE inhibitory activity, provided the SAR established for the parent series was observed. Thus, 3d was less potent than the more lipophilic analogues 3e-g. Even though the activity of compounds as CaIPDE inhibitors showed limited correlation with their binding at the [³H]rolipram binding site, potent [³H]rolipram binding compounds were also good CaIPDE inhibitors. In addition, the selectivity of these compounds for CaIPDE was determined by assaying them with a calcium-dependent PDE preparation.⁹ In all cases, IC₅₀ values for inhibition of calcium-dependent PDE were much greater than 10 μM.

Characterization of selected members of this series in animal models of chronic disease afforded the results shown in Table II. Potent in vitro activity translated well

(6) Durant, G. J. *Chem. Ind.* 1965, 1428.

(7) In analogy with the preparation of the corresponding initial intermediate in the synthesis of nitraquazone, see: Lehmsstedt, K.; Schrader, K. *Chem. Ber.* 1937, 70, 838. Ullman, F. *Liebigs Ann. Chem.* 1907, 355, 330.

(8) Commercially available from Aldrich Chemical Co., see: Shioiri, T.; Ninomiya, K.; Yamada, S. *J. Am. Chem. Soc.* 1972, 94, 6203.

(9) Davis, C. *Biochim. Biophys. Acta* 1984, 797, 354. The CaIPDE preparation described herein which was used in these assays is believed to be a mixture of several isozymes with similar high affinity for cAMP (Davis, C., personal communication).

M

Table II. In Vivo Characterization of PDE Inhibitors 3 and 4

no.	mouse reserpine-induced hypothermia ^a	mouse swim test ^b	rat foot edema ^c
3a			19
3b	<0.32		5
3c	0.1	>32	25
3e	0.01	0.01	50*
3f	0.003	1.0	
3g	0.001	1.0	
rolipram	0.00003	1.78	41
nitroquazone	0.001	3.2	43*

^a Minimal effective dose for inhibition of reserpine-induced hypothermia in mice, in mg/kg po. ^b Minimal effective dose for inhibition of immobility in the mouse forced swim test, in mg/kg po. ^c Percent inhibition of carrageenan-induced rat foot edema at 32 mg/kg po, except where indicated* at 3.2 mg/kg po.

Table III. Physical Data

no.	formula	method	mp, °C	% yield	analysis
1a	C ₁₄ H ₁₁ NO ₄	A	277-279	69	C, H, N
1b	C ₁₅ H ₁₀ N ₂ O ₄ ·1/2H ₂ O	A	275-280	72	C, H, N
2a	C ₁₆ H ₁₄ NO ₄	B	oil	61	C, H, N
2b	C ₁₆ H ₁₄ N ₂ O ₄	B	105-106	13	C, H, N
3a	C ₁₆ H ₁₆ N ₂ O ₄	C	135-136	22	C, H, N
3b	C ₁₆ H ₁₇ N ₂ O ₄	C	274-276	8	C, H, N
3c	C ₂₀ H ₁₈ N ₂ O ₄	C	151-153	45	C, H, N
3d	C ₁₇ H ₁₆ N ₃ O ₄ ·1/2H ₂ O	C	186-187	4	C, H, N
3e	C ₂₀ H ₁₇ N ₃ O ₄	C	157-160	31	C, H, N
3f	C ₂₁ H ₂₁ N ₃ O ₄	D	149-151	5	C, H, N
3g	C ₂₃ H ₂₃ N ₃ O ₄	D	157-159	20	C, H, N
4a	C ₂₀ H ₁₆ N ₂ O ₄ ·1/2H ₂ O		260-262	95	C, H, N
4b	C ₂₀ H ₁₆ N ₃ O ₃ ·1/2H ₂ O		250-251	87	C, H, N

to animal models for depression, and 3e, which in addition to potent inhibitory activity against both central and peripheral CaIPDE enzymes has potent [³H]rolipram binding activity, also showed antiinflammatory activity comparable to that of nitroquazone. This result is remarkable in light of the rapid metabolic hydrolysis of 3e to the corresponding acid by rat plasma;¹⁰ 3e is thus active in vivo presumably by virtue of its very potent in vitro activity. Thus, although proving a liability in terms of bioavailability, the use of an ester as a bioisostere for the nitro or lactam group has provided a valuable hypothesis for the development of new CaIPDE inhibitors and may find applications in other systems as well.

Conclusion

Recent work on CaIPDE has suggested that it is involved in controlling basal levels of cAMP in various cell types; it thus may play a role in the long-term pathology of chronic disease.¹¹ This hypothesis is supported by the finding that rolipram is effective in the treatment of depression.¹² Our finding that a completely different structural class of CaIPDE inhibitors, exemplified by compound 3e, is effective in animal models of depression and inflammation adds fresh support to this hypothesis. Future reports from these laboratories will present an account of our efforts to evaluate the potential for such applications of CaIPDE inhibitors.

Experimental Section

Melting points were obtained on a Hoover melting point apparatus and are uncorrected. NMR spectra were obtained on a Varian XL-300 or a Bruker AM-300 spectrometer. IR spectra were obtained on Perkin-Elmer 283B or 1420 spectrometers. Mass

spectra were obtained on a Finnegan 4610 mass spectrometer, and high-resolution mass spectra were obtained on an AE-9 instrument. TLC analyses were carried out on EM Kieselgel 60 F₂₅₄ 5 × 20 cm plates. Elemental analyses were carried out by the Analytical Laboratory of Pfizer Central Research and are within ±0.4% of theory unless otherwise noted.

Method A. 2-[(3-Carboxyphenyl)amino]benzoic Acid (1a). To a 500-mL round-bottomed flask equipped with condenser and N₂ inlet were added 15.7 g (0.10 mol) of 2-chlorobenzoic acid, 23.3 g (0.17 mol) of 3-aminobenzoic acid, 23.5 g (0.17 mol) of potassium carbonate, 50 mg of copper powder, and 40 mL dimethylformamide. The mixture was heated to reflux, and three 50-mg portions of methanol-washed copper(I) bromide were added. The reaction was refluxed for 3.5 hr, cooled, and poured into 1 N HCl. The mixture was stirred for 10 min and filtered, and the filtered solid washed with water, methanol, and ether, and dried to afford 19 g (74%) of a gray solid: mp 277-279 °C; ¹H NMR (DMSO-*d*₆) δ 6.86 (t, 1 H), 7.3-8.0 (m, 7 H); IR (KBr) 1684 (C=O) cm⁻¹; MS *m/z* 257 (100, parent), 239 (44), 222 (28), 221 (76), 195 (69), 167 (55), 166 (44), 165 (42), 139 (27). Anal. (C₁₄H₁₁NO₄) C, H, N.

2-[(3-Carboxyphenyl)amino]nicotinic acid (1b) was prepared according to method A in 81% yield: mp 275-280 °C; ¹H NMR (DMSO-*d*₆) δ 6.90 (m, 1 H), 7.43 (t, *J* = 8, 1 H), 7.58 (d, *J* = 8, 1 H), 7.94 (m, 1 H), 8.29 (m, 2 H), 8.42 (m, 1 H), 10.56 (bs, 1 H, N-H), 12.5 (bs, 2 H, CO₂H); IR (KBr) 1720, 1652 (C=O) cm⁻¹; MS *m/z* 258 (96, parent), 257 (100), 239 (82), 168 (65), 66 (66). Anal. (C₁₅H₁₀N₂O₄·1/2H₂O) C, H, N.

Method B. Methyl 2-[(3-Carbomethoxyphenyl)amino]benzoate (2a). The acid from the previous step was taken up in 250 mL of methanol and the solution saturated with HCl, refluxed 40 h, cooled, and evaporated. The residue was chromatographed on silica gel using methylene chloride as eluent to afford 17.46 g (61% overall) of a yellow oil: ¹H NMR (CHCl₃) δ 3.898 (s, 3 H), 3.900 (s, 3 H), 6.77 (m, 1 H), 7.2-7.4 (m, 4 H), 7.73 (m, 1 H), 7.95 (m, 1 H), 7.98 (m, 1 H), 9.55 (bs, 1 H, NH); IR (KBr) (C=O) 1730 cm⁻¹. Anal. (C₁₆H₁₅NO₄) C, H, N.

Methyl 2-[(3-carbomethoxyphenyl)amino]nicotinate (2b) was prepared according to method B in 13% yield: mp 105-106 °C; NMR (CDCl₃) δ 3.91 (s, 3 H), 3.93 (s, 3 H), 6.74 (m, NH, 1 H), 7.38 (m, 1 H), 7.70 (m, 1 H), 8.00 (m, 1 H), 8.3 (m, 3 H), 10.27 (bs, 1 H); IR (KBr) 1713 (CO), 1682 (CO), 1602, 1585 cm⁻¹; MS (*M*⁺) *m/z* 286 (92, parent), 285 (100). Anal. (C₁₅H₁₄N₂O₄) C, H, N.

Method C. 1-(3-Carbomethoxyphenyl)-3-ethylquinazoline-2,4-dione (3a). To a 25-mL round-bottomed flask equipped with condenser and N₂ inlet were added 3.0 g (10.5 mmol) of methyl 2-[(3-carbomethoxyphenyl)amino]benzoate, 0.83 mL (10.5 mmol) of ethyl isocyanate, 1 mg of camphorsulfonic acid, and 3 mL of xylene. The solution was refluxed for 6 days, cooled, and evaporated. The residue was taken up in ethyl acetate and the product was crystallized by adding isopropyl ether at boiling until most of the ethyl acetate had been replaced and then cooling the solution to 0 °C, affording 0.76 g (22%) of a crystalline solid: mp 135-136 °C; ¹H NMR (DMSO-*d*₆) δ 1.20 (t, *J* = 6, 3 H), 3.88 (s, 3 H), 4.00 (q, *J* = 6, 2 H), 6.41 (d, 1 H), 7.1-8.0 (m, 7 H); IR (KBr) 1722, 1663 (C=O) cm⁻¹; MS *m/z* 324 (87, parent), 325 (27), 296 (29), 222 (38), 221 (100), 194 (63), 166 (48), 165 (30), 139 (20), 111 (67), 83 (45), 70 (22), 59 (25). Anal. (C₁₈H₁₆N₂O₄) C, H, N.

1-(3-Carbomethoxyphenyl)quinazoline-2,4-dione (3b) was prepared according to method C in 8% yield: mp 274-276 °C; ¹H NMR (DMSO-*d*₆) δ 3.90 (2, 3 H), 6.45 (d, 1 H), 7.2-8.2 (m, 7 H), 11.77 (bs, 1 H); IR (KBr) 1685 (C=O) cm⁻¹; MS *m/z* 296 (100, parent), 297 (25), 221 (71), 194 (25), 82 (24), 69 (22). Anal. (C₁₆H₁₂N₂O₄) C, H, N.

1-(3-Carbomethoxyphenyl)-3-benzylquinazoline-2,4-dione (3c) was prepared according to method C in 45% yield: mp 151-153 °C; ¹H NMR (DMSO-*d*₆) δ 3.90 (s, 3 H), 5.19 (s, 2 H), 6.48 (d, 1 H), 7.2-8.2 (m, 12 H); IR (KBr) 1728, 1706, 1660 (C=O) cm⁻¹; MS 387 (11), 386 (51, parent), 222 (22), 221 (74), 194 (30), 166 (28), 91 (100), 59 (23). Anal. (C₂₃H₁₈N₂O₄) C, H, N.

1-(3-Carbomethoxyphenyl)-3-ethylpyrido[2,3-*d*]pyrimidine-2,4-dione (3d) was prepared according to method C in 6% yield: mp 186-187 °C; ¹H NMR (DMSO-*d*₆) δ 1.16 (t, *J* = 6, 3 H), 3.83 (s, 3 H), 3.97 (q, *J* = 6, 2 H), 7.31 (m, 1 H), 7.66 (m, 2 H), 8.03 (m, 2 H), 8.4-8.5 (m, 2 H); IR (KBr) 1719, 1671 (C=O)

(10) We thank Dr. L. Tremaine for this unpublished data.

(11) Donaldson, J.; Brown, A. M.; Hill, S. J. *Biochem. Pharmacol.* 1988, 37, 715.

(12) Hebenstreit, G. F.; Fellerer, K.; Fichte, K.; Geyer, N. *Pharmacopsychiatry* 1989, 22, 156.

cm^{-1} ; MS m/z 325 (51, parent), 324 (100), 253 (23), 168 (22). Anal. ($\text{C}_{17}\text{H}_{15}\text{N}_3\text{O}_4 \cdot 1/2\text{H}_2\text{O}$) C, H, N.

1-(3-Carbomethoxyphenyl)-3-benzylpyrido[2,3-*d*]pyrimidine-2,4-dione (3e) was prepared according to method C in 31% yield: mp 157–160 °C; ^1H NMR ($\text{DMSO}-d_6$) δ 3.84 (s, 3 H), 5.12 (s, 2 H), 7.2–8.1 (m, 10 H), 8.45 (m, 2 H); IR (KBr) 1723 ($\text{C}=\text{O}$), 1677 ($\text{C}=\text{O}$), 1594 cm^{-1} ; MS m/z 387 (100, parent), 283 (31), 253 (11), 226 (16), 195 (14), 181 (11.5), 168 (18), 91 (28). Anal. ($\text{C}_{22}\text{H}_{17}\text{N}_3\text{O}_4$) C, H, N.

Method D. 1-(3-Carbomethoxyphenyl)-3-(cyclopentylmethyl)pyrido[2,3-*d*]pyrimidine-2,4-dione (3f). To a 35-mL round-bottomed flask equipped with a condenser and N_2 inlet were added 1.0 g (3.496 mmol) of methyl 2-[(3-carbomethoxyphenyl)amino]nicotinate, 0.44 g (3.496 mmol) of cyclopentylacetic acid, 4 mL of dry xylene, 0.83 mL (3.846 mmol) of diphenyl phosphorazidate, and 0.54 mL (3.846 mmol) of triethylamine. The reaction was heated to 70 °C, whereupon a gas was evolved, and 5 mg of camphorsulfonic acid was added once gas evolution had ceased. The reaction was heated at reflux for 2.5 days, cooled, and chromatographed on silica gel using ethyl acetate in methylene chloride as eluent to give 67 mg (5.1% yield) of a white solid: mp 149–151 °C; ^1H NMR ($\text{DMSO}-d_6$) δ 1.2–1.7 (m, 8 H), 2.3–2.4 (m, 1 H), 3.87 (s, 3 H), 3.91 (d, 2 H), 7.3–8.5 (m, 7 H); IR (KBr) 1721 ($\text{C}=\text{O}$), 1677 ($\text{C}=\text{O}$), 1593 cm^{-1} ; MS m/z 379 (19, parent) 299 (20), 298 (100), 296 (101), 267 (10), 266 (50), 253 (10), 195 (16), 168 (12), 167 (17). Anal. ($\text{C}_{21}\text{H}_{21}\text{N}_3\text{O}_4$) C, H, N.

1-(3-Carbomethoxyphenyl)-3-(norbornylmethyl)pyrido[2,3-*d*]pyrimidine-2,4-dione (3g) was prepared according to method D in 20% yield: mp 157–159 °C; ^1H NMR (CDCl_3) δ 1.0–1.2 (m, 6 H), 1.3–1.5 (m, 4 H), 2.0–2.1 (m, 1 H), 3.7–4.0 (m, 2 H), 3.81 (s, 3 H), 7.1–8.5 (m, 7 H); IR (CHCl_3) 1700 ($\text{C}=\text{O}$), 1650 ($\text{C}=\text{O}$), 1580 cm^{-1} ; MS m/z 405 (5, parent), 299 (20), 298 (100), 266 (33), 195 (13), 167 (14), 95 (17), 85 (11), 83 (19), 67 (15). Anal. ($\text{C}_{23}\text{H}_{23}\text{N}_3\text{O}_4$) C, H, N.

1-(3-Carboxyphenyl)-3-benzylquinazoline-2,4-dione (4a). To a 500-mL round-bottomed flask equipped with condenser and N_2 inlet were added 3.86 g (10 mmol) of 1-(3-carbomethoxyphenyl)-3-benzylquinazoline-2,4-dione, 24.1 g (180 mmol) of lithium iodide, and 250 mL of dimethylformamide. The reaction was refluxed for 36 h, cooled, and added to 1 N HCl. The mixture was stirred for 20 min and filtered, and the solid washed with water and dried to afford 3.55 g (95%): mp 260–262 °C; ^1H NMR ($\text{DMSO}-d_6$) δ 5.18 (s, 2 H), 6.49 (d, 1 H), 7.2–8.2 (m, 12 H); IR (KBr) 1667 ($\text{C}=\text{O}$) cm^{-1} ; MS m/z 373 (26), 372 (100, parent), 239 (18), 221 (27), 195 (17), 91 (18). Anal. ($\text{C}_{22}\text{H}_{16}\text{N}_2\text{O}_4 \cdot 1/2\text{H}_2\text{O}$) C, H, N.

1-[3-(*N*-Methylcarbamoyl)phenyl]-3-benzylquinazoline-2,4-dione (4b). To a 100-mL three-necked round-bottomed flask equipped with septum and N_2 inlet were added 0.50 g (1.34 mmol) of 1-(3-carboxyphenyl)-3-benzylquinazoline-2,4-dione, 0.16 mL (1.34 mmol) of *N*-methylmorpholine, 10 mL of methylene chloride, and 1.5 mL of dimethylformamide. The solution was cooled to –10 °C and 0.17 mL (1.34 mmol) of isobutyl chloroformate added. The reaction was stirred at –10 °C for 10 min, then methylamine gas was bubbled through the solution for 10 min. The reaction was allowed to warm to room temperature and stirred for 3 days. It was taken up in methylene chloride, washed with 1 N HCl and brine, dried, and evaporated. The residue was chromatographed on silica gel using methylene chloride/ethyl acetate as eluent to afford 0.45 g (87%) of a white, crystalline solid after trituration with isopropyl ether: mp 250–251 °C; ^1H NMR ($\text{DMSO}-d_6$) δ 2.79 (m, 3 H), 5.16 (s, 2 H), 6.43 (m, 1 H), 7.2–8.1 (m, 12 H), 8.53 (m, 1 H); IR (KBr) 1702, 1657 ($\text{C}=\text{O}$) cm^{-1} ; MS m/z 395 (12, parent), 222 (32), 221 (100), 166 (38), 165 (40), 91 (99), 85 (21), 83 (53). Anal. ($\text{C}_{23}\text{H}_{19}\text{N}_3\text{O}_3 \cdot 1/2\text{H}_2\text{O}$) C, H, N.

Biological Methods. Measurement of [^3H]Risperidone Binding. Fresh rat brain was homogenized in 20 volumes of ice-cold 50 mM Tris/1.2 mM MgCl_2 (pH 8.0) for 20 s (position 6.5 on a Polytron). The resulting homogenate was centrifuged at 15000 rpm for 20 min. The pellet was resuspended in 20 volumes of the Tris buffer and centrifuged as before. The final homogenate was resuspended in Tris buffer to give a protein concentration of 0.5 mg/mL. The binding assay was run with 1.0 mL of the tissue suspension that was incubated with 0.1 mL of [^3H]risperidone and 0.2 mL of test compound at various concentrations.

Measurement of Phosphodiesterase Activity. CaIPDE-A. Phosphodiesterase activity was determined with 0.1 mL of a reaction medium containing 50 mM Tris-HCl/5 mM MgCl_2 (pH 7.5) buffer and [^3H]cAMP (NEN NET-275). The final concentration of cAMP was 1.0 μM containing 400000 dpm of [^3H]cAMP. Vehicle or test compound (10 μL) and 10 μL of fresh calcium-independent phosphodiesterase (courtesy of Dr. Craig Davis, University of South Carolina¹³) or boiled enzyme were added to 80 μL of substrate in the Tris/ MgCl_2 buffer. The incubation was carried out at 37 °C for 8 min and stopped by placing in a boiling water bath. Carrier 5'-AMP (0.5 mL of 5 mM 5'-AMP in 0.1 M Hepes/0.1 M NaCl buffer (pH 8.0) was added and the reaction mixture was chromatographed on a polyacrylamide-boronate affinity gel (Bio-Rad Affi-Gel 601 boronate gel). The unhydrolyzed [^3H]cAMP was eluted from the gel with 7.5 mL of the Hepes-NaCl buffer. The [^3H]5'-AMP product was eluted with 7 mL of 50 mM NaOAc buffer (pH 4.8). Aliquots (1.0 mL) of the latter eluates were counted in a liquid scintillation counter to determine their relative content of radioactive 5'-AMP.

CaIPDE-B. Lung tissue from guinea pigs was placed in a homogenization buffer (20 mM Bistris, 5 mM 2-mercaptoethanol, 2 mM benzamide, 2 nM EDTA, 50 mM sodium acetate, pH 6.5) at a concentration of 10 mL/g of tissue. The tissue was homogenized with a Tekmar Tissumizer at full speed for 10 s. Phenylmethanesulfonyl fluoride (PMSF, 50 mM in 2-propanol) was added to the buffer immediately prior to homogenization to give a final PMSF concentration of 50 μM . The homogenate was centrifuged at 12000g for 10 min at 4 °C. The supernatant was filtered through gauze and glass wool and then applied to a 17 $\times$ 1.5 cm column of DEAE-Sephacel CL-6B, pre-equilibrated with homogenization buffer, at 4 °C.¹ After the supernatant had passed through the column, the column was washed with homogenization buffer, and PDE eluted with a linear gradient of 0.05–1.0 M sodium acetate. Fractions containing CaIPDE activity, as determined by inhibition by rolipram, were pooled (2 mL of ethylene glycol was added to each fraction) and stored at –20 °C. The assay was then carried out by incubating 25 μL of CaIPDE solution, 25 μL of test compound solution in 4% DMSO in water, 25 μL of test buffer (50 mM Tris, 10 mM MgCl_2 , pH 7.5), and 25 μL of [^3H]cAMP solution (final concentration of [^3H]cAMP in assay mixture was 1 μM). The tubes were incubated at 37 °C for 10 min, and the reaction was stopped by placing the tubes in a boiling water bath for 2 min. Wash buffer (0.5 mL, 0.1 M Hepes/0.1 M NaCl, pH 8.5) was added to each tube in an ice bath. The contents of each tube were applied to an Affi-Gel column (boronate affinity gel, 1.2-mL bed volume) previously equilibrated with wash buffer. [^3H]cAMP was eluted with 2 $\times$ 6 mL wash buffer, and [^3H]5'-AMP eluted with 6 mL of 0.25 M acetic acid. After vortexing, 1 mL of the eluate was added to 3 mL of Atomlight scintillation fluid, vortexed, and counted for tritium.

Reserpine Hypothermia Test. In accordance with the antidepressant screening method of Askew,¹⁴ as modified by Koe and co-workers,¹⁵ mice were individually housed at 18–20 °C in plastic chambers with cardboard bottoms. The animals were injected with reserpine (2 mg/kg sc), and retained at 18–20 °C for 18 h. Rectal temperatures were then measured, after which the animals were treated with either saline or test drug solution. Rectal temperatures were again determined, typically at 0.5, 1.0, 2.0, and 14 h after the second injection. Reserpine-pretreated mice given vehicle exhibit rectal temperatures of 20–21 °C 3 h after injection with vehicle, while antidepressant-treated animals display a temperature increase of several degrees centigrade.

Mouse Swim Test. In accordance with the method of Porsolt and co-workers,¹⁶ as modified by Wallach and Hedley,¹⁷ mice were pretreated with test compounds dissolved in saline and administered po in a volume of 1 mL/100 g body weight. One hour after dosing, the animals were placed in 1-L beakers containing water at 22–24 °C, such that their tails could not touch the beaker bottom. After a 2-min habituation time, the mice were observed

(13) See, also: Davis, C. W.; Daly, J. W. *J. Cycl. Nucleotide Res.* 1979, 5, 65.

(14) Askew, B. M. *Life Sci.* 1963, 4, 725.

(15) Koe, B. K. *J. Pharmacol. Exp. Ther.* 1983, 226, 686.

(16) Porsolt, R. *Arch. Int. Pharmacodyn.* 1977, 229, 327.

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10 times (once every 30 s) to determine whether or not they were mobile. Antidepressant drugs reduce the score in this test in a dose-dependent fashion.

Rat Foot Edema Test. The procedure of Winter¹⁸ was modified as follows: Adult male, fasted, unanesthetized, Charles River CD Sprague-Dawley rats of 170-205 g body weight were numbered and weighed, and an ink mark was placed on the right lateral malleolus. Each paw was immersed in mercury exactly to the ink mark. The mercury was contained in a plastic cylinder connected to an Omega pressure transducer. The output from the transducer was fed through a Buxco organ volume monitor. The volume of mercury displaced by the immersed paw was read, then printed on a Texas Instruments Silent 700. Drugs were given by gavage in a 25 mL/kg volume. One hour after drug administration, edema was induced by injection of 0.05 mL of a 2-

week-old 1% solution of carrageenan in distilled water into the plantar tissue of the marked paws. Immediately thereafter, the volume of the injected paw was measured. The increase in foot volume 3 h after the injection of carrageenan constitutes the individual response. Compounds were considered active if they inhibited the edema response by 30% or more in a group of six rats.

Acknowledgment. We would like to thank Drs. F. J. Vinick, N. A. Saccomano, and J. E. Macor for their chemical and intellectual support.

Registry No. 1a, 27693-87-0; 1b, 114918-39-7; 2a, 114918-37-5; 2b, 114918-35-3; 3a, 130985-15-8; 3b, 130985-16-9; 3c, 114934-47-3; 3d, 130985-17-0; 3e, 114918-24-0; 3f, 114918-31-9; 3g, 114918-32-0; 4a, 114934-48-4; 4b, 114934-49-5; CalPDE, 9036-21-9; PhCH₂NCO, 3173-56-6; 2-chlorobenzoic acid, 118-91-2; 2-chloronicotinic acid, 2942-59-8; ethyl isocyanate, 109-90-0; 2-norbornylacetic acid, 1007-01-8; cyclopentylacetic acid, 1123-00-8; 3-aminobenzoic acid, 99-05-8.

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Synthesis and Anticonvulsant Activity of 1-Acyl-2-pyrrolidinone Derivatives

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Several 1-acyl-2-pyrrolidinone derivatives were synthesized as derivatives of γ -aminobutyric acid (GABA), and their pharmacological activities and stabilities were investigated. The derivatives showed anticonvulsant effect on picrotoxin-induced seizure at a dose of 200 mg/kg. In particular, 1-decanoyl-2-pyrrolidinone (7) and 1-dodecanoyl-2-pyrrolidinone (8) had a high activity. The anticonvulsant activity showed a dose dependency. Some of 1-acyl-2-pyrrolidinone derivatives prolonged sleeping time which was induced by sodium pentobarbital and showed a recovery from disruption of the memory of passive avoidance response, which was induced by an electroconvulsive shock. As shown by the results of the stability study of 1-acetyl-2-pyrrolidinone (1), it was degraded in an acidic buffer and an alkaline buffer although 2-pyrrolidinone was stable. 1-Acyl-2-pyrrolidinone derivatives were degraded in liver and brain homogenates of mouse and rat. They showed a degradation rate in rat plasma. Conversion of 8 to GABA in mouse liver homogenate was demonstrated. These results suggested that the pharmacological activity of 1-acyl-2-pyrrolidinone is probably due to the release of GABA by hydrolysis of derivatives although further work is necessary.

Several neurological and neuropsychiatric disorders such as epilepsy and Huntington's disease have been reported to be associated with a decrease in γ -aminobutyric acid (GABA) levels in the central nervous system (CNS).¹⁻³ These observations suggest that increased levels of GABA in the CNS may be useful in the treatment of such neuropsychiatric disorders. However, attempts to use GABA in clinical trials failed due to the extremely high doses required to force GABA across the blood-brain barrier.^{4,5}

Numerous derivatives of GABA, including alkyl ester of GABA,^{6,7} γ -acetylenic GABA,⁸ aliphatic and steroid esters of GABA,⁹⁻¹¹ Schiff bases of GABA,¹² soft drug of GABA,¹³ and isonicotinoyl GABA,¹⁴ have been developed in the hope of facilitating the uptake of GABA into the brain.

Callery et al.^{15,16} suggested another possibility, use of 2-pyrrolidinone as a GABA prodrug. 2-Pyrrolidinone, the lactam of GABA, is more lipophilic than GABA and penetrates readily into the CNS. However, attempts to increase whole-brain GABA concentrations with single large doses of 2-pyrrolidinone have been unsuccessful.⁴ The failure may be explained by a slow hydrolysis of 2-pyrrolidinone to GABA.¹⁶

The tight amide bond of 2-pyrrolidinone may be weakened by introduction of an acyl function to the 1-position. Also the lipophilicity of drug may be controlled by the chain length of the acyl function to be introduced. Therefore, several 1-acyl-2-pyrrolidinone derivatives having

various alkyl chains were synthesized and their GABA-like pharmacological activities such as anticonvulsant activity and sedative activity were evaluated. Antianesthetic activity

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Chemotherapeutic potential of phosphodiesterase inhibitors

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The application of molecular cloning has revealed the phenomenal diversity and complexity of the phosphodiesterase isoenzyme family. Thus, more than 30 human phosphodiesterases are now known; all are apparently necessary for the seemingly simple task of hydrolysing the 3'-ester bond of either cyclic adenosine monophosphate or cyclic guanosine monophosphate. The availability of phosphodiesterase isoenzymes as pure recombinant proteins has greatly facilitated the identification of potent, selective inhibitors. The potential of these inhibitors to therapeutically exploit the molecular diversity of the phosphodiesterases has progressed significantly. A number of drugs are in clinical trials for asthma, and Viagra has become the first selective phosphodiesterase inhibitor to be approved by the US Food and Drug Administration.

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Current Opinion in Chemical Biology 1998, 2:472-481

<http://biomednet.com/elecref/1367593100200472>

© Current Biology Publications ISSN 1367-5931

Abbreviations

AC	adenylate cyclase
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
NO	nitric oxide
PDE	phosphodiesterase
PKA	protein kinase A
Sc	catalytic site
Sr	high-affinity rolipram-binding site

Introduction

The cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are ubiquitous second messengers responsible for transducing the effects of a variety of extracellular signals, including hormones, light and neurotransmitters. These two molecules influence a vast array of processes including pro-inflammatory mediator production and action, ion channel function, muscle contraction, learning, differentiation, apoptosis, lipogenesis, glycogenolysis and gluconeogenesis. Given the multitude of cellular responses that cAMP and cGMP can elicit, it is clear that to achieve specificity of signal transduction the cell must be able to tightly regulate the magnitude and duration of cAMP/cGMP elevation, and also in particular the cellular location. Mammalian cells have evolved a complex and highly conserved complement of enzymes in order to generate, recognise and inactivate the cyclic nucleotides. For example, there are nine

isoforms of adenylyl cyclase (AC), plus additional splice variants produced during mRNA processing, responsible for the generation of cAMP from ATP [1**]. Similarly, protein kinase A (PKA) consists of multiple forms of regulatory and catalytic subunits, the cellular location of which is determined by their interaction with members of a large family of A-kinase-anchoring proteins [2], found associated with an array of subcellular structures.

Inactivation of cAMP/cGMP is achieved by hydrolysis of the 3'-ester bond catalysed by the cyclic-nucleotide-dependent phosphodiesterases (PDEs), of which more than 30 have been identified [3]. If cells did not process PDEs intracellular cAMP levels would rapidly become uniform. These enzymes therefore provide a key ability for the cell to generate nonuniform intracellular distribution of cAMP/cGMP and hence differentially activate distinct compartmentalised protein kinase species.

The elevation of cAMP beyond the activation threshold for PKA is clearly governed by either or both the activation of AC and inhibition of PDEs (Figure 1). To be of therapeutic use, cAMP elevation has to be achieved with defined cellular selectivity. The molecular diversity described above offers scope for selective intervention and it is the potential opportunities provided by the PDEs that will be discussed here.

Phosphodiesterase isoforms

Cyclic nucleotide PDEs are a large multigene family comprising ten families of PDE isoenzymes designated 1-10. These families contain two or more related but distinct gene products (designated A, B, C and so on) and alternative mRNA processing gives rise to multiple splice variants for each gene. This molecular diversity provides an array of more than 30 enzymes with distinct substrate specificity, kinetic characteristics, regulatory properties and cellular and subcellular distributions [1**]. The PDEs share a similar structural organisation comprising two functional domains (the regulatory and catalytic domains). The catalytic domain represents the region of greatest homology between the PDE families (>75% homology at the amino acid level) and determines substrate and inhibitor specificity.

With respect to substrate, PDEs 4, 7 and 8 are highly specific for cAMP, whereas PDEs 5, 6 and 9 are selective for cGMP. PDE3 binds cAMP and cGMP with similar affinity but hydrolyses cGMP relatively poorly. Thus this enzyme can be regarded functionally as specific for cAMP, with cGMP acting as a negative modulator, via competitive binding, at the active site. PDEs 1 and 2 hydrolyse both cyclic nucleotides, although with PDE1 the relative efficiencies vary with isoenzyme subtype.

The amino-terminal regulatory domain is highly heterogeneous, reflecting the different cofactor dependence of the PDE families. This region can contain binding domains for Ca^{2+} /calmodulin (CaM) (PDE1), noncatalytic cGMP (PDE2, 5 and 6) and transducin (PDE6). In addition this amino-terminal region contains membrane-targeting domains (PDE3 and 4) and protein kinase phosphorylation sites (PDEs 1, 3, 4 and 5). These phosphorylation sites can regulate catalytic activity and/or subcellular location.

The combination of substrate and cofactor specificity allows crosstalk between cAMP and cGMP systems. For example, elevation of cGMP in platelets by either nitrovasodilators or PDE5 inhibition results in the inhibition of PDE3 and a concomitant increase in cAMP [4]. Conversely, in adrenal glomerulosa cells, atrial natriuretic factor elevates cGMP levels but inhibits cAMP-stimulated aldosterone synthesis via cGMP-mediated activation of PDE2 [5].

In order for PDE inhibition to be effective, cAMP has to be elevated above the threshold for activation of PKA. This, in part, is determined by the basal activity of AC, which is isoform-dependent. Hence, in certain cells such as adipocytes, basal AC activity is high and cAMP levels are maintained at basal levels through correspondingly high PDE activity. In adipocytes, therefore, PDE inhibition produces a rapid rise in cAMP above the PKA activation threshold. In contrast, hepatocytes display very low basal AC activity and hence PDE inhibition alone fails to activate PKA; however, if AC is activated in these cells PDE inhibitors will act synergistically to elevate cAMP beyond the PKA activation threshold. This is of particular importance at sites of inflammation, where the action of locally produced anti-inflammatory mediators such as prostaglandin E_2 (PGE_2) can be enhanced by inhibition of PDEs. Thus the functional consequences of PDE inhibition are dependent not only on the isoform of PDE but also on the activation status and isoform of AC to which it is linked.

In addition, most PDE inhibitors are competitive with substrate and thus their therapeutic profile is dependent on both the Michaelis-Menton equilibrium constant (K_M) and the substrate concentration in which they are operating. Thus, inhibitors that show high inhibition constant (K_i) values for PDE isoforms effectively undergo administration of selectivity ratio inside the cell under conditions of variable substrate concentration, especially if the PDE isoforms differ significantly in substrate K_M .

The above considerations give an idea of the difficulties in obtaining specific intervention via PDE inhibitors; however, the potential therapies offered by PDE inhibitors have encouraged the pharmaceutical industry to invest considerable resources in obtaining highly-isoenzyme selective PDE inhibitors. The progress made in this regard is discussed below.

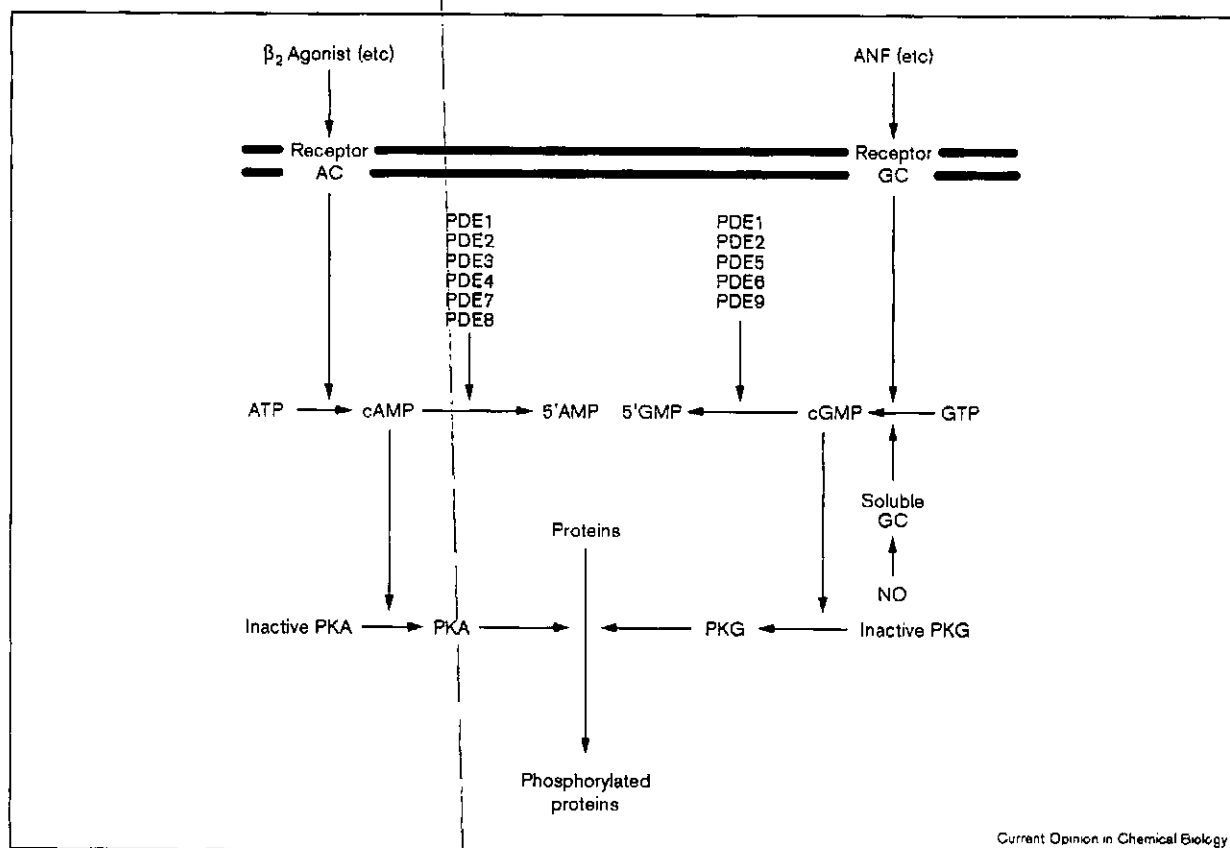
Phosphodiesterase 1 -

Three PDE1 isoenzymes (PDE1A, B and C) are encoded by separate genes with additional isoforms generated via alternative mRNA splicing [6]. The catalytic activity of these PDE1 isoforms is regulated via two CaM-binding domains, which allows for control by Ca^{2+} , although each isoform appears to have a distinct Ca^{2+} threshold for activation. In addition, whereas PDE1C hydrolyses cAMP and cGMP equally, PDE1A and B hydrolyse cGMP preferentially [7]. Although little is known about the control of expression of the three PDE1 isoforms, it is clear that they exhibit defined tissue and cellular localisation [8]. PDE1B is expressed in both brain and lymphocytes and its expression appears to be up-regulated following mitogenic stimulation. At a recent conference DR Repaske, personal communication) it was reported that there was no obvious phenotype in the PDE1B knockout (KO) mouse. Blood levels of lymphocytes were normal and these cells responded to mitogenic stimulation *in vitro*. Interestingly, although there were no overt central nervous system effects in the KO mouse, the level of CaM-stimulated PDE activity in the brain was reduced by 50%, indicating that there had not been a compensating increase in PDE1A or PDE1C.

Unfortunately, the lack of specific PDE1 inhibitors means that the functional role of PDE1 isoenzymes remains speculative. Vinpocetine (Richter Gedon VG, Budapest, Hungary; Figure 2) remains the principal inhibitor of PDE1, whereas the other common PDE1 inhibitors, the phenothiazines, act indirectly via their binding to CaM [9]. Both vinpocetine and the phenothiazines lack specificity of action to explore the functional role of PDE1. Initially it was claimed that PDE1 inhibitors were effective vascular relaxants. With the availability of purified cloned enzymes, however, it is now known that such inhibitors are in fact equally active against PDE5. Indeed it remains very much the case today that potent inhibitors of PDE1 are also active against PDE5; however, selective PDE5 inhibitors (discussed below) have been used to try and separate the roles of the two isoenzymes. Thus it has been suggested that PDE1 inhibitors may prevent intimal hyperplasia following angioplasty on the basis that SCH 51866 (a PDE1 and 5 inhibitor [Schering-Plough, New Jersey, USA]; see Figure 2) but not E4021 (a PDE5 inhibitor [Eisai Co, Tokyo, Japan]; Figure 2) is effective in the rat carotid artery injury model [10]. Given that cooperativity, as discussed above, is a common feature with PDEs, however, this subtractional analysis is open to question and a conclusive view on the functional role of PDE1 must await the development of selective inhibitors.

Unfortunately a recent survey of the patent literature [11*] indicated that there is a lack of apparent progress and effort with respect to PDE1 inhibitors. This is disappointing, given the diversity of PDE1 genes and given that their likely role in cross-talk between calcium and cyclic nucleotide signalling pathways suggests they

Figure 1



The cyclic nucleotides showing the role of PDEs. Receptor ligation activates either membrane AC or GC (guanylate cyclase), while soluble GC can be activated by nitric oxide (NO). The cyclases catalyse the conversion of ATP to cAMP and GTP to cGMP, which uncouples the regulatory subunits from PKA and PKG (protein kinase G), respectively. The activated kinases phosphorylate specific intracellular proteins, altering their activities to permit signal transduction to deliver a specific physiological response. ANF, atrial natriuretic factor.

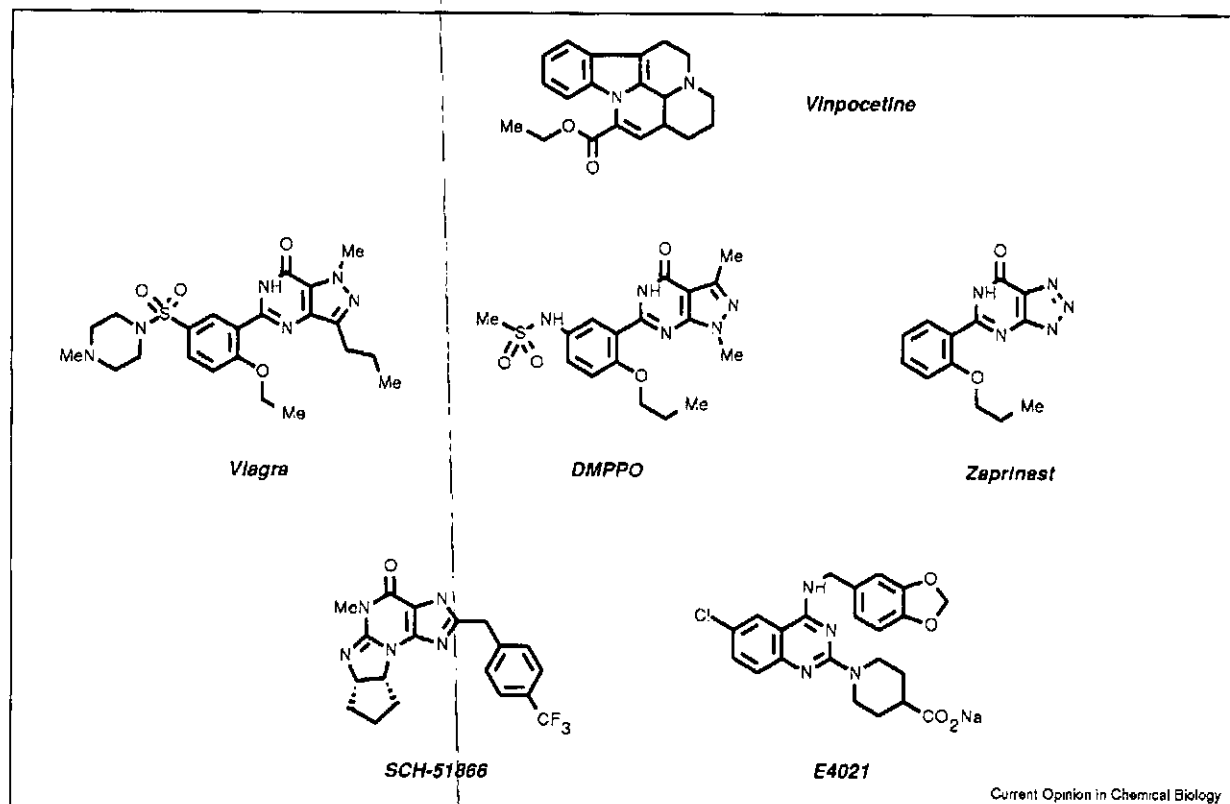
may be useful targets for therapeutic interaction with respect to disorders of the central nervous system and cardiovascular and immune systems.

Phosphodiesterase 2

The three cGMP-stimulated PDEs, PDE2A1, A2 and A3, are the products of a single gene but differ at their amino termini as a result of alternative exon splicing [12]. The PDE2 isoforms exhibit differential tissue and subcellular distribution. Membrane-bound enzyme is found in the brain and heart, whereas the enzyme is soluble in liver and platelets. Interestingly, although found in endothelial cells, PDE2 mRNA was not expressed in vascular smooth muscle. The presence of PDE2 in T cells is of interest especially as down-regulation of PDE2 activity has been reported in thymocytes following ligation of the T cell antigen receptor [13]. Indeed it has been suggested that in thymocytes, control of cAMP metabolism can switch from PDE4 to PDE2 depending on the intracellular concentration of cGMP [13]. Similarly, in platelets the role of PDE2 is highly dependent on the prevailing cyclic

nucleotide concentration [14]: at low cAMP concentrations PDE2 activity is dependent on cGMP, whereas at high cAMP PDE2 plays the major role in cAMP hydrolysis whether cGMP is present or not. The apparent interdependency of cGMP and cAMP concentrations in the heart suggest a role for PDE2 and PDE3 inhibitors in the treatment of angina, hypertension and heart failure. Although significant progress has been made in identifying selective inhibitors of PDE3, however (see below), only a limited effort appears to have been made in synthesising novel PDE2 inhibitors. EHNA (erythro-9-(2-hydroxy-3-nonyl)adenine) exhibits selectivity for this isoenzyme [15] at moderate potency (that is, shows a 50% inhibition concentration [IC₅₀] of 1 μ M). EHNA is also a potent inhibitor of adenosine deaminase [15], however, and thus has the potential to induce the accumulation of adenosine, which, acting via its receptor, can modulate cAMP levels. The potential synergistic action of cGMP and adenosine may be, in theory at least, of therapeutic benefit in antiarrhythmia, for example, but limits the use of EHNA in establishing the physiological role of PDE2.

Figure 2



Inhibitors of PDE1 and PDE5.

Nevertheless, ELNA has been used to identify a role for PDE2 in regulating the basal calcium current in human, in contrast to rat, atrial myocytes [16].

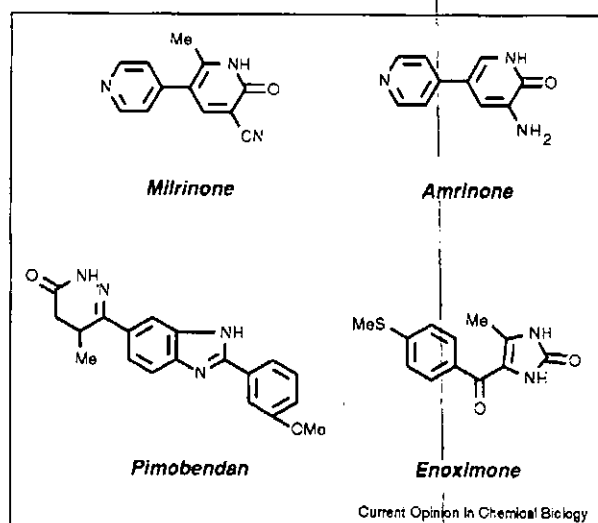
Clearly much has to be done to understand the physiological relevance of PDE2 but, nevertheless, this isoenzyme appears worthy of further attention from the pharmaceutical industry.

Phosphodiesterase 3

The two human isoforms of PDE3, PDE3A and PDE3B, are the products of different genes located on chromosomes 12 and 11, respectively [17]. The biology of PDE3 has been recently reviewed [18,19]. The structural organisation of PDE3 is of particular note: although the catalytic domains of PDE3A and 3B are similar both contain a different 44 amino acid insert. This insertion not only distinguishes each isotype but also the PDE3 catalytic domains from all the other PDE isoenzyme families. A future understanding of the role of this catalytic insert offers the potential for selective inhibition of the two PDE3 isoenzymes. This is of particular interest given the distinct tissue and cellular distribution of PDE3A and 3B mRNAs; PDE3A is particularly abundant in platelets and in heart and vascular smooth muscle, whereas PDE3B is present in adipocytes and T lymphocytes [18,20].

A plethora of selective PDE3 inhibitors have been identified in the past seven years [21,22] and have been shown to be potent vasodilators and inotropic agents with antiplatelet activity [22]. Thus, initially PDE3 inhibitors were seen as a novel class of inodilators and as such of high potential in the treatment of heart failure; however, the Prospective Randomised Milrinone Survival Evaluation (PROMISE) trial [22] showed that repeated oral doses of the PDE3 inhibitor milrinone increased mortality by heart failure and almost immediately curtailed further development of this class of drug. A limited number of inhibitors such as milrinone, aminone (Elf Sanofi, Paris, France; Figure 3) and enoximone (Hoechst Marion Roussel, Cincinnati, Ohio, USA; Figure 3) have been approved for acute short-term intravenous treatment of heart failure although patients are closely monitored for evidence of increased ventricular arrhythmias [23]. The inotropic effects of PDE3 inhibitors are thought to be via cAMP-mediated increases in intracellular Ca^{2+} . Dual action agents such as pimobendan (Boehringer Ingelheim, Ingelheim am Rhein, Germany; Figure 3) that exhibit both PDE3 inhibition and calcium-sensitising properties have been identified [24] in an attempt to reduce the degree of PDE inhibition required. It would appear, however, that such agents offer no advantage over milrinone [25].

Figure 3



Inhibitors of PDE3.

One area of potential use for PDE inhibitors that has yet to be fully explored is the modulation of T lymphocyte function. This could be of particular significance given that, as discussed earlier, PDE3B, and not the cardiac isoenzyme PDE3A, is found in T lymphocytes. Identification of isoenzyme-specific inhibitors would therefore overcome the problems encountered with milrinone, for example. In human T lymphocytes PDE3 and PDE4 show the predominant cyclic nucleotide hydrolysing activities [26]. Although poorly active on their own, PDE3 inhibitors act synergistically with PDE4 inhibitors to potently inhibit T cell receptor mediated cytokine production and mitogenic proliferation [26,27].

Phosphodiesterase 4

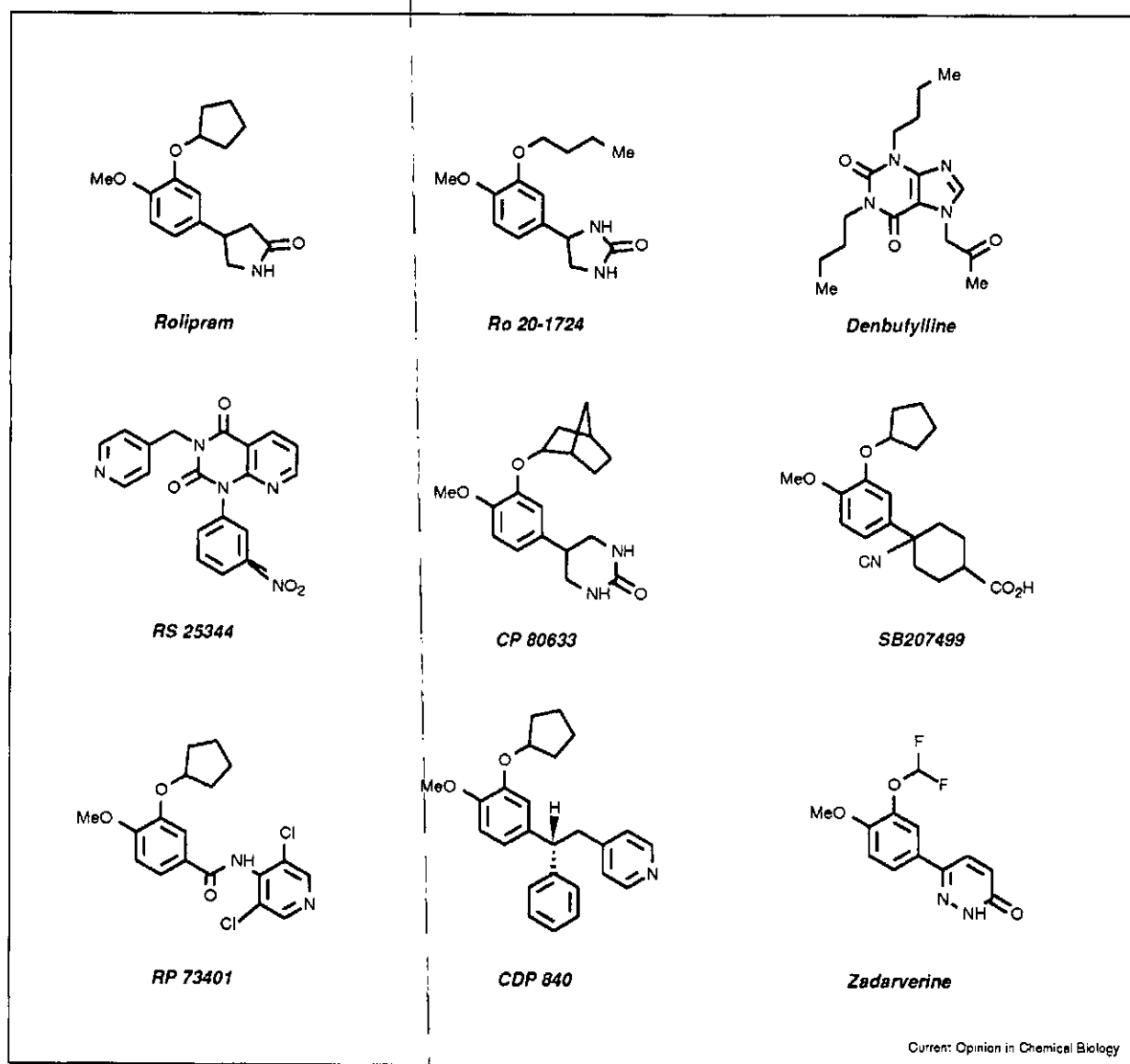
The application of molecular cloning during the 1990s revealed the extraordinary diversity of PDE4 isoenzymes. There are four PDE4 genes encoding distinct isoforms (A, B, C and D) in humans. Additional diversity arises either from alternative initiation sites and/or alternative splicing among the 5' exons yielding isoforms with distinct amino-terminal domains [28]. The early identification of rolipram ([29]; Schering AG, Berlin, Germany; Figure 4) as a selective PDE4 inhibitor has enabled the role of PDE4 to be investigated in a variety of cell types. It is now clear that PDE4 controls the breakdown of cAMP in many inflammatory cells and that inhibition of this isotype is associated with the suppression of immune and inflammatory cells. In addition cAMP mediates airway smooth muscle relaxation. PDE4 inhibitors are relatively poor bronchodilators compared to β -adrenoceptor agonists, however. Consequently, PDE4 inhibitors are the subject of intense interest by the pharmaceutical industry as a

novel class of anti-inflammatory drugs, particularly for the treatment of asthma. Extensive preclinical investigations have provided much data to support this optimism and this data has been the subject of numerous recent reviews [28,30,31^{••},32[•]].

Early clinical trials with prototype PDE4 inhibitors such as rolipram [33] and denbufylline ([34]; SmithKline Beecham, King of Prussia, Pennsylvania, USA; Figure 4) proved inconclusive because side effects such as nausea and emesis were observed before a statistically significant therapeutic effect was established. Despite the progress made in obtaining structurally diverse PDE4 inhibitors [35[•]], this side effect profile remains the limiting factor in the clinical development of this class of drug. Given the structural diversity of PDE4 inhibitors it would appear that nausea and emesis are mechanistic to cAMP elevation by this class of compounds. The molecular diversity of the PDE4 family, however, together with an understanding of the precise inhibitor-enzyme interactions, may permit the development of drugs that have increased therapeutic benefit. Whereas no PDE4 isoenzyme selective inhibitors have been identified, significant progress has been made in dissecting the interactions of certain inhibitors with the PDE4 isoenzymes.

It has been known for some time that rolipram exhibits differential binding to PDE4 isoenzymes. Thus [³H]-rolipram exhibits high-affinity binding (i.e. it exhibits a dissociation constant [K_d] of 1 nM) for certain PDE4 isoenzymes. The affinity of rolipram for this site (Sr) on the PDE4 (exhibits high affinity for rolipram and is detected by binding assays) is some 50–1000-fold higher than the inhibitory K_i at the catalytic site (Sc) [28] on the PDE4 (detected as a result of inhibition of catalytic activity). This differential activity of rolipram may explain the variable potency of rolipram relative to inhibitors such as RP73401 (Rhône Poulenc Rorer, Dagenham, UK; see Figure 4) and CDP840 (Celltech Therapeutics, Slough, UK; see Figure 4), which exhibit similar Sr and Sc affinity in *in vitro* and *in vivo* functional assays [36^{••}]. This has led to speculation that activity at the Sr site is responsible for the nausea and emetic profile of PDE4 inhibitors [37]. The exact relationship between the Sr and Sc sites remains in dispute; however, we believe that the recent data [38[•],39,40[•],41] suggest that all PDE4 isoenzymes contain a single inhibitor site that is competitive with cAMP but that is modified by the adoption of two distinct conformations. These two conformers differ at or near the substrate-binding site such that certain inhibitors (CDP840, RP73401, SB207499 [SmithKline Beecham, King of Prussia, Pennsylvania, USA; Figure 4] and cAMP exhibit similar affinities for both conformers, whereas the potency of rolipram and other inhibitors such as RS25344 (Hoffman-La Roche, Basle, Switzerland; Figure 4) and denbufylline, is highly dependent on the conformation of the enzyme.

Figure 4



Inhibitors of PDE4.

We have recently reported the characterisation of CDP840 [42], a potent PDE4 inhibitor that is effective in a clinical model of asthma without adverse events [43]. CDP840 is equally active at both the Sr and Sc sites and its potency at the Sr site is comparable with that of rolipram [42]. This, and our observations (unpublished) on a range of structurally diverse inhibitors, suggest that potency at the Sr site may in fact not be an absolute determinant for nausea and emesis. Our preliminary data [38,42] supports the recent elegant and thorough mechanistic study [40] of rolipram-PDE4 interaction. This study demonstrates that rolipram acts as a slow-binding inhibitor at the Sr site in contrast to its normal kinetics at the Sc site. A consequence

of slow-binding inhibition [44] is that such inhibitors are not as influenced as non-slow-binding inhibitors by high substrate concentrations (i.e. rolipram would be expected to be more effective than inhibitors such as CDP840 and so on, in inhibiting the Sr conformation of PDE4 under conditions of high intracellular cAMP). The significance of this hypothesis has yet to be proved but it is of interest to note that, as discussed earlier, the efficacy of PDE4 inhibitors is highly dependent on the activity status of AC [45,46].

In an attempt to limit side effects, PDE4 inhibitors such as RP73401 and zardaverine (Byk Gulden, Konstanz,

Germany; Figure 4) were administered by inhalation, but clinical results were disappointing [47,48]. Topical application of CP80633 (Pfizer, Groton, Connecticut, USA; Figure 4) led to significantly reduced inflammation in skin lesions of atopic dermatitis patients [49], however, confirming previous observations with Ro20-1724 (Hoffman-La Roche, Basle, Switzerland; see Figure 4) in the treatment of psoriatic lesions [50].

Phosphodiesterase 5

In contrast to PDEs 1 and 2, PDE5 catalyses the hydrolysis of cGMP with absolute specificity, and with only one protein identified, lacks their isoform diversity. Although there is still much to learn concerning the function and regulation of this PDE, recent information has provided an insight into the specificity of the substrate-binding site [51,52]. PDE5 contains two allosteric cGMP-binding domains that are arranged in tandem at the amino-terminal portion of the protein. Binding of cGMP at the allosteric site does not influence catalytic activity directly but appears to regulate the ability of the enzyme of be phosphorylated by PKA [53]. Phosphorylation is a regulating mechanism common to all PDEs [54]; however, the significance for PDE5 is, as yet, undefined but is likely to involve control of protein-protein interactions or cellular localisation.

As discussed earlier, cGMP is the signal transducer for both nitric oxide (NO)-mediated endothelial relaxation and arterial natriuretic factor (ANF)-mediated diuresis. Elevation of cGMP via PDE inhibition can thus be expected to stimulate endothelial relaxation and diuresis, a combined activity with potential in the treatment of hypertension and congestive heart failure. Similarly, benefit might be expected in coronary artery disease where endothelial-dependent vasodilation is impaired and in angina pectoris due to antiplatelet and antithrombotic activity of PDE5 inhibitors.

PDE5 exhibits a more limited tissue distribution than PDE1 and 2; it is particularly prevalent in vascular smooth muscle [54]. This, coupled to its specificity for cGMP, has identified PDE5 as a target of considerable interest for the pharmaceutical industry.

The availability of inhibitors such as zaprinast, with comparable potency against PDE1 and 5, provided a good pharmacophore reference. Progress in modifying zaprinast (Rhone poulenc Rorer, Dagenham, UK; Figure 2) to remove the PDE1 activity was rapid and resulted in the identification of two highly potent PDE5 inhibitors (K_i 3 nM), Viagra (Sildenafil, UK92480) and DMPP0 by Pfizer [55]; Groton, Connecticut, USA; see Figure 4) and GlaxoWellcome (Stevenage, UK) [56], respectively. Compounds that are structural diverse from zaprinast have been reported, with E4021 [57] being of particular interest.

These potent inhibitors of PDE5 have been profiled extensively in preclinical models of cardiovascular disease

and the results reviewed elsewhere [58]. From these studies it is apparent that PDE5 inhibitors profoundly reduce pulmonary arterial pressure compared to mean arterial pressure and exhibit minimal effects on heart rate. This indicates that PDE5 inhibitors may be a novel class of selective pulmonary vasodilators. It is also apparent that PDE5 inhibitors have little direct effect but rather potentiate the activity of endogenous or exogenous guanylate cyclase activators.

While the role for PDE5 inhibitors in cardiovascular therapy has yet to be clinically established, the US Food and Drug Administration has approved Viagra for the treatment of male impotence and erectile dysfunction. Penile erection is initiated through relaxation of smooth muscle fibres of the corpora cavernosa allowing an increase in blood flow to the penis and opening of the sinusoid spaces [59]. The most important factor for relaxation of the corpora cavernosa is considered to be NO operating via its second messenger, cGMP. Until recently NO was thought to be of neurogenic origin; however, mice lacking neurogenic NO synthetase (NOS) exhibit normal erectile function [60]. Interest has now focused on endothelial NOS and/or other mediators released from nerves or endothelium [60]. Whatever the source of NO, it is clear that it is the ability of Viagra to potentiate the elevation of intracellular cGMP that is key to its therapeutic benefit. This is in accord with the observation that the main PDE activity in human corpora cavernosa is PDE5 with lesser activities attributable to PDE2 and 3 [61]. Data obtained using PGE₁ and forskolin indicate that penile smooth muscle relaxation can be achieved via the cAMP pathway, however [60]. Thus Viagra may also act to elevate cAMP by cGMP-mediated inhibition of PDE3. Viagra did not alter cAMP in rabbit corpora cavernosa, however [62], although the PDE profile of the tissue was not reported.

In the phase III trials for Viagra, 3% of the patients experienced a blue-green tinge to their vision, presumably caused by a transient reversible effect on retinal function. In this context it is of interest to note that Viagra, in common with E4021 and DMPP0, is only weakly selective (tenfold) for PDE5 compared to PDE6, the photoreceptor PDE, which is the effector enzyme in the phototransduction cascade [63]. Mutations inhibitory to retinal PDE6 led to degeneration of that tissue and blindness in both humans and animals [64]. The commercial success of Viagra has prompted extensive interest in PDE5 within the pharmaceutical industry. A clear objective of the third generation PDE5 inhibitors will be absolute specificity for PDE5. This is likely to be of particular value in the cardiovascular area, where experience with Viagra [64] suggests that dose escalation may be required.

Phosphodiesterase 7

Five years after its identification [65] the function of this enzyme remains enigmatic. Known to be expressed

as two splice variants (PDE7A1 and A2) from a single gene [66,67], the mRNA of both variants is ubiquitously expressed in many tissues. Protein expression is far more restricted, however, suggesting that the functional role of PDE7 necessitates that its translation be highly regulated. PDE7A1 activity and protein has been identified in T lymphocytes.

As discussed earlier, experience with inhibitors of PDE3 and 4 and T lymphocyte activation has identified the importance of elevating cAMP in the appropriate intracellular location. Given the side effect profiles of PDE3 and 4 inhibitors, inhibition of PDE7 may be of benefit in the treatment of certain immune disorders. Until a selective PDE7 inhibitor of adequate potency is identified, however, the functional role of this isoenzyme remains unknown.

Novel phosphodiesterases

Recently, bioinformatic screening of databases for homology to the catalytic domains of known PDEs has identified two new isoenzyme families PDE8 [68] and PDE9 [69,70]. PDE8A is a cAMP-selective enzyme of low K_M (150 nM), insensitive to the nonselective PDE inhibitor, IBMX (3-isobutyl-1-methyl-xanthine), but inhibited by dipyridimole (IC_{50} ~5 μ M). PDE9A is a high affinity, cGMP-specific PDE with a K_M of 170 nM for cGMP. It is insensitive to a variety of standard inhibitors but is weakly inhibited by zaprinast. Interestingly, PDE9A lacks a domain homologous with the cGMP-binding allosteric domains of PDEs 2, 5 and 6. The mRNA of this isoenzyme is most highly expressed in kidney.

In addition, at a recent conference (K Loughney, personal communication) another PDE family, PDE10, again identified via bioinformatics, was reported. As yet, no published data for PDE10 has appeared.

The identification of these three new PDE families serves to emphasise the scope and diversity of these enzymes. Whether they prove to be of therapeutic potential awaits a definition of their functional roles and, as always, the identification of potent selective inhibitors.

Conclusions

The diversity and complexity of the PDE superfamily presents an enormous potential for novel therapeutics across a broad spectrum of disease states. Experience with PDE3 and 4 enzymes has shown, however, that the attainment of potent selective inhibitors alone is not sufficient to deliver therapeutic benefit. A far more detailed understanding of the diverse intracellular environments in which these isoenzymes operate and the mechanisms by which they interact and cooperate is required. In addition, structural data on the PDEs, together with inhibitor mechanistic studies, are necessary to provide information on the potential for greater isoenzyme selectivity. Such information will afford the opportunity to incorporate

an improved degree of cellular selectivity into the next generation of inhibitors.

Despite the many failures of PDE inhibitors in clinical trials, it should be recognised that significant progress has been made. In the treatment of asthma, CDP840 has demonstrated that it is possible to attenuate the allergen-induced pulmonary late phase in asthmatic patients without producing side effects. Given that the late phase reaction is associated with tissue inflammation and damage, the inhibition of PDE4 is likely to exhibit anti-inflammatory action with minimal direct bronchodilator effect. In addition, two other PDE4 inhibitors, SB207499 and LAS31025 are progressing in phase II and III clinical trials, respectively. Clinical data on these are eagerly awaited. Similarly, topical application of the PDE4 inhibitor CP80633 has demonstrated efficacy in atopic dermatitis and may find utility in psoriasis.

Finally, the huge commercial success of the PDE5 inhibitor Viagra has, for many, vindicated their optimism in the concept of PDE-based therapy. Whether the success of Viagra will be repeated by PDE inhibitors in other areas remains to be seen. It is certain, however, that the exponential increase in information on the molecular organisation, regulation and biological function of PDEs will open additional routes to the production of novel therapeutics.

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Identification of purine inhibitors of phosphodiesterase 7 (PDE7)

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Received 26 August 2003; revised 27 February 2004; accepted 8 March 2004

Abstract—A series of purine based inhibitors of PDE7 has been derived from screening lead **1a**. The synthesis, structure–activity relationships (SAR), and selectivity against several other PDE family members are described.
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Phosphodiesterases (PDEs) hydrolyze the second messenger molecules cAMP and cGMP to affect cellular signaling. At least 11 families of PDEs exist, some of which (PDE3, 4, 7, and 8) are specific for cAMP, and others (PDE5, 6, and 9) for cGMP. Additional family members (PDE1, 2, 10, and 11) have dual specificity. PDEs are involved in a myriad of important physiological functions and as such continue to be a major target for pharmacological intervention on the part of the pharmaceutical industry.¹ A recent publication demonstrated a role for PDE7A in the activation and/or proliferation of T cells.² Resting T lymphocytes express mainly PDE3 and PDE4. However, upon activation, T cells dramatically up-regulate PDE7A1 and appear to principally rely on this isozyme for regulation of cAMP levels. Suppression of PDE7 up-regulation by antisense oligonucleotides inhibited proliferation and IL-2 production, and maintained elevated levels of intracellular cAMP in CD3xCD28 stimulated T cells. PDE7A has also been demonstrated to be up-regulated in human B-lymphocytes.³ PDE7A3, a splice variant of PDE7A1 is also reported to be upregulated in activated CD4⁺ T cells.⁴ This expression profile suggests inhibitors of PDE7A would have broad application as an immunosuppressant. Inhibition of PDE7 has been suggested to be of use in chronic obstructive pulmonary disease and asthma.⁵ Additionally PDE7A1 and PDE7A2 have been shown to have significant expression in rat brain

although no function has been ascribed to their presence in that tissue.⁶

PDE7B, the second member of the PDE7 family, shares 70% amino acid homology with PDE7A in the catalytic domain. It is expressed in a wide variety of tissues and has been shown to discriminate among several general PDE inhibitors.^{7,8}

To date there have been a few low micromolar inhibitors of PDE7 described in the general literature,^{9,10} including one guanine system.¹¹ There has also been a report of PDE7 inhibitors, which have an effect in T cells although these compounds are not entirely selective for PDE7.¹²

Our initial approach to develop inhibitors of PDE7 was prompted by the discovery of lead **1a** from the BMS compound collection (see Fig. 1). The SPA screening assay utilized a whole cell lysate of the Hut78 T cell line as a surrogate for the use of recombinant PDE7A1.¹³ A representative panel of known PDE inhibitors were

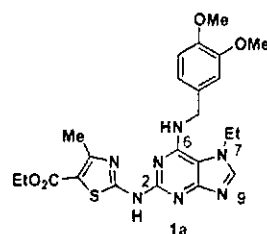


Figure 1. Screening lead **1a**.

Keywords: Phosphodiesterase 7; PDE7; Inhibitor.

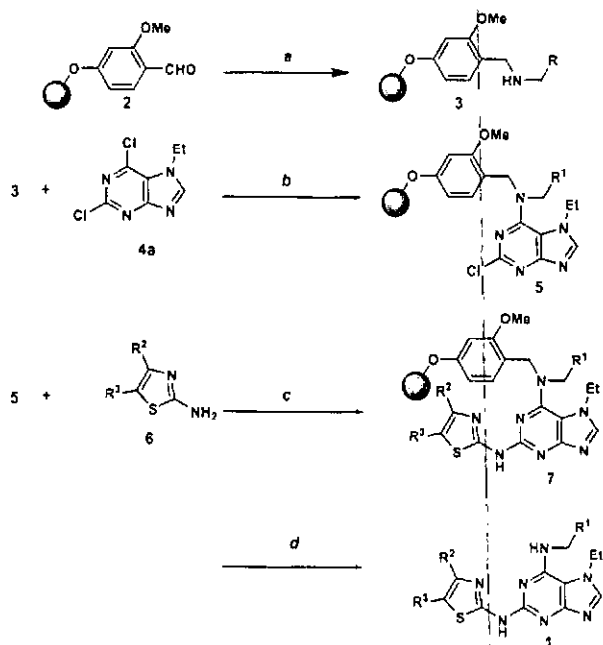
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Table 1

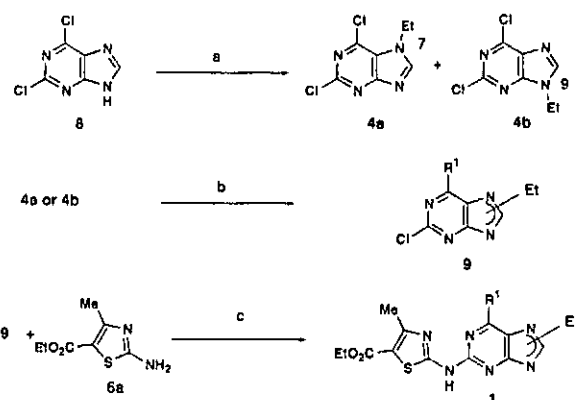
	PDE1 IC ₅₀ (μM) (PDE1/PDE7)	PDE2 IC ₅₀ (μM) (PDE2/PDE7)	PDE3 IC ₅₀ (μM) (PDE3/PDE7)	PDE4 IC ₅₀ (μM) (PDE4/PDE7)	PDE5 IC ₅₀ (μM) (PDE5/PDE7)	PDE7 IC ₅₀ (μM)
Zardaverine	>10	>10	0.11	0.21	>10	>10
8-Methoxy IBMX	1.80	>10	>10	>10	3.02	>10
Cilostamide	>10	>10	0.01	>10	7.15	>10
Rolipram	>10	>10	>10	0.74	>10	>10
Sildenafil	>10	>10	7.0	4.6	0.011	>10
1a	0.22 (1.5)	1.7 (11)	0.58 (3.5)	1.1 (7.3)	0.27 (1.8)	0.15

examined for inhibition of PDE activity in this cell line (see Table 1). Of the PDE inhibitors examined, none demonstrated activity less than 10 μM against PDE7 in our assay. In contrast lead compound **1a** had a PDE7 IC₅₀ of 0.15 μM. The selectivity against other PDEs was poor, ranging between 1.5- and 11-fold. This lack of selectivity is interesting. The compound inhibited recombinant PDE7 in a competitive fashion with respect to cAMP (data not shown). One might expect a sub-micromolar active site inhibitor to discriminate between PDE's to a greater degree with respect to preferred substrate (i.e., cAMP or cGMP) or level of active site homology, however for this lead (**1a**) this was not the case. A focused chemistry effort was initiated to determine if the potency and selectivity could be improved.

One advantage to this lead series was that it had been prepared via a solid-phase synthetic route, which would allow rapid expansion of some SAR. The solid-phase chemistry involved reaction of SASRIN aldehyde resin **2**, with the desired amine under reductive amination conditions. Reaction with the appropriate 2,6-dichloro-



Scheme 1. Reagents and conditions: (a) SASRIN aldehyde resin, amine, ZnCl₂, BH₃·pyridine, THF, rt, 16 h; (b) Hunigs base, NMP, 85 °C, 16 h; (c) Pd₂(dba)₃, (+)-TolBINAP, *t*-BuONa, dioxane/toluene, 110 °C, 16 h; (d) TFA, dichloromethane.



Scheme 2. Reagents and conditions: (a) Na₂CO₃, EtI, acetone, reflux 3 h (total yield 72–80%); (b) Hunigs base, amine, NMP, 85 °C, 16 h (85–93%); or NaH, and arylsulfonamide (40–45%) or alcohol (50–65%); (c) Pd₂(dba)₃, 2-(di-*t*-butylphosphino)biphenyl, anhyd K₃PO₄, DMA, 110 °C, 2–4 h (35–51%).

7-ethylpurine, provided the 6-substituted purine **5**. Amination at the 2-position was accomplished using Buchwald conditions.¹⁴ The final products **1** were obtained by cleavage from the resin using TFA (Scheme 1).

In order to obtain compounds with greater diversity at the 6-, and 7/9-positions on the purine ring, and avoid complications with solid phase TFA deprotection, solution phase chemistry was employed as depicted in Scheme 2. 2,6-Dichloropurine (**8**) was alkylated with ethyl iodide to provide of **4a** and **4b** in approximately a 1 to 4 ratio. After separation by flash column chromatography, the chlorine at the 6-position of the purine could be readily displaced with amines to produce intermediate **9**. Alternatively the chlorine could be displaced with the sodium salt of either sulfonamides or alcohols to produce intermediate **9**. Thiazole **6a** was originally coupled to **9** in low (12%) yield under the Buchwald conditions utilized for the resin bound synthesis. A wide variety of conditions were examined in an effort to improve the coupling yield with this amine. The use of a new ligand¹⁵ for palladium with potassium phosphate as the base in DMA improved the reproducibility and yields to a range of 35–50%. The analogues prepared were examined for PDE inhibition.

The SAR for the inhibition of PDE catalytic activity are summarized in Table 2. Low inhibitor selectivity between PDE7 and PDE4 might be anticipated based on several considerations. PDE4 was originally classified as a high affinity cAMP specific enzyme based on a *K_m* for

Table 2. SAR of 1b–l with respect to PDE inhibition. PDE#/PDE7 represents IC₅₀ ratio

1b-m

Compd	R ¹	R ²	Et position	PDE7 IC ₅₀ μM	PDE1/ PDE7	PDE2/ PDE7	PDE3/ PDE7	PDE4/ PDE7	PDE5/ PDE7
1b			7	0.11	19	NT	11	NT	0.38
1c			7	0.10	1.7	14	33	24	15
1d			7	0.15	2.8	3.6	17	1.3	4.6
1e			7	0.94	1.3	2.3	2.5	3.8	2.9
1f			7	0.055	18	25	130	22	0.90
1g			7	0.011	86	86	270	50	1.0
1h			9	0.010	200	45	>5000	16	13
1i			9	1.6	NT	NT	0.11	0.046	0.55
1j			9	0.37	NT	NT	21	NT	4.1
1k			7	0.82	12	11	12	7.0	1.1
1l			7	15% @ 1 μM	NT	NT	NT	NT	NT

cAMP of approximately 4 μM. The K_m for cAMP for PDE7 has been determined to be 0.2 μM. Additionally the K_m for cAMP for PDE8 is reported to be even lower (0.05 μM).¹⁶ Another selectivity consideration is the degree of homology in the enzyme active site among the

PDE family members (see Fig. 2). It is interesting to note that the compounds reported here generally demonstrated low selectivity against PDE5 despite this enzyme's high K_m for cAMP (150 μM) and low homology to PDE7.

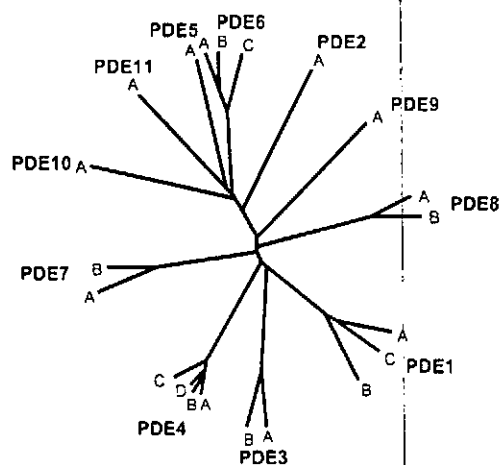


Figure 2. Dendrogram of PDE active site homology.¹⁸

Cursory examination of the lead structures **1a** and the analogues **1b** through **1e** suggests similarity with the 3,4-dialkoxyphenyl motif observed in many PDE4 inhibitors.¹⁷ A logical approach to improve selectivity against PDE4 would be to vary **R**¹.

A significant (16- to 50-fold) improvement in PDE4 selectivity and an increase in PDE7 potency was observed when the phenyl substitution pattern was changed in analogues **1f–h**. These analogues also demonstrated improved selectivity against PDE1, PDE2, and PDE3. Analogue **1h** demonstrated a significantly improved selectivity against PDE3 when compared to the 7-ethyl analogue **1g**. Additionally **1h** was approximately 100-fold selective against PDE6 (data not shown) and greater than 1000-fold selective against PDE8 (data not shown). Based on the selectivity data presented it is difficult to draw any correlation between the degree of PDE homology and inhibitor selectivity for this set of compounds. Unfortunately **1g** and **1h** have an aqueous solubility at pH 6.5 of less than 5 µg/mL. A suitable formulation for iv dosing could not be identified. Oral dosing of **1h** at 100MPK as a suspension produced peak plasma concentrations of less than 150 nM. A sulfonamide or oxygen linker were tolerated (**1i**, **1j**). Analogue **1i**, which does not contain an ester (e.g., **1a**) or amide (**1k**) is significantly less potent. Additional SAR studies on the thiazole are needed to better understand the observed loss in potency.

In summary we have reported the identification of a potent purine based inhibitor of PDE7 (**1a**) by screening the BMS compound collection. An exploratory chemistry effort demonstrated that the potency and PDE selectivity could be improved (e.g., **1h**). Future reports from our laboratory will describe efforts to further improve the selectivity of these lead compounds for PDE7 over other PDE family members and address issues such as solubility, which will permit the in vivo evaluation of PDE7 inhibitors.

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- Inhibition of PDE activity in Hut78 cell lysate was determined using an SPA specific for cAMP (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturers instructions with minor modifications. Enzyme assays were performed at room temperature in the presence of 50 mM Tris-HCl, pH 7.5, containing 8.3 mM MgCl₂, 1.7 mM EGTA, and 0.5 mg/mL BSA. Each assay was performed in a 100 µL reaction volume in 96 well microtitre plates containing the above buffer. 0.3 µL of Hut78 cell lysate treated with 2 µM Zardaverine to inhibit PDE3 and PDE4, 0.05 µCi of [5',8-³H] Adenosine 3',5'-cyclic phosphate as an ammonium salt for 20 min. The reaction was terminated by the addition of 50 µL PDE SPA beads (1 mg) water with 10 mM cold cAMP (Sigma, St. Louis MO). The reaction mix was allowed to settle for 20 min before counting in a Top Count-NXT scintillation counter (Packard BioScience, Meriden, CT). For selectivity studies, the assay was essentially unchanged except that ³H-cyclic GMP was used as the substrate for PDE1, PDE5, and PDE6. The following PDEs/activators and enzyme sources were used: PDE1, bovine (Sigma St. Louis); calmodulin: PDE2, rat kidney; cGMP: PDE3, human platelet; PDE4, rat kidney; PDE5, human platelet, and PDE6, bovine retina.
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