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Clarke, Modet & Cº

COLOMBIA

2657/PCT.

**SUPERINTENDENCIA DE INDUSTRI
DIVISION DE NUEVAS CREACIONES**
E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
No. 04-052629-00007-0000
C.C. 39.779.452 de Usaquen
T.P. 68.228
Cod. 5376
/srr

Re: Expediente No. **04.052.629**
Solicitud de Patente a nombre de
NOVARTIS AG

Yo, JIMENA ESCOBAR URIBE, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 No. 86-53, piso 6, obrando como apoderada en este negocio, atentamente solicito a usted se sirva conceder una prórroga de 30 días para dar respuesta al oficio No. 10750 notificado por fijación en lista el 22 de agosto de 2008, relacionado con la solicitud de patente que se está tramitando bajo el expediente de la referencia.

Acompaño el recibo Nr. 08-86,357 de 4 de noviembre de 2008, correspondiente a la tasa de la prórroga del término.

Señor Superintendente,


JIMENA ESCOBAR URIBE
C.C. 39.779.452 de Usaquen
T.P. 68.228
Cod. 5376
/srr

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800170089-2

RECIBO OFICIAL DE CAJA : 08 - 86,357
FECHA : NOVIEMBRE 4 DE 2008

NO. 04-052629-00007-0000
DIRECCION NACIONAL DE REGISTRO Y CONTROL
DIRECCION NACIONAL DE REGISTRO Y CONTROL

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VAL. PAGO
CLARKE MODET Y CO. COLOMBIA	CONSIGNACION	BANCO DE BOGOTA	062754387	95151114	31/10/2008	39,200,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-08	PRORROGA DE TERMINOS	75,000.00
		TOTAL :	\$ 75,000.00

SON: SETENTA Y CINCO MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 04-052629- -00008-0000

Fecha: 2008-12-30 15:02:00 Dep. 2020 NUECREACIONI
Tra. 11 PATENTE(Y) Eve. 379 FASENACIONALI
Act. 444 RESPUESTAREQUE Folios: 23

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **04-052.629**

Solicitud de patente a nombre de **NOVARTIS AG.**

Yo, JIMENA ESCOBAR URIBE, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **NOVARTIS AG**, domiciliada en Basilea Suiza solicitante de la patente de la referencia, en respuesta al oficio No. 10750 notificado por fijación en lista el 22 de agosto de 2008, relacionado con el concepto técnico de fondo No. 1784, me permito manifestar:

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo capítulo reivindicatorio, que reemplaza el presentado con mi memorial del 14 de mayo de 2008 y en el cual se han realizado las siguientes modificaciones:

1.1 En la reivindicación 1 se introdujo la expresión R1 es un grupo aromático monovalente de hasta 10 átomos de carbono, de acuerdo con la primera página de la descripción, con el fin de brindar soporte a los compuestos que contienen grupos fenilo fusionados con anillos heterocíclicos.

1.2 En las reivindicaciones 2 y 3 se especificaron las posibles veces que puede estar sustituido el grupo fenilo para el radical R1. El sustento para esta modificación se encuentra en la página 4, líneas 23 a 25, página 6, líneas 17 a 20 y página 7, líneas 14 a 16 de la descripción.

1.3 Se reemplazó la expresión “átomos heterogéneos” por “heteroátomos”.

Respecto a estas modificaciones, atentamente manifiesto que las mismas no amplían la materia objeto de la solicitud estudiada y se enfocan con el fin de

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brindar cabal cumplimiento al requisito de claridad de la solicitud expuesto por el señor examinador.

Para este efecto, me permito acompañar el recibo No. 08-96,186 del 12 de diciembre de 2008 con el cual queda cubierta la tasa correspondiente a la modificación de una solicitud de patente en cuanto a sus reivindicaciones.

2. Claridad de la solicitud.

2.1 Descripción

Con relación a la memoria descriptiva, respetuosamente me permito decir que contrario a lo afirmado por ese despacho, la misma es explícita en cuanto a las ventajas de la invención con relación al estado del arte. De hecho, como se explicó en mi memorial anterior, la invención en su parte descriptiva muestra un ensayo de inhibición de Isocenzima PDE4 en la página 15 desde la línea 4 hasta la página 19, línea 9, donde se explica de manera detallada que este ensayo se hace mediante la clonación de la PDE humana, la expresión de la misma, su purificación y los análisis de tinción Western con los cuales se demuestra que compuestos como el 1, 3, 10, 12, 14, y 15, que son ejemplos no limitantes pero sí representativos de la invención, mostraron una concentración de IC₅₀ determinada a partir de las curvas de inhibición-concentración de 38, 9, 25, 21, 57, y 9 nM, respectivamente, para la inhibición de PDE4D, es decir, que es de esperarse que todos los compuestos exhiban selectividad para la inhibición de la isoenzima PDE4D más que para las isoenzimas PDE4A, PDE4B y PDE4C.

En este orden de ideas, resulta necesario hacer énfasis a que la Decisión 486 no hace ninguna alusión en cuanto a que se deban “probar” todos los compuestos cobijados por el capítulo reivindicatorio. Es más, el artículo 28, establece en su literal e) que la invención incluirá información en cuanto a “una descripción de la mejor manera conocida por el solicitante para ejecutar o llevar a cabo a la práctica la invención, utilizando ejemplos y referencias a los dibujos, de ser éstos pertinentes;” (el resaltado es mío). En consecuencia, no es procedente por parte de ese despacho exigir que se presenten datos de todos los compuestos reclamados, pues la jurisprudencia señala que son ejemplos y sus resultados los que deben aparecer señalados en la descripción como se establece en el literal c) del artículo 28 de la Decisión 486. Por lo tanto, sugerimos retirar esta objeción.

Aún más, como se manifestó en mi respuesta del 14 de mayo de 2008, el artículo 18 de la Decisión 486 deja en claro que es legítimo presentar a manera de muestra la mejor forma de llevar a cabo la invención y no se establece en ninguna parte de este artículo, que se deban presentar resultados de todos los compuestos porque no sería una acción de ejemplificar e involucraría la petición por parte de ese despacho de una prueba diabólica, puesto que se estaría solicitando al inventor

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proporcionar pruebas que demuestren que no es cierto que no todos los compuestos tienen actividad biológica, lo cual en derecho se conoce como una **prueba inquisitorial**, es decir, probar lo que no es. En este caso, ese despacho se encuentra solicitando al inventor que demuestre que no es cierto que no todos los compuestos presenten actividad demostrable como así lo quiere ver ese despacho.

2.2 Reivindicaciones

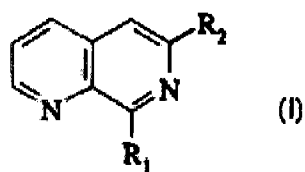
Sobre este particular, me permito decir que las reivindicaciones se encuentran debidamente sustentadas y adicionalmente con las modificaciones anteriores las expresiones de las reivindicaciones dependientes de la cláusula 1 tienen soporte adicional en la reivindicación 1 modificada.

En consecuencia, respetuosamente me permito decir que los argumentos expuestos en el concepto técnico No. 1784 para desvirtuar la claridad, el sustento y la veracidad de la invención, es una forma de obstaculizar el trámite de la consecución del privilegio de invención para las solicitudes en estudio y no resulta ser una razón válida a la luz de la normatividad estipulada en la Decisión 486 de la Comunidad Andina.

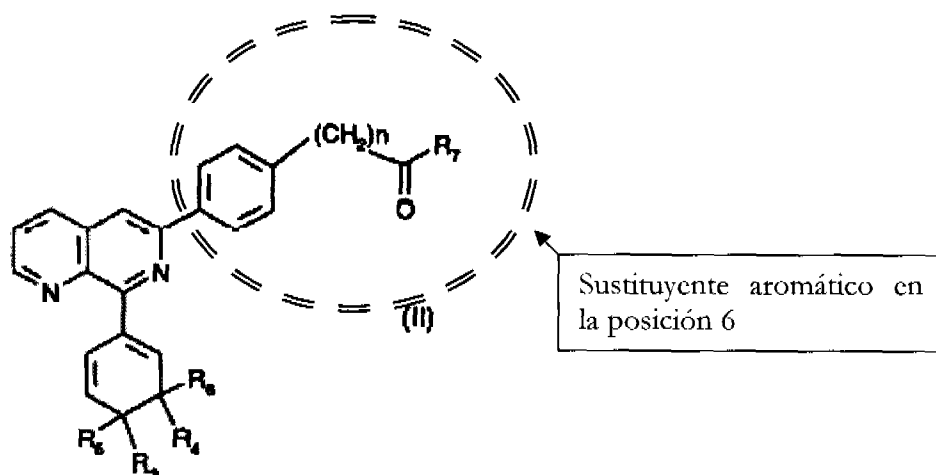
3. Nivel inventivo.

El examinador establece en su concepto técnico No.1784 que tanto los compuestos de la fórmula (I) como el proceso para la preparación de los mismos carecen de nivel inventivo en vista de las enseñanzas del documento D1 (WO 98/18796).

Sobre el particular, nuevamente me permito decir que la anterioridad reporta 8-aril-1,7-naftiridinas que son inhibidores PDE IV de la fórmula



y especialmente, compuestos de la fórmula (II)



el cual posee un sustituyente aromático en la posición 6 de la naftiridina como inhibidores PDE4. Por el contrario, la presente invención se encuentra dirigida al problema técnico de proporcionar inhibidores de la isoenzima de fosfodiesterasa selectivos para la isoenzima tipo 4A para las enfermedades inflamatorias, especialmente para aquellos trastornos de las vías respiratorias.

Dicho problema se resuelve mediante los novedosos compuestos de la solicitud en estudio que son derivados de 8-aryl-1,7-naftiridina de fórmula (I) en los cuales la posición 6 se encuentra sustituida por un grupo cicloalquilo de 3 a 8 átomos de carbono que está insustituido o sustituido por uno o más sustituyentes seleccionados de alquilo de 1 a 4 átomos de carbono, carboxilo, alcoxicarbonilo de 1 a 8 átomos de carbono y aminocarbonilo.

Ese despacho puede constatar que la anterioridad no revela ni sugiere la posibilidad de reemplazar los grupos allí mencionados para la posición 6 (grupo hidroxilo, amino, trifluorometansulfonilo, alilo, alquilo, alquenilo, alquinilo, alcoxilo, arilo, aralquilo, ariloxilo, arilamino, diarilamino, alcamino, dialcamino, alcarilo, arilamindo o alcamido) por un grupo cicloalquilo de 3 a 8 átomos de carbono como el revelado en la solicitud en estudio para la misma posición, razón por la cual una persona medianamente versada en la técnica no encontraría evidente los compuestos de la presente invención a partir de lo revelado en D1, toda vez que este documento no sugiere que al realizar cambios de esta naturaleza en la posición 6, pudieran alcanzar ventajas técnicas en nuevos compuestos.

En este sentido, una persona medianamente versada en la técnica no habría encontrado motivación alguna a partir de lo revelado en D1 para llevar a cabo una sustitución como la mostrada para la posición 6 del anillo de naftiridina de la presente invención, a partir de sustituyentes tan diferentes como los divulgados en D1. Estos sustituyentes a todas luces alejarían a la persona con conocimientos promedio en la técnica, de compuestos como los revelados en la invención en estudio, ya que en D1 los mismos tienen sustituyentes aromáticos y no cicloalquílicos.

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Aún más, al analizar la clase de compuestos preferidos y revelados bajo la fórmula (II) en la anterioridad, se puede constatar que la sugerencia de D1 es que las modalidades preferidas allí mencionadas son grupos fenílicos para-sustituídos con grupos $(CH_2)_nC(=O)R^7$ donde n es 1 ó 0. Esto dista bastante de los compuestos de la presente invención, toda vez que, en la posición 6 de la estructura central los sustituyentes R^2 como los mostrados en la tabla 1, página 39 y 40 de la descripción de la invención en estudio, son todos sistemas cíclicos alquílicos de seis miembros con un grupo ácido carboxílico terminal.

Por lo tanto, la persona medianamente versada en la materia no encontraría evidente la modificación en la posición 6 del sistema bicíclico, toda vez que para nada son equiparables o equivalentes los grupos fenílicos a los grupos cicloalquílicos mostrados en la invención en estudio.

En este sentido, nuevamente dirijo la atención de ese despacho a las páginas 16 a 18 de D1 donde se muestra la tabla 1:

Example No.	R ₁	R ₂
22		-
23		-
24		-
25		
26	-	
27	-	
28		
29		-
30		
31		-
32		

Example No.	R ₁	R ₂
15		
16		-
17		-
18		
19	-	
20	-	
21		-

Example No.	R ₁	R ₂
33		-

En ella, se sugieren como sustituyentes preferidos para la posición 6 del sistema bicíclico de la fórmula I los grupos NH₂, fenílico, grupo -OSO₂CH₃, grupo -C=CH₂ ó grupo -C≡H. Esto no conduciría de manera evidente a las sustituciones planteadas en la solicitud en estudio.

El solicitante en vista de insistencia de ese despacho en cuanto a pruebas comparativas, desca manifestar que el documento D1 (WO 98/18796) revela derivados de 8-aril-1,7-naftiridina como inhibidores de PDE4 y la potencia del compuesto más eficaz y potente encontrada dentro de esta clase fue hallada para exceder los inhibidores de PDE4 conocidos Ariflo, LAS 31025 y V11294A. Estos

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resultados son reportados en el artículo de la revista *Journal of Pharmacology and Experimental Therapeutics*, 301, 241-248 del cual se adjunta una copia informal.

En este orden de ideas, a pesar que esta potencia del compuesto preferido (compuesto 11 en la publicación de HERSPERGER R y colaboradores titulada como “Pd-catalised cross-coupling reactions for the synthesis of 6,8-disubstituted 1,7-naphthyridines: A novel class of Potent and selective phosphodiesterase type 4D inhibitors”, documento citado en fase internacional, y el compuesto 14 del documento D1), se encontró que son compuestos altamente insolubles en agua, es decir, del orden de 0.0023 mg/ml a un pH de 6.8 y adicionalmente, proporciona una limitada biodisponibilidad. En contraste, los compuestos de la presente invención son el producto de uno de un número de intentos para mejorar la solubilidad y la biodisponibilidad de manera significativa sin la pérdida de la potencia.

La siguiente tabla resume la solubilidad y la biodisponibilidad para muchos de los compuestos de la presente invención en comparación con los compuestos preferidos de D1 y el artículo citado en fase internacional. La solubilidad termodinámica se determinó en una solución acuosa amortiguada a pH de 6.8 y la biodisponibilidad se determinó en ratas.

compuesto	Solubilidad pH 6.8 (g/l)	Biodisponibilidad (%)
11 del documento citado en fase internacional	0.0023	10
Ejemplo 2	<u>0.56</u>	<u>92</u>
Ejemplo 10	-	78
Ejemplo 11	>4	-
Ejemplo 12	<u>0.92</u>	<u>73</u>
Ejemplo 14	-	<u>107</u>
Ejemplo 15	<u>2.0</u>	<u>131</u>

En este sentido, ese despacho puede corroborar que es claro a partir de la tabla anterior que los compuestos de la solicitud en estudio, como una representación de los compuestos probados son significativamente más solubles y presentan una biodisponibilidad mucho más alta que el compuesto preferido revelado en el documento D1/D2.

En consecuencia, no hay ninguna sugerencia en el documento D1 que pudiera conducir a una persona medianamente versada en la técnica a predecir que los compuestos de la solicitud en estudio podrían presentar estas propiedades mejoradas. Por lo tanto, los datos anteriormente presentados apoyan la existencia del nivel inventivo en los compuestos reclamados en la solicitud en estudio.

De otra parte, con relación al proceso revelado en la reivindicación 9, me permito señalar que dicho proceso tiene nivel inventivo sobre lo revelado en D1 dado que

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produce compuestos inventivos como los reclamados en condiciones específicas no enseñadas ni sugeridas por la anterioridad D1.

En conclusión, me permito manifestar que a la luz de los argumentos anteriormente expuestos, los datos presentados y de las modificaciones realizadas en el capítulo reivindicatorio, los compuestos de la presente invención sí tienen nivel inventivo sobre las enseñanzas de D1, razón por la cual solicito de manera atenta se proceda a conceder el privilegio de patente para la invención de la referencia.

Señor Superintendente,


JIMENA ESCOBAR URIBE
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Còd.5376

Pharmacological Profile of a Novel Phosphodiesterase 4 Inhibitor, 4-(8-Benzo[1,2,5]oxadiazol-5-yl-[1,7]naphthyridin-6-yl)-benzoic Acid (NVP-ABE171), a 1,7-Naphthyridine Derivative, with Anti-Inflammatory Activities

ALEXANDRE TRIFILIEFF, DANIEL WYSS, CHRISTOPH WALKER, LAZZARO MAZZONI, and RENE HERSPERGER

Novartis Horsham Respiratory Centre, Horsham, United Kingdom (A.T., D.W., C.W.); and Novartis Pharma AG, Basel, Switzerland (L.M., R.H.)

Received October 8, 2001; accepted December 5, 2001 This article is available online at <http://jpet.aspetjournals.org>

ABSTRACT

We investigated the pharmacology of a new class of phosphodiesterase 4 (PDE4) inhibitor, 6,8-disubstituted 1,7-naphthyridines, by using 4-(8-benzo[1,2,5]oxadiazol-5-yl-[1,7]naphthyridin-6-yl)-benzoic acid (NVP-ABE171) as a representative compound and compared its potency with the most advanced PDE4 inhibitor, undergoing clinical trials, Ariflo (*cis*-4-cyano-4-(3-cyclopentyloxy-4-methoxyphenyl)-*r*-1-cyclohexanecarboxylic acid). NVP-ABE171 inhibited the activity of phosphodiesterase 4A, 4B, 4C, and 4D with respective IC_{50} values of 602, 34, 1230, and 1.5 nM. Ariflo was about 40 times less potent. In human cells, NVP-ABE171 inhibited the eosinophil and neutrophil oxidative burst, the release of cytokines by T cells, and the tumor necrosis factor- α release from monocytes, in the nanomolar range. Ariflo presented a similar inhibition profile but was 7 to 50 times less potent. In BALB/c mice challenged with

lipopolysaccharide, NVP-ABE171 inhibited the airway neutrophil influx and activation with an ED_{50} in the range of 3 mg/kg. Ariflo was inactive up to a dose of 10 mg/kg. In ovalbumin sensitized Brown Norway rats, NVP-ABE171 inhibited the lipopolysaccharide-induced airway neutrophil influx and activation (ED_{50} of 0.2 mg/kg) and the ovalbumin-induced airway eosinophil influx and activation (ED_{50} of 0.1 mg/kg). Ariflo was about 100 times less potent in both models. In the ovalbumin model, NVP-ABE171 had a duration of action of more than 24 h. NVP-ABE171 is a novel PDE4 inhibitor that shows activity both in vitro on human inflammatory cells and in vivo in animal models of lung inflammation. This compound class may have potential for the treatment of airway inflammatory conditions such as asthma and chronic obstructive pulmonary diseases.

3',5'-Cyclic nucleotide phosphodiesterases (PDEs) are families of hydrolases that catalyze the hydrolysis of cAMP and cGMP, and thereby terminate their role as second messengers in mediating cellular responses to various mediators. Eleven families of PDE enzymes, sharing a conserved catalytic domain and exhibiting distinct substrate specificity and regulatory properties, have been identified (Soderling and Beavo, 2000). Among the cAMP-specific isoenzymes, PDE4s have received particular attention after the recognition that these are the primary enzymes responsible for the metabolism of cAMP in inflammatory and immune cells (Muller et al., 1996). Because PDE4 inhibitors have been shown to be potent anti-inflammatory agents in various animal models (Teixeira et al., 1997), they have been proposed as a new therapeutic approach for a variety of inflammatory diseases such as asthma and chronic obstructive pulmonary diseases

(Barnes, 1999). Despite the large effort of the pharmaceutical industries to identify selective PDE4 inhibitors in the last decade, only a few of them have shown efficacy in patients, probably due to the fact that the clinical usefulness of these compounds is limited by adverse effects (i.e., nausea and emesis) (Teixeira et al., 1997). However, promising data from clinical trials with Ariflo, the most advanced PDE4 inhibitor in clinical development, support the concept that a dissociation of side effects from therapeutic benefit can be achieved with this class of drug (Torphy et al., 1999).

Recently, different isogenes and splice variants of human PDE4 have been described (Beavo, 1995). The four gene products (PDE4A, 4B, 4C, and 4D) are characterized by their selective, high-affinity hydrolysis of cAMP and sensitivity to inhibition by rolipram. According to an analysis of a number of human peripheral blood cells and cell lines, mRNA of these

ABBREVIATIONS: PDE, phosphodiesterase; NVP-ABE171, 4-(8-benzo[1,2,5]oxadiazol-5-yl-[1,7]naphthyridin-6-yl)-benzoic acid; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; PBS, phosphate-buffered saline; IL, interleukin; IFN, interferon; ELISA, enzyme-linked immunosorbent assay; TNF, tumor necrosis factor; LPS, lipopolysaccharide; Ariflo, *cis*-4-cyano-4-(3-cyclopentyloxy-4-methoxyphenyl)-*r*-1-cyclohexanecarboxylic acid; V 11294A, 3-(3-cyclopentyloxy-4-methoxybenzyl)-6-ethylamino-8-isopropyl-3H-purine hydrochloride; YM976, 4-(3-chlorophenyl)-1,7-diethylpyrido[2,3-*d*]pyrimidin-2(1H)-one; LAS 31025, 3-(*p*-chlorophenyl)-1-propylxanthine; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.

isoforms is expressed in the majority of the immune and inflammatory cells (Muller et al., 1996). In particular, PDE4A, 4B, and 4D were predominantly expressed in eosinophils and neutrophils, whereas PDE4C mRNA was not detected in any of the inflammatory cells tested. Our research efforts in finding novel PDE4 inhibitors were based upon a new approach. We wanted to find orally active, second-generation PDE4 inhibitors displaying different selectivity profiles against the PDE4 isozymes (Hersperger et al., 2000). In this report we investigate the *in vitro* and *in vivo* anti-inflammatory properties of 4-(8-benzo[1,2,5]oxadiazol-5-yl-[1,7]naphthyridin-6-yl)-benzoic acid (NVP-ABE171), an inhibitor for the PDE4B and 4D isozymes. Ariflo, V 11294A, and LAS 31025 were used as comparison compounds.

Materials and Methods

Inhibition of Nucleotide Phosphodiesterase Isoenzymes. Phosphodiesterase activity was determined as previously described using cAMP or cGMP as substrate (Engels et al., 1995). Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and diluted to the required concentration with the assay buffer containing DMSO at a final concentration of 0.5%. DMSO itself, at the concentration used, did not affect any of the PDE activity.

PDE1 was purified from human lung obtained from patients undergoing surgery for lung cancer. Tissue specimens were homogenized in Tris buffer, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 7 mg/ml phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin (TES).

PDE2 and 5 were purified from platelet concentrate obtained from the local blood transfusion center. Platelets were suspended in TES buffer and sonicated four times for 15 s at 4°C. Both preparations were centrifuged (120,000g for 60 min) and filtrated over a 0.22- μ m filter. The resulting homogenate was applied to a 20-ml column of Q-Sepharose (Amersham Biosciences AB, Uppsala, Sweden) equilibrated in sucrose-free TES buffer. Upon elution with a gradient of 0 to 0.45 M NaCl, PDE1 was obtained as the calcium-calmodulin-stimulated peak of activity. PDE2 was obtained as the peak of activity by using 100 μ M cAMP and PDE5 as a peak of activity by using 100 μ M cGMP. Purification of human PDE3 and cloning and expression of human PDE4A, 4C, 4D, and rat PDE4B were performed as previously described (Hersperger et al., 2000). Pooled active fractions of each enzyme were made in ethylene glycol and stored at -20°C.

Permeability across Caco-2 Cells. Caco-2 cells, a colonic adenocarcinoma cell line, were seeded at a density of 10^5 cells/cm² onto transwell filter inserts (1 cm²) and held in culture for 21 days. Test compounds were added to the donor compartment and aliquots were taken from the acceptor compartment and replaced with equal volume of blank medium at different time points. [³H]Propranolol was used as a reference compound for transcellular transport. Levels of NVP-ABE171 were analyzed by high-performance liquid chromatography/UV detection.

Measurement of Intracellular cAMP Elevation in HaCat Cells. cAMP elevation studies were conducted in human keratinocyte cell line, HaCat. Cells (2.5×10^5) were allowed to adhere overnight to 24-well tissue culture plates at 37°C in 0.5 ml of medium [Dulbecco's modified Eagle's medium-H21 containing 5% fetal calf serum (FCS) and 2 mM L-glutamine]. Medium was removed and fresh medium containing 0.03 μ M isoprenaline (Sigma-Aldrich, Poole, Dorset, UK) was added for 4 h. The reaction was stopped by the addition of 2 volumes of cold ethanol (-20°C). After a 15-min incubation at 4°C, the samples were centrifuged (450g for 10 min) and levels of cAMP in the supernatant measured by cAMP enzyme-linked immunosorbent assay kits (Amersham plc, Little Chalfont, Buckinghamshire, UK). For all the cell-based assays, test compounds or vehicle were preincubated for 15 min at 37°C.

Oxidative Burst from Human Eosinophils and Neutrophils. Blood was obtained from normal individuals. Granulocytes were separated from mononuclear cells by Ficoll-Hypaque gradient centrifugation. Erythrocytes were lysed by two cycles of hypotonic lysis and the remaining granulocytes were either used as enriched neutrophil preparation (>95% neutrophils) or incubated with anti-CD16-coated immunomagnetic particles. Magnetically labeled neutrophils were then depleted by passing the granulocytes through a magnetic cell separation column, which resulted in a more than 98% pure eosinophil preparation. Purified eosinophils or neutrophils (5×10^6 /well in RPMI-1640 containing 0.1% human serum albumin) were stimulated with plate-bound human IgG (Sandoz-globin-coated 96-well microtiter plates, 50 μ g/well) or 1 μ M N-formyl-methionyl-leucyl-phenylalanine, respectively, and the ability of cells to generate superoxide anions during an oxidative burst was measured using a Hamamatsu MTP reader. The IgG-induced changes in lucigenin chemiluminescence was monitored over 40 min and results were calculated as percentage of IgG- or N-formyl-methionyl-leucyl-phenylalanine-induced luminescence in the absence of compounds.

Proliferation and Cytokine Production of Human Peripheral Blood Lymphocytes. Mononuclear cells were isolated from blood of normal individuals by Ficoll-Hypaque gradient centrifugation (20 min at 800g). The interphase was collected, washed twice in phosphate-buffered saline (PBS), and resuspended in RPMI-1640 supplemented with 10% FCS. Cell density was adjusted to 10^6 cell/ml. One hundred microliters of the mononuclear cells suspension was placed in 96-well culture plates and 50 μ l of either medium or compounds in the indicated concentrations was added. After a 10-min preincubation, cells were stimulated with 50 μ l of anti-CD3 monoclonal antibodies (OKT-3, 100 ng/ml), incubated for 42 h at 37°C in a humidified incubator with 5% CO₂. Supernatants were harvested after 20 h of incubation. IL-4, IL-5, and IFN- γ were measured by sandwich ELISA by using two monoclonal antibodies recognizing different epitopes of the specific cytokine. Antibodies used for measuring cytokine levels were purchased from PharMingen (San Diego, CA). In all cases, binding of the second antibody was analyzed by stepwise incubation with streptavidin-alkaline phosphatase conjugate (Sigma-Aldrich) and 4-nitrophenylphosphate disodium salts (Sigma-Aldrich). Optical density was measured at 405 nm and cytokine concentration was calculated based on the results from serial dilutions of standard recombinant human IL-4, IL-5, and IFN- γ , respectively. The sensitivity of the cytokine ELISAs was 10 pg/ml.

TNF- α Production by Human Peripheral Blood Mononuclear Cells. Mononuclear cells were isolated as described above and resuspended in RPMI-1640 supplemented with 10% FCS. Cell density was adjusted to 10^6 cell/ml. Cells were stimulated with 10 μ g/ml lipopolysaccharide (LPS; *Salmonella typhosa*) and 50 ng/ml IFN- γ and supernatants were harvested after 20 h of incubation at 37°C in a humidified incubator with 5% CO₂. Concentration of TNF- α in the supernatants was measured by sandwich ELISA by using two monoclonal antibodies recognizing different epitopes of the specific cytokine (mAb357/101-4 and biotinylated 2-179/E11; Novartis, Basel, Switzerland). Binding of the second antibody was analyzed by stepwise incubation with streptavidin-alkaline phosphatase conjugate (Mabtech, Stockholm, Sweden) and 4-nitrophenylphosphate disodium salt (Sigma-Aldrich). Optical density was measured at 405 nm and cytokine concentration was calculated based on the results from serial dilutions of standard recombinant human TNF- α .

Animals. The animals were housed in plastic cages in air-conditioned room at 24°C in a 12-h light/dark cycle. Food and water were available *ad libitum*. The studies reported herein conformed to the UK Animals (scientific procedures) Act 1986.

LPS-Induced Lung Inflammation in BALB/c Mice. Female BALB/c mice (5 weeks old) were treated intranasally, under halothane/oxygen/nitrous oxide anesthesia, with 0.3 mg/kg LPS (*Salmonella typhosa*; Sigma-Aldrich) in 50 μ l of sterile PBS or with sterile PBS alone. Three or 24 h after the provocation, anesthesia was

induced with 60 mg/kg i.p. pentobarbitone sodium. After anesthesia, the abdominal cavity was opened and the animal exsanguinated by withdrawal of blood from a major blood vessel. The trachea was cannulated and bronchoalveolar lavage was performed by injecting 4×0.3 ml of PBS into the lung via the trachea. The fluid was immediately withdrawn and the cell suspension stored on ice. Total cell count was measured and cytospin preparation (Shandon Scientific Ltd, Cheshire, UK) prepared. Cells were stained with Dif-Quick (Baxter Dade AG, Duding, Switzerland) and a differential count of 200 cells performed using standard morphological criteria. The remaining lavage fluid was centrifuged at 200g for 10 min, and the supernatant was either used fresh or aliquoted and stored at -80°C .

Ovalbumin-Induced Lung Inflammation in Brown Norway Rats. Animals were sensitized as described (Hannon et al., 2001). Briefly, 20 $\mu\text{g}/\text{ml}$ ovalbumin was mixed with 20 mg/ml aluminum hydroxide and injected (0.5 ml/animal s.c.) coincidentally with Acular pertussis adsorbat vaccine (0.2 ml/animal i.p.; diluted 1:4 with 0.9% saline). Injection of ovalbumin, together with adjuvant, was repeated 14 and 21 days later. On day 28, sensitized animals were restrained in plastic tubes and exposed for 1 h to an aerosol of 3.2 mg/ml ovalbumin by using a nose-only exposure system. Animals were killed 48 h later with 250 mg/kg i.p. pentobarbital. The lungs were lavaged using three aliquots (4 ml) of Hanks' solution, recovered cells were pooled, and the total volume of recovered fluid adjusted to 12 ml by addition of Hanks' solution. Differential cell counts were done as described for the mice.

LPS-Induced Lung Inflammation in Brown Norway Rats. Ovalbumin-sensitized animals were challenged intratracheally with LPS (*Salmonella typhosa*, 10 $\mu\text{g}/\text{kg}$), on day 28 and killed 24 h later with 250 mg/kg, i.p. pentobarbital. The lungs were lavaged as described above.

Bronchoalveolar Lavage Soluble Mediators Measurements. Myeloperoxidase activity was measured on fresh bronchoalveolar lavage supernatant using a 96-well plate format colorimetric assay. Fifty microliters of the samples, in duplicate, were mixed with 100 μl of the substrate buffer for 5 min at room temperature (50 mM sodium phosphate, pH 6.0, containing, 0.5% hexadecyltrimethylammonium bromide, 0.167 mM *O*-dianiside dihydrochloride, and 0.4 mM H_2O_2). The reaction was stopped with 100 μl of 5% sodium azide in distilled water and the optical density read at 450 nm. Results were expressed as units per milliliter by using a standard curve established with human leukocyte myeloperoxidase (Sigma-Aldrich).

Eosinophil peroxidase activity was measured on fresh bronchoalveolar lavage supernatant by using a 96-well plate format colorimetric assay. Bronchoalveolar lavage (50 μl) was mixed with 100 μl of substrate (1 mM *o*-phenylenediamine dihydrochloride, 1 mM H_2O_2 , 0.1% Triton X-100, dissolved in 50 mM Tris-HCl pH 7.7) in a 96-well flat-bottomed microtiter plate and incubated for 30 min at room temperature. The reaction was stopped by adding 50 μl of 4 M H_2SO_4 , and absorbance was measured at 492 nm in a microtiter plate absorbance spectrophotometer. The concentration of eosinophil peroxidase activity was calculated as milliunits per milliliter according to the activity of serial dilutions of a standard horseradish peroxidase (Sigma-Aldrich).

The concentration of protein in bronchoalveolar lavage fluid supernatants was measured by a colorimetric assay (Bio-Rad DC protein assay) as described by the manufacturer (Bio-Rad, Hercules, CA). TNF- α levels were measured on bronchoalveolar lavage supernatant by using commercially available ELISA kits (Genzyme, Cambridge, UK).

Drug Administration. For in vivo testing, compounds were dissolved in DMSO (4% final concentration) and diluted with Neoral placebo for administration by gavage. Compounds or vehicle were applied 1 h before and 6 h after the LPS exposure in the mice, 1 h before the LPS exposure in the rat, and 1 h before and 24 h after the ovalbumin challenge in the rat. For the duration of action experiment, in the rat ovalbumin challenge model, animals were treated from 1 to 24 h before the challenge.

Data Analysis. Data are expressed as mean $\pm$ S.E.M. Statistical comparisons were performed using a Kruskal-Wallis test with Bonferroni's correction for multiple comparison, and a *P* value of less than 0.05 was considered significant. Depending on the efficacy of the compounds in the various test systems, corresponding IC or ED values for half-maximum inhibition were calculated from dose-dependent curves by nonlinear regression analysis. For cAMP elevation, EC_{200} was calculated as the concentration that doubled (200%) the level of cAMP induced by isoprenaline.

Results

Inhibition of Purified PDE Isozymes. Ariflo, V 11294A, LAS 31025, and NVP-ABE171 were synthesized by the Department of Chemistry (Novartis Pharmaceuticals, Horsham, UK). The structure of NVP-ABE171 is shown in Fig. 1. NVP-ABE171 and Ariflo dose dependently inhibited metabolic activity of the PDE4 isozymes; however, NVP-ABE171 was about 40-fold more potent than Ariflo. V 11294A and LAS 31025 inhibited the PDE4 isozymes only in the micromolar range. None of the compounds were active on all the other PDEs tested (i.e., PDE1, 2, 3, and 5) (Table 1).

Caco-2 Permeability and Intracellular cAMP Accumulation in HaCat Cells. In the Caco-2 assay NVP-ABE171 had a permeability coefficient value of $208.7 \pm 24.1 \times 10^{-5}$ cm/s ($n = 4$), well above the reference compound pro-

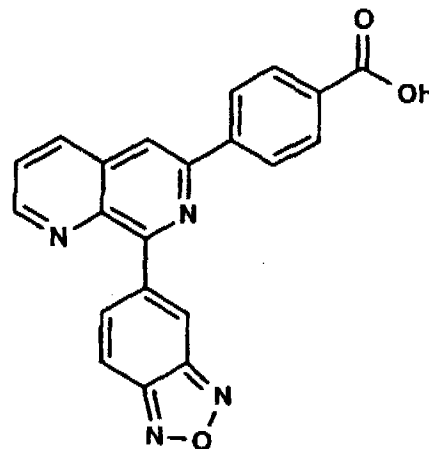


Fig. 1. Structure of NVP-ABE171.

TABLE 1

Inhibition of human phosphodiesterase activity by NVP-ABE171 and related compounds

Data are expressed as mean $\pm$ S.E.M. from the number of experiments indicated in parentheses.

	IC_{50}			
	NVP-ABE171	Ariflo	V 11294A	LAS 31025
	nM			
PDE4A	602 $\pm$ 25 (4)	398 $\pm$ 7 (4)	446 $\pm$ 11 (3)	2614 $\pm$ 48 (3)
PDE4B	34 $\pm$ 0.5 (3)	288 $\pm$ 7 (3)	616 $\pm$ 7 (3)	1452 $\pm$ 52 (3)
PDE4C	1230 $\pm$ 39 (3)	813 $\pm$ 13 (4)	1698 $\pm$ 44 (3)	1995 $\pm$ 38 (3)
PDE4D	1.5 $\pm$ 0.1 (4)	63 $\pm$ 2 (4)	645 $\pm$ 14 (3)	965 $\pm$ 8 (3)
PDE1	>10,000 (2)	>10,000 (2)	>10,000 (2)	>10,000 (2)
PDE2	>10,000 (2)	>10,000 (2)	>10,000 (2)	>10,000 (2)
PDE3	>10,000 (2)	>10,000 (2)	>10,000 (2)	>10,000 (2)
PDE5	>10,000 (2)	>10,000 (2)	>10,000 (2)	>10,000 (2)

TABLE 2

Effects of NVP-ABE171 and related compounds on functional responses of human inflammatory cells
Data are expressed as mean $\pm$ S.E.M. from the number of experiments indicated in parentheses.

	IC ₅₀		
	NVP-ABE171	Ariflo	V 11294A
	nM		
Eosinophil function			
Oxidative burst (IgG)	6.0 $\pm$ 0.2 (4)	104.7 $\pm$ 5.3 (3)	712 $\pm$ 19 (3)
T-Cell cytokines release			
INF- γ (anti-CD3)	10.0 $\pm$ 0.2 (5)	257.1 $\pm$ 7.6 (4)	1905 $\pm$ 63 (3)
IL-4 (anti-CD3)	16.6 $\pm$ 0.3 (5)	389.0 $\pm$ 8.5 (4)	2089 $\pm$ 51 (3)
IL-5 (anti-CD3)	28.2 $\pm$ 0.5 (5)	199.5 $\pm$ 9.2 (4)	2754 $\pm$ 94 (3)
Monocytes cytokine release			
TNF- α (INF- γ + LPS)	67.6 $\pm$ 1.0 (3)	3467 $\pm$ 64 (3)	4365 $\pm$ 49 (3)

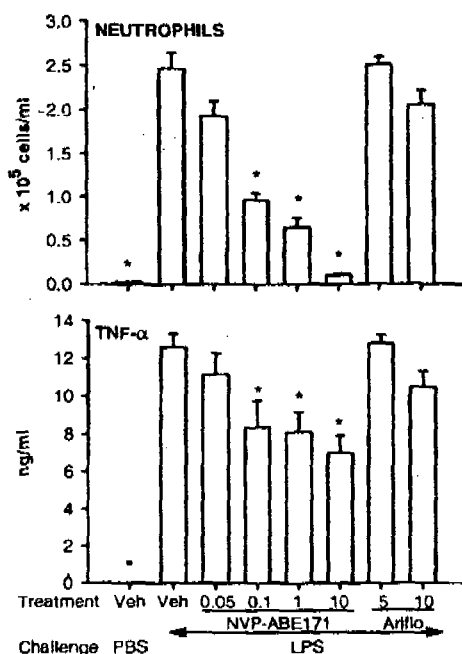


Fig. 2. Effect of NVP-ABE171 and Ariflo on LPS-induced airway inflammation 3 h after the intranasal challenge in mice. NVP-ABE171 (0.05–10 mg kg⁻¹) and Ariflo (5–10 mg kg⁻¹) were given orally 1 h before the LPS exposure. Three hours after the LPS challenge, animals were sacrificed and bronchoalveolar lavage was performed. Results are expressed as means $\pm$ S.E.M. of two different experiments, each including 6 to 10 animals/group. Statistical significance (*, $P < 0.05$) was assessed against the vehicle-treated (Veh) and LPS-challenged animals. Doses are expressed in milligrams per kilogram.

pranolol ($52.6 \pm 9.5 \times 10^{-5}$ cm/s, $n = 4$). As expected for a PDE4 inhibitor, NVP-ABE171 enhanced the accumulation of cAMP in human HaCat cells induced by isoprenaline with an EC₂₀₀ value of 3.5 ± 0.9 nM ($n = 3$). Because the other compounds are well characterized PDE4 inhibitors, they were not tested in these assays.

Inhibition of Cell Function in Vitro. Because of its very low potency in the enzyme-based assay, LAS 31025 was not studied further. In line with the enzyme inhibition data, V 11294A was a very weak inhibitor of the inflammatory cells' activation (Table 2).

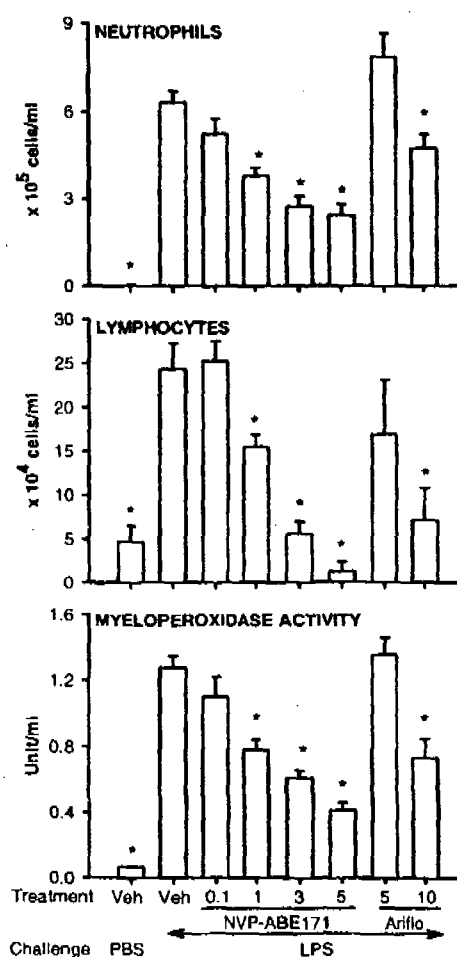


Fig. 3. Effect of NVP-ABE171 and Ariflo on LPS-induced airway inflammation 24 h after the intranasal challenge in mice. NVP-ABE171 (0.1–5 mg kg⁻¹) and Ariflo (5–10 mg kg⁻¹) were given orally 1 h before and 6 h after the LPS exposure. Twenty-four hours after the LPS challenge, animals were sacrificed and bronchoalveolar lavage was performed. Results are expressed as means $\pm$ S.E.M. of two to three different experiments, each including 6 to 10 animals/group. Statistical significance (*, $P < 0.05$) was assessed against the vehicle-treated (Veh) and LPS-challenged animals. Doses are expressed in milligrams per kilogram.

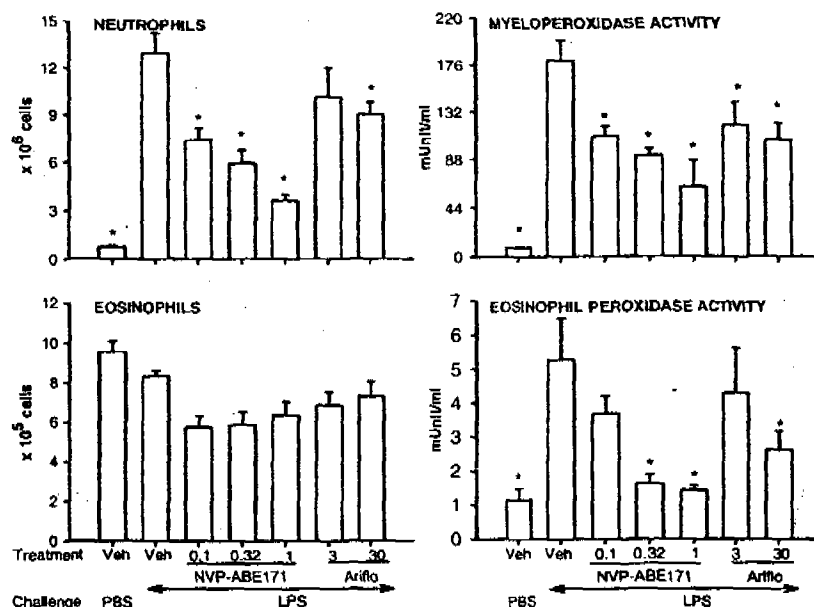


Fig. 4. Effect of NVP-ABE171 and Ariflo on LPS-induced airway inflammation 24 h after the intratracheal challenge in rats. NVP-ABE171 (0.1–1 mg kg⁻¹) and Ariflo (3 and 30 mg kg⁻¹) were given orally 1 h before the LPS exposure. Twenty-four hours after the LPS exposure, animals were sacrificed and bronchoalveolar lavage was performed. Results are expressed as means $\pm$ S.E.M. of two different experiments, each including 5 to 10 animals/group. Statistical significance (*, $P < 0.05$) was assessed against the vehicle-treated (Veh) and LPS-challenged animals. Doses are expressed in milligrams per kilogram.

NVP-ABE171 fully suppressed all the measured functional responses of human eosinophils, T cells, and monocytes with IC₅₀ values in the nanomolar range (Table 2). Ariflo also fully suppressed these responses, but showed lower potencies (7 to 50 times less potent) than NVP-ABE171 (Table 2). In contrast to the other cell types' functions, NVP-ABE171 and Ariflo inhibited the neutrophil oxidative burst to a maximum of about 50% under the conditions used. Therefore, for quantitative analysis IC₂₅ values were calculated (NVP-ABE171, 1.5 ± 0.09 nM; and Ariflo, 65.5 ± 5.4 nM, $n = 4$). Because V 11294A and LAS 31025 were very weak PDE4 inhibitors *in vitro*, we decided not to pursue their characterization *in vivo*.

Inhibition of LPS-Induced Lung Inflammation in Mice. At 3 h postprovocation, LPS induced a marked increase in neutrophil numbers and TNF- α levels in the bronchoalveolar lavage. No changes in the other cell types (macrophages, eosinophils, and lymphocytes) were observed (data not shown). Oral administration of NVP-ABE171, 1 h before the challenge, induced a dose-dependent inhibition of the neutrophil influx with an ED₅₀ value of 0.08 mg/kg (Fig. 2). The LPS-induced TNF- α release was only partially inhibited by NVP-ABE171 and no ED₅₀ could be calculated (Fig. 2). Ariflo at both 5 and 10 mg/kg was inactive on all the parameters studied (Fig. 2).

At 24 h postprovocation, LPS induced a marked increase in neutrophil and lymphocyte numbers. No changes in the other cell types (macrophages and eosinophils) were observed (data not shown). Oral administration of NVP-ABE171, 1 h before and 6 h after the challenge, induced a dose-dependent inhibition of these two parameters with an ED₅₀ value of 2.8 and 1.5 mg/kg for the neutrophil and lymphocyte influx, respectively (Fig. 3). In parallel with the number of neutrophils, the levels of myeloperoxidase activity as a measure of neutrophil degranulation were also dose dependently inhibited by NVP-ABE171 with an ED₅₀ value of 3.2 mg/kg (Fig. 3). Ariflo was

able to inhibit all the parameters studied only at 10 mg/kg (Fig. 3).

Inhibition of LPS-Induced Lung Inflammation in Brown Norway Rat. Oral administration of NVP-ABE171 given 1 h before LPS exposure dose dependently inhibited the infiltration and activation, as measured by myeloperoxidase activity levels, of neutrophils recovered in the bronchoalveolar lavage 24 h after LPS challenge. The ED₅₀ value for both parameters was 0.2 mg/kg (Fig. 4). In addition to its neutrophil infiltration-suppressing activity, the compound also reduced the activity of eosinophil peroxidase activity recovered in the bronchoalveolar lavage of LPS-challenged animals (ED₅₀ value of 0.13 mg/kg) without affecting the number of eosinophils (Fig. 4). Oral administration of Ariflo (3 and 30 mg kg⁻¹) given 1 h before LPS challenge inhibited the infiltration and activation of neutrophils recovered in bronchoalveolar lavage fluid 24 h after LPS challenge, displaying a flat dose relationship. Reductions in the activity of eosinophil peroxidase activity were also seen (Fig. 4).

Inhibition of Ovalbumin-Induced Lung Inflammation in Rat. Forty-eight hours after ovalbumin challenge, there was a pronounced infiltration of eosinophils into the airways of sensitized Brown Norway rats, accompanied by increases in bronchoalveolar lavage eosinophil peroxidase activity and proteins. Oral administration of NVP-ABE171, 1 h before and 24 h after antigen challenge, induced dose-dependent inhibition of the numbers of eosinophils, eosinophil peroxidase activity, and plasma-derived protein levels in bronchoalveolar lavage (Fig. 5). The ED₅₀ value for the changes in eosinophil numbers was 0.1 mg/kg. Similar ED₅₀ values were obtained for the inhibition of eosinophil peroxidase activity (0.1 mg/kg) and protein levels (0.2 mg/kg) in bronchoalveolar lavage.

NVP-ABE171 also reduced the numbers of neutrophils and lymphocytes in bronchoalveolar lavage after oral administra-

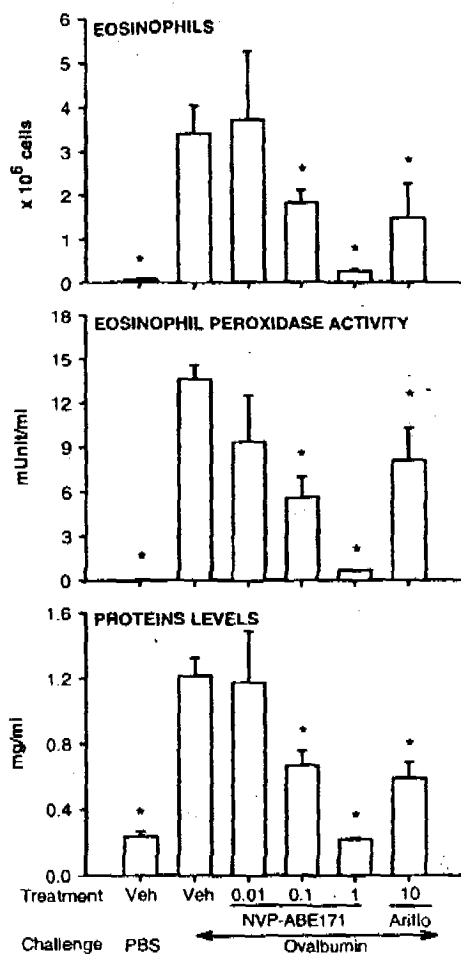


Fig. 5. Effect of NVP-ABE171 and Ariflo on the ovalbumin-induced airway inflammation 48 h after the aerosol challenge in rats. NVP-ABE171 (0.01 – 1 mg kg^{-1}) and Ariflo (10 mg kg^{-1}) were given orally 1 h before and 24 h after the ovalbumin exposure. Forty-eight hours after the ovalbumin exposure, animals were sacrificed and bronchoalveolar lavage was performed. Results are expressed as means $\pm$ S.E.M. of two different experiments, each including 5 to 10 animals/group. Statistical significance (* , $P < 0.05$) was assessed against the vehicle-treated (Veh) and ovalbumin-challenged animals. Doses are expressed in milligrams per kilogram.

tion 1 h before and 24 h after allergen challenge. Again, the ED_{50} values calculated from the dose-response curves were similar to those required to inhibit eosinophil infiltration (data not shown).

Ariflo, at 10 mg/kg, was substantially less active in this model than NVP-ABE171, although the effects were qualitatively similar (Fig. 5). Thus, by using eosinophil numbers in bronchoalveolar lavage as the readout, the reduction seen with Ariflo after oral administration of 10 mg/kg 1 h before and 24 h after allergen challenge was 44%.

Duration of Action of NVP-ABE171 in Ovalbumin-Induced Lung Inflammation in Rat. The ovalbumin-induced increase in eosinophil numbers, eosinophil peroxidase

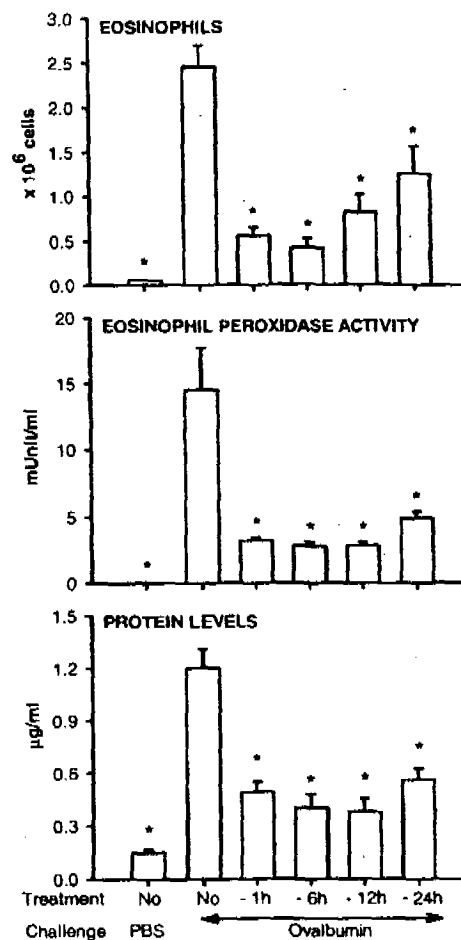


Fig. 6. Duration of action of NVP-ABE171 on the ovalbumin-induced airway inflammation in rats. NVP-ABE171 (0.4 mg kg^{-1}) was given orally from 1 to 24 h before the ovalbumin challenge. Twenty-four hours after the ovalbumin exposure, animals were sacrificed and bronchoalveolar lavage was performed. Results are expressed as means $\pm$ S.E.M. of one experiment including 6 to 10 animals/group. Statistical significance (* , $P < 0.05$) was assessed against the untreated (No) and ovalbumin-challenged animals.

activity, and protein levels recovered 24 h after the allergen challenge was significantly inhibited by 0.4 mg/kg NVP-ABE171 administered 1, 6, 12, and 24 h before the challenge (Fig. 6). A similar duration of action on the reduction in total cells, neutrophils, and lymphocytes was also evident (Table 3). Ariflo (30 mg/kg) was inactive when given 6 h before the challenge (data not shown).

Discussion

Chronic obstructive pulmonary disease and asthma are two inflammatory disorders of the airways. Chronic obstructive pulmonary disease is characterized by airway obstruction that is slowly progressive and irreversible. This disease encompasses two related conditions: chronic bronchitis and emphysema. Chronic bronchitis is characterized by mucus

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hypersecretion and increased risk of chest infections. Approximately 15% of smokers develop progressive airflow limitation that is largely due to enzymatic destruction of elastin fibers in the lung parenchyma (emphysema). Both conditions have an inflammatory component that is characterized by infiltration of neutrophils and macrophages into the lungs (Jeffery, 1998). Bronchial asthma is a chronic inflammatory disease of the airways, a prominent feature of which is an intense infiltration of eosinophils and mononuclear cells (Djukanovic et al., 1990).

PDE4 inhibitors have been shown to be potent anti-inflammatory agents in various animal models (Teixeira et al., 1997) and also have shown clinical efficacy in both asthma and chronic obstructive lung diseases (Torphy et al., 1999). Analysis of a number of human cell lines or defined blood cell populations revealed clear differences in the PDE4 isotype expression pattern in the immune system (Muller et al., 1996). As such, PDE4C could not be detected in any of the immune cells, whereas PDE4A and 4B expression were seen in the majority of the immune cells. PDE4D was mainly expressed in eosinophils and to a lesser extent in neutrophils. Based on all this information, we designed PDE4 inhibitors displaying different selectivity profiles against the PDE4 isozymes (Hersperger et al., 2000) and as a result of this strategy, NVP-ABE171, a selective inhibitor for PDE4B and 4D, was selected for further profiling.

Because PDE4 inhibitors have the potential to be efficacious in both asthma and chronic obstructive pulmonary disease, we next tested the efficacy of NVP-ABE171 on different human leukocytes known to be involved in these two diseases. As such, the asthmatic airway inflammation is driven by T cells and eosinophils are believed to be one of the terminal effector cells in this disease (Djukanovic et al., 1990). On the other hand, in patients with chronic obstructive pulmonary disease the inflammatory cells involved are neutrophils and macrophages (Jeffery, 1998). In agreement with the enzyme-based data, Ariflo was about 10 to 50 times less potent than NVP-ABE171, depending on the cell types used. Compared with the other leukocytes investigated, monocytes and neutrophils seem to be more resistant to inhibition by both compounds. Indeed, for both compounds, the potency on monocytes was lower compared with eosinophils or T cells and the neutrophil oxidative burst could be inhibited to a maximum of 50%. This has already been reported for other PDE4 inhibitors (Hatzelmann and Schudt, 2001) and one can argue that it may be a therapeutic advantage, because both macrophages and neutrophils are important immune cells in the host defense process.

We next investigated the *in vivo* efficacy of NVP-ABE171

and Ariflo in animal models of lung inflammation by using both mice and rats. Because V 11294A and LAS 31025 were weak PDE4 inhibitors *in vitro* we decided not to characterize them *in vivo*. Because drugs that are aimed at treating chronic inflammation in humans should be administered daily, orally active compounds are highly desirable as therapeutics. We therefore tested the effect of NVP-ABE171 by the oral route. Because chronic obstructive pulmonary disease is characterized by an increase in the activation and/or numbers of neutrophils and macrophages, it was important to test NVP-ABE171 in a macrophage-dependent *in vivo* model. To do so, we used the LPS-induced lung inflammation model that has been shown to be dependent upon macrophage activation, and more importantly, to be inhibited by rolipram, the archetype of PDE4 inhibitors (Goncalves de Moraes et al., 1998). In both mice and rats, NVP-ABE171 potently inhibited the neutrophil influx and their activation observed 24 h after the LPS challenge. Ariflo also inhibits these parameters, however, with a much lower potency. Because it has been shown that treatment with cAMP-elevating agents inhibits the TNF- α production in a murine LPS model (Goncalves de Moraes et al., 1996), it was somewhat surprising to see that NVP-ABE171 was not very potent at inhibiting the LPS-induced TNF- α release in the mice. This observation is in line with the results obtained in our cell-based assay showing that from all the inflammatory cells tested, monocytes were the least responsive to inhibition by NVP-ABE171. Furthermore, it has been shown *in vitro* that to efficiently inhibit the LPS-induced TNF- α release from macrophages, an inhibition of PDE4 and PDE3 was necessary (Gantner et al., 1997). Nevertheless, we clearly showed that NVP-ABE171, in two different species (mice and rats) is a powerful inhibitor of both the migration of neutrophil into the airways and their activation. It is interesting to note that NVP-ABE171 was effective in suppressing the neutrophilia at both 3 and 24 h postchallenge, whereas Ariflo inhibited the neutrophil influx only at the late time point. We do not have any explanation for this phenomenon. However, in line with our results, Ariflo has been reported to be less potent in inhibiting the early phase (2 h) of the LPS-induced airway inflammation in rat compared with the latest phase (24 h) (Spond et al., 2001).

Ovalbumin inhalation to actively sensitized rats is a well characterized and recognized model for asthma (Renzi et al., 1993). This acute inflammatory model is characterized by an influx of leukocytes and plasma accumulation into the airways, two conditions seen in human asthma (Djukanovic et al., 1990). NVP-ABE171 dose dependently and potently inhibited the allergen-induced increase in airway plasma pro-

TABLE 3

Duration of action of NVP-ABE171 (0.4 mg kg⁻¹) on ovalbumin-induced increase in total cell, lymphocyte, and neutrophil in rat airways. Results are expressed as means $\pm$ S.E.M.

Treatment/Challenge	n	Cell Counts (10 ⁶)		
		Total Cells	Lymphocytes	Neutrophils
Non-treated/PBS	5	4.80 $\pm$ 0.38*	0.27 $\pm$ 0.02*	0.14 $\pm$ 0.02*
Non-treated/ovalbumin	10	10.60 $\pm$ 1.92	1.95 $\pm$ 0.38	1.52 $\pm$ 0.29
-1 h/ovalbumin	10	4.66 $\pm$ 0.45*	0.54 $\pm$ 0.08*	0.38 $\pm$ 0.07*
-6 h/ovalbumin	10	4.40 $\pm$ 0.33*	0.52 $\pm$ 0.07*	0.38 $\pm$ 0.06*
-12 h/ovalbumin	10	5.40 $\pm$ 0.66*	0.78 $\pm$ 0.13*	0.69 $\pm$ 0.15*
-24 h/ovalbumin	10	6.54 $\pm$ 0.91*	1.14 $\pm$ 0.17*	0.77 $\pm$ 0.19*

Statistical significance (* $P < 0.05$) was assessed against the vehicle-treated and ovalbumin-challenged animals.

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tein accumulation. The inhibitory mechanism of action of such compounds on plasma exudation is still unclear; however, other PDE4 inhibitors have been reported to inhibit plasma leakage *in vivo* through the inhibition of the endothelial cell contraction (Ortiz et al., 1996). NVP-ABE171 was also very potent at inhibiting the influx of inflammatory leukocytes and their activation. Because PDE4 inhibitors are known to have a powerful modulating activity on virtually all cells involved in the inflammatory process (Teixeira et al., 1997), it is quite difficult to dissect out their precise anti-inflammatory mechanism of action in this model. However, the main effect of NVP-ABE171 in this model is rather likely to be due to the inhibition of T-cell activation, which are the cells known to drive the inflammatory process in this model (Underwood et al., 1997). In addition to its greater potency compared with Ariflo, NVP-ABE171 also has a much longer duration of action. As such, in the Brown Norway rat, the inhibitory effect of Ariflo was lost when given 6 h before the allergen challenge (data not shown), whereas NVP-ABE171 had a duration of action of more than 24 h. Because Ariflo is administered twice a day in the clinic, these data suggest that NVP-ABE171 could be a once-a-day drug.

During the preparation of this manuscript, the preclinical data for roflumilast (3-cyclopropylmethoxy-4-difluoromethoxy-*N*-[3,5-dichloropyrid-4-yl]-benzamide), a PDE4 inhibitor in clinical development administered once a day, were published (Bundschuh et al., 2001; Hatzelmann and Schudt, 2001). Compared with Ariflo, this compound was reported to be 50 to 100 times more potent in *in vitro* assays (Hatzelmann and Schudt, 2001) and about 50 times more potent in inhibiting the ovalbumin-induced lung inflammation in the Brown Norway rat (Bundschuh et al., 2001). In this model, its duration of action was reported to be more than 18 h (Bundschuh et al., 2001). YM976 is another PDE4 inhibitor in clinical development and a comprehensive package of preclinical data has been published for this compound (Aoki et al., 2000a,b, 2001). Compared with Ariflo, YM976 has been shown to be about 50 times more potent *in vitro*. However, when tested *in vivo*, YM976 was only 10 times more potent than Ariflo (Aoki et al., 2000). Although we did not compare NVP-ABE171 with roflumilast and YM976 in the present study, based on the published data, NVP-ABE171 seems to be 10 times more potent than YM976 *in vivo* and at least as potent as roflumilast with a similar duration of action *in vivo*.

In summary, the preclinical data presented in this report indicate that NVP-ABE171 is a potent orally active PDE4D inhibitor with a long duration of action *in vivo*. Thus, PDE4 inhibitors from the 6,8-disubstituted 1,7-naphthyridine class may have potential for the treatment of inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease.

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