

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
RAD: 11-164618- -6	FECHA: 2013-10-25 17:23:37
DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 4
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
ALICIA LLOREDA RICAURTE

Asunto: Radicación: 11-164618- -6
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 20422
 Folios: 4

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2010/034600				
Fecha de Presentación Internacional	12/05/2010				

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:	Radicado 11 164618 00000	30/Noviembre/2011
Reivindicaciones:	Radicado 11 164618 00000	30/Noviembre/2011
	Radicado 11 164618 00003	30/Noviembre/2011
Dibujos:	Radicado 11 164618 00000	30/Noviembre/2011
Prioridad:	US 61/177,972	13/Mayo/2009
	US 61/224,745	10/Julio/2009
	US 61/238,760	01/Septiembre/2009

2. Análisis de la Prioridad

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

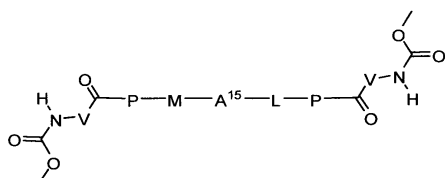
3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. Con relación a la presente solicitud se realizó el pago de las reivindicaciones adicionales y se estudiarán las reivindicaciones 1 a 25 de acuerdo al Radicado 11 164618 00003.

Título de la solicitud: “Compuestos Antivirales”

El problema planteado en esta solicitud consiste en la necesidad de proporcionar compuestos para ser empleados en el tratamiento del VHC en mamíferos.

Para resolver este problema técnico la solicitud en estudio proporciona un compuesto de la fórmula P-M-A-L-P y composiciones que lo contienen.



El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07D 401/14.

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 17 y 23 a 25 no es patentable, de acuerdo con el Art 14. Aunque las reivindicaciones 17, 23 y 25 se encabecen como un compuesto o composición reclaman un uso.

5. De la solicitud de patente

Título

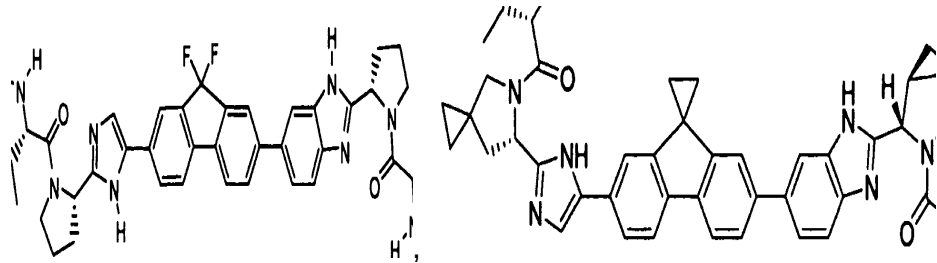
El título planteado en la presente solicitud es general y no define el objeto reclamado. Se sugiere concretar el título teniendo en cuenta las características estructurales de los compuestos de la solicitud.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 a 14 no son concisas porque las definiciones de V, P, M, A¹⁵, L y P están en términos géneros funcionales, se sugiere restringir la solicitud a una razonable generalización del tipo de sustituyentes que han sido probados, es decir, que se encuentran debidamente sustentados de acuerdo a lo presentado en la tabla 1. Es necesario indicar también el número de átomos de cada sustituyente. La generalización

en la definición de los diferentes sustituyentes permitiría incluir combinaciones que no se encuentran sustentadas.

Tabla 1. Ejemplos sustentados de los sustituyentes

Sustituyentes	Ejemplos
V	Alquilo C3-C4
P-M-A ¹⁵ -L-P	

Las reivindicaciones 16 y 18-22 reclaman una composición y dentro del capítulo descriptivo no se definen ejemplos concretos que sustenten dicha pretensión. Se sugiere eliminar las reivindicaciones en referencia.

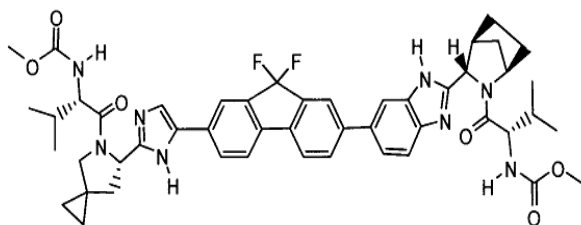
Las reivindicaciones 7 a 10 y 15 reclaman un profármaco. Dicha característica no está sustentada en el capítulo descriptivo, por lo cual se sugiere eliminarla.

Las reivindicaciones 19 a 22 no son claras porque los ingredientes de una composición no pueden ser definidos por su actividad farmacológica y que no existe evidencia de su obtención ni de ningún efecto generado por la misma, tal como sinergia, disminución de dosis, entre otras. Además, las mismas hacen referencia a un método de tratamiento. Se sugiere eliminarlas.

Se requiere al solicitante hacer las aclaraciones necesarias.

6. Unidad de Invención.

La solicitud no cumple con el requisito de unidad de invención porque la fórmula Markush reclamada presenta hipervariabilidad, ya que se ajusta a una fórmula A-B-C-D, lo que no permite definir un concepto común inventivo. De acuerdo, a la materia presentada en el capítulo reivindicatorio se observa una estructura básica, como se indica a continuación:



Se sugiere presentar las solicitudes divisionales que considere necesarias, o 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 presentación de la solicitud de la cual se invoca prioridad, es decir, mayo 13 de 2009. Consultadas las fuentes de información con que se cuenta, se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	WO2008021927	"Hepatitis C Virus Inhibitors"	21/Febrero/2008
D2	WO2009020828	"Crystalline form of methyl ((1S)-1-(((2S)-2-(5-(4'-(2-((2S)-2-(methoxycarbonyl)amino)-3-methylbutanoyl)-2-pyrrolidinyl)-1H-imidazol-5-yl)-4-biphenyl)-1H-imidazol-2-yl)-1-pyrrolidinyl)carbonyl)-2-methylpropyl)varbamate dihydrochloride salt	12/Febrero/2009

El documento D1⁽¹⁾ divulga compuestos, composiciones y métodos de tratamiento para infecciones del virus de la hepatitis C (HCV). (Resumen).

El documento D2⁽²⁾ divulga un compuesto y composiciones farmacéuticas para tratar infecciones del virus de la hepatitis C (HCV). (Resumen).

8. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

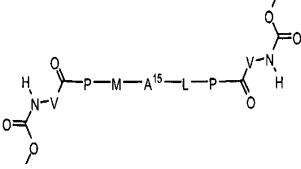
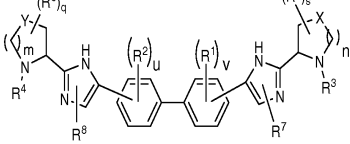
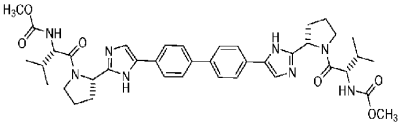
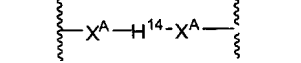
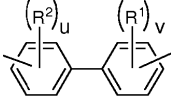
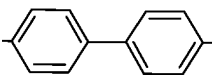
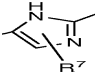
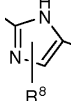
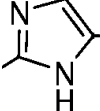
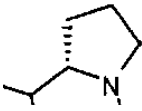
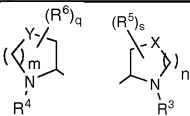
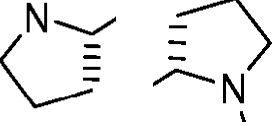
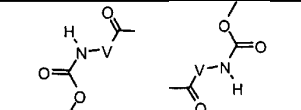
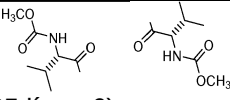
La reivindicación 1 consiste en: "Un compuesto de fórmula (I) donde V es alquilo, L es

1 http://worldwide.espacenet.com/publicationDetails/originalDocument;jsessionid=C044A6D5742676A9BE8E65E75F7BDAB8.espacenet_levelx_prod_3?FT=D&date=20080221&DB=&&CC=WO&NR=2008021927A2&KC=A2&ND=1&locale=en_EP

2. <https://www.google.com/search?tbm=pts&hl=es&q=WO2009020828>

benciimidazolilo, M un anillo de 5 miembros (...)"

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Compuestos de fórmula: 	Compuestos de fórmula I:  (Pág. 2 líneas 25 a 29)	Compuestos de forma N-2:  (Pág. 35 línea 6)
A15 es:  X ^A : ausente H14: carbociclo triciclo fusionado	 Siendo u y v: cero (Pág. 3 línea 2)	 (Pág. 35 línea 6)
L: bencimidazolilo	 R7: hidrógeno (Pág. 2 líneas 25 a 29 y Pág. 3 líneas 16 a 29)	 (Pág. 35 línea 6)
M: anillo heterociclo de cinco miembros	 R8: hidrógeno (Pág. 2 líneas 25 a 29 y Pág. 3 línea 16)	 (Pág. 35 línea 6)
P: 	 q y s: cero m y n: uno (Pág. 2 líneas 25 a 29 y Pág. 3 líneas 10 y 19 a 24)	 (Pág. 35 línea 6)
V: alquilo 	R3y R4: R9-C(O)-, y R9 es NR ^c R ^d carbonilo (Pág. 2 líneas 25 a 29 y Pág. 3 líneas 10 y 19 a 24)	 (Pág. 35 línea 6)

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque en el caso del documento D1 divulga compuestos, composiciones y métodos de tratamiento para infecciones del virus de la hepatitis C (HCV). (Resumen). Por su parte, el documento D2⁽²⁾ divulga un compuesto y composiciones farmacéuticas para tratar infecciones del virus de la hepatitis C (HCV). (Resumen).

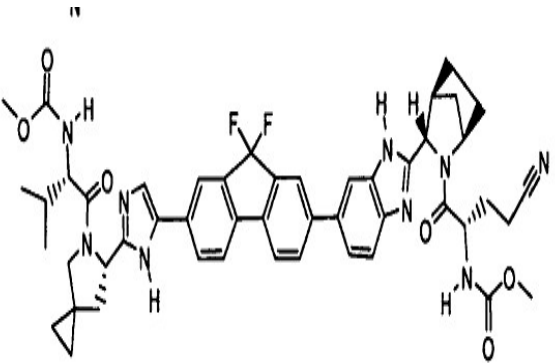
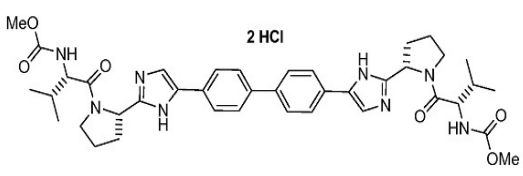
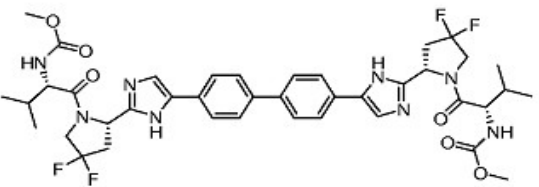
La diferencia entre la invención, definida en la reivindicación 1 y compuesto de fórmula I divulgado en el documento D1 consiste en que el compuesto reclamado incluye en la parte central de sus estructura un triciclo fusionado y un benzoimidazolilo mientras que

la estructura del compuesto divulgado en el documento D1 carece de un triciclo fusionado y un benzoimidazolilo.

El efecto que logra incluir en la parte central de la estructura del compuesto de fórmula (I) un triciclo y un benzoimidazolilo consiste en que se aumenta la eficiencia biológica del compuesto.

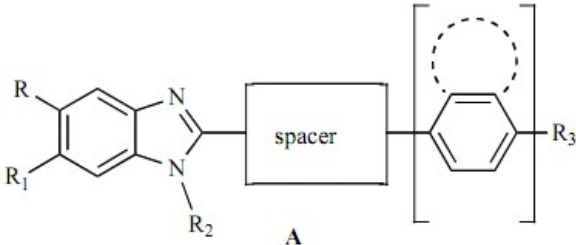
Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: Cómo modificar la estructura del compuesto de fórmula general I para aumentar su eficiencia biológica.

A continuación se muestra una comparación entre la estructura de dos compuestos divulgados en el documento D1 y un compuesto reclamado por la presente solicitud junto con su actividad inhibitoria EC50

Solicitud (Pág. 947)	D1
 Actividad EC50 0,006 nM	Example 24-23  Actividad EC50: 10-999 pM (Pág. 156 y 629)  OL-7 Actividad EC50: 10-999 pM (Pág. 503 y 632)

A pesar que, el documento D1 no incluya en su estructura un triciclo y un benzoimidazolilo la actividad inhibitoria HCV EC50 correspondiente a los compuestos divulgados en éste documento se encuentran comprendidos entre un rango de 1nM a 999 pM (Pág. 627, líneas 19 a 22). Por su parte, la presente solicitud define que la actividad antiviral EC50 para sus compuestos está comprendida en un rango 0,0023 nM a >44,44 nM (Pág. 1197 a 1267). Vitale et. Al., en el 2008⁽³⁾ divulga compuestos con actividad antiviral sobre virus de la familia flaviviridae, en donde dichos compuestos incluyen un espacio el cual puede ser sustituido por difenilo, un derivado de estirilo, un grupo naftilo, anillos de 5 a 6 átomos y fenilbenziimidazoles (Pág. 607).

3. Medicinal Chemistry, 2-Arylbenzimidazoles as antiviral and antiproliferative agents Part-1, Villate, G, et al., 2008(4) Pag. 605-615.



Por lo anterior, la utilización de puentes estructurales en compuestos antivirales sólo constituye una estrategia en el desarrollo de compuestos y los mismos no definen por sí mismos la actividad inhibitoria sobre el virus de la hepatitis C y mediante diversas combinaciones de sustituyentes en el puente se alcanzan resultados comparables de inhibición. Es así que, la presente solicitud no define resultados sorprendentes frente al estado de la técnica en relación a la inclusión de un benzoimidazolilo y un triciclo.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el el benzoimidazolilo y el triciclo en la estructura divulgada en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 2-16 y 18 a 22 no mencionan características técnicas esenciales sobre el compuesto de fórmula I, tampoco se pueden considerar inventivas.

9. Conclusión

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

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO2008021927	1-16 y 18 a 22	Nivel inventivo
D2	WO2009020828	1-16 y 18 a 22	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 del día siguiente 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.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

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 2013-10-29

Elaboró: German Antonio Clavijo Cohen
Revisó: Jeny Paola Villate Becerra

2-Arylbenzimidazoles as Antiviral and Antiproliferative Agents-Part 1

G. Vitale^a, A. Carta^a, M. Loriga^a, G. Paglietti^{a,*}, P. La Colla^{b,*}, B. Busonera^b, D. Collu^b and R. Loddo^b

^aDipartimento Farmaco Chimico Tossicologico, Università di Sassari, Via Muroni, 23-07100 Sassari (Italy); ^bDipartimento di Scienze e Tecnologie Biomediche, Sezione di Microbiologia e Virologia Generale e Biotecnologie Microbiche, Università di Cagliari; Cittadella Universitaria, 09042 Monserrato (CA), Italy

Abstract: Being involved in an anti-Flaviviridae Project, and because of the role played by benzimidazole derivatives as promising inhibitors of the HCV helicase and RNA polymerase, as well as of the Zn finger transcription factor, we synthesized a new series of 2-arylbenzimidazoles and evaluated them for antiviral activity, as well as for antiproliferative activity.

Compounds were tested in cell-based assays against viruses representative of: i) two of the three genera of the *Flaviviridae* family, i.e. *Flaviviruses* and *Pestiviruses*; ii) other RNA virus families, such as *Retroviridae*, *Picornaviridae*, *Paramyxoviridae*, *Rhabdoviridae* and *Reoviridae*; iii) two DNA virus families (*Herpesviridae* and *Poxviridae*). Compounds **15**, **28** and **29** resulted moderately active only against Yellow Fever Virus (a *Flavivirus*) (range 6-27 μ M), whereas none of the title benzimidazoles showed any antiviral activity at concentrations not cytotoxic for the resting cell monolayers.

Compounds were also tested for antiproliferative activity against a panel of exponentially growing cell lines derived from human haematological and solid tumors. Several new benzimidazoles turned out active. Among them, compound **27** was the most potent against human haematologic and solid tumor cells and turned out to be as potent as Etoposide and more potent than 6-mercaptopurine (6-MP), used as reference antitumor agents.

Key Words: Antiviral activity, flaviviridae, antiproliferative activity, arylbenzimidazoles.

INTRODUCTION

Benzimidazoles represent a class of heterocycles which are receiving much interest in the field of anti-infective therapies. Up to 1980 the most extensively marketed benzimidazoles spanned from pharmaceuticals (mainly human and veterinary antihelmintic, a few antineoplastic, antihistaminic, vasodilator and psychoactive agents), insecticides to fungicides [1]. After 1980 benzimidazole derivatives have emerged as a new class of commercially successful anti-ulcer agents. Among these, Omeprazole (AstraZeneca) is still on the top ten worldwide marketed drugs. A special attention was dedicated to benzimidazole derivatives as promising antiviral agents [2].

Benzimidazole (**1**) (Fig. 1), in fact, may be considered a 1,3-dideazapurine and may replace this base in the process involving formation of nucleosides in viral replication.

Various benzene ring substituted benzimidazoles, bearing a carbocyclic ring at position 1 instead of a normal sugar, have been considered as nucleoside analogues of purines. For example, compounds of structure **2** are reported to be active against hepatitis B virus at 0.74 μ M [3], while compound **3** exhibited an *in vitro* inhibiting activity against both DNA and RNA virus [2].

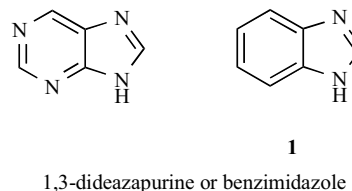


Fig. (1). Structure of purine and benzimidazole (**1**).

Successively, compounds **4** and **5** revealed to be 100-fold more potent [4] against the same strain of virus (Fig. 2).

However, emerging strategies using benzimidazole derivatives as antiviral agents broke through after the discovery of hepatitis C virus (HCV) and identification of its genome that took advantage of enormous efforts spent in both disclosing and fighting the HIV replication phases. Since chronic infections with hepatitis C virus is a serious worldwide public health problem, the demand for effective HCV antiviral therapeutics is at present very high. Thus, a recent review has defined the status of the research in this field, where benzimidazole derivatives play an important role as promising anti-HCV agents [5]. Viropharma patented a series of compounds of formula **6-9** as inhibitors of NS3 helicase, with IC₅₀ ranging from 0.7-10 μ M [6] (Fig. 3).

Two similar series of benzimidazole derivatives were patented independently by Japan Tobacco, Bristol-Meyers-Squibb and among these compounds **10** [7] and **11** [8] were reported as strong inhibitors of NS5B RNA polymerase. More recently, Japanese Authors described compound **12** [9]

*Address correspondence to these authors at the Dipartimento Farmaco Chimico Tossicologico, via Muroni 23/a, 07100 Sassari, Italy; Tel: +39-079228704; Fax: +39-079-228720; E-mail: paglietti@uniss.it

Dipartimento di Scienze e Tecnologie Biomediche, Sezione di Microbiologia e Virologia Generale e Biotecnologie Microbiche, Università degli Studi di Cagliari, Cittadella Universitaria, 09042 Monserrato (Cagliari), Italy; E-mail: placolla@unica.it

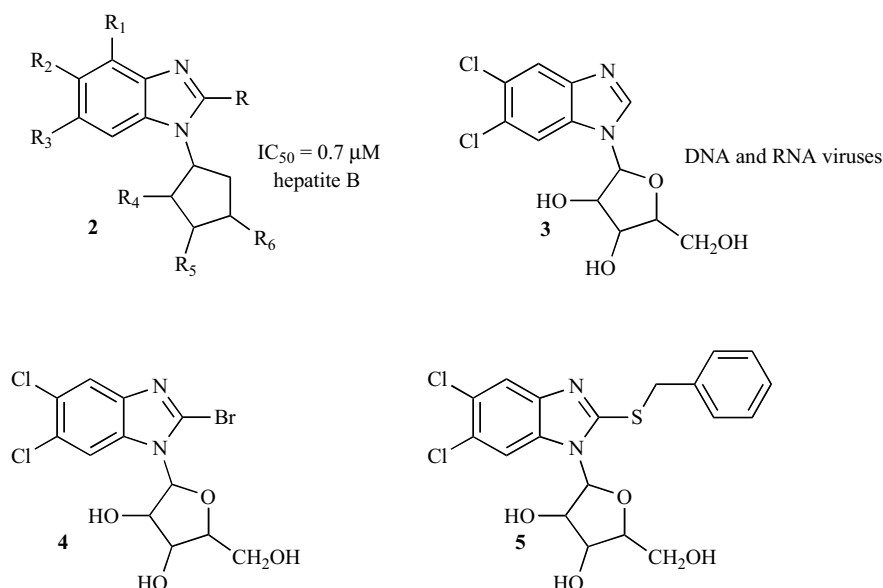


Fig. (2). Benzimidazoles active as antiviral agents.

as an improvement of this type of NS5B RNA polymerase inhibitors (Fig. 4).

Our interest in the description of the biological and pharmacological properties of benzimidazoles started long time ago, when we reported analgesic-ant inflammatory [10, 11] and choleric [12,13] activities, as well as a chlorpromazine-like activity in the conditioned avoidance response

[14,15]. In the last five years several teams coordinated by one of us (P. La Colla) have been involved in a national MIUR program aimed at the development of novel anti-HCV agents. Within this project, two groups belonging to different Universities (Genova and Sassari, Italy) jointly afforded a program for the preparation of HCV non-nucleoside inhibitors based on the benzimidazole structure. Thus, taking into account the recent disclosure of their activity as inhibitors of

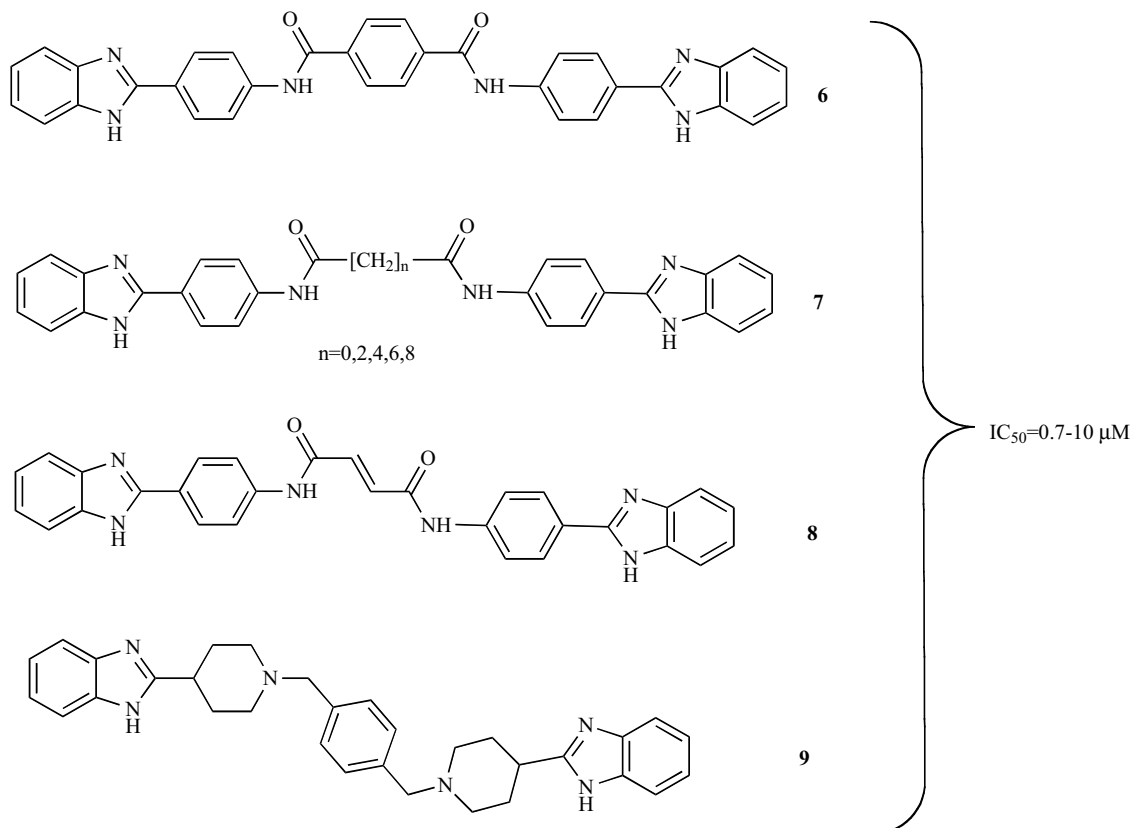


Fig. (3). Benzimidazoles patented as inhibitors of NS3 helicase.

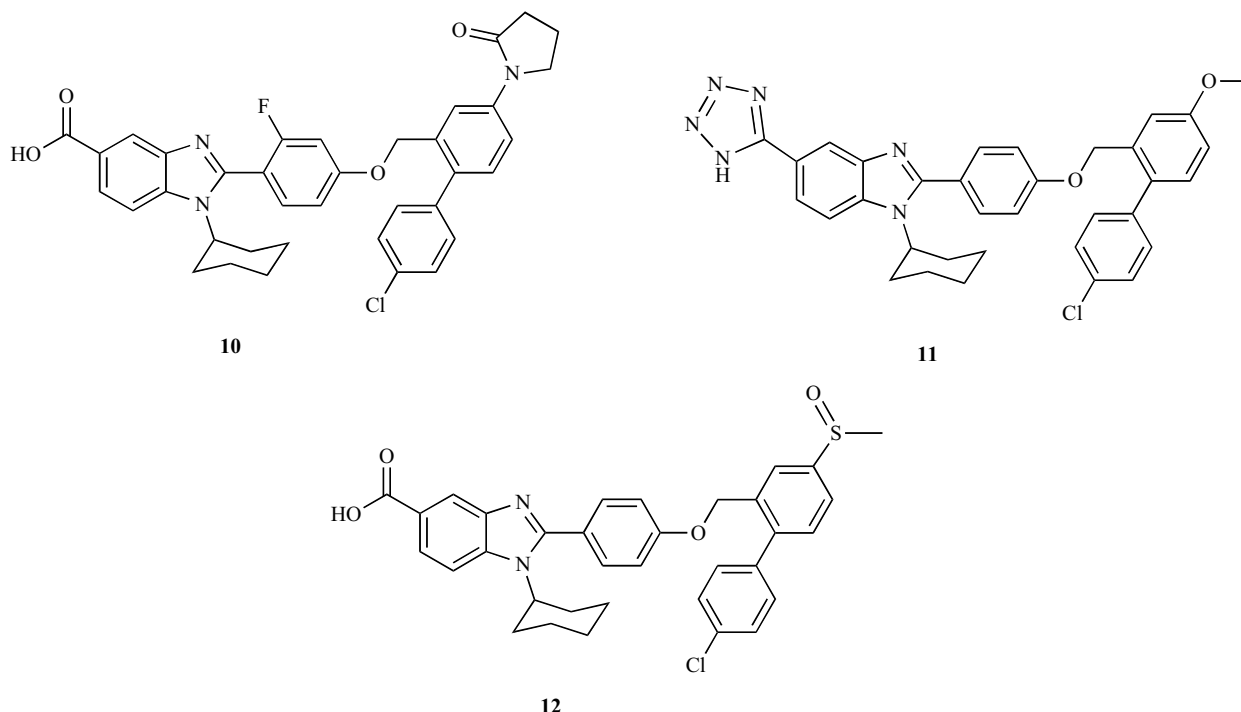


Fig. (4). Benzimidazoles patented as NS5B RNA polymerase inhibitors.

the HCV helicase and RNA polymerase [6-9], our group considered that part of this program could be oriented towards a further development of the benzimidazoles chemistry.

In this context, two convergent strategies were developed. On the one hand, we synthesized compounds of structure A (Fig. 5), derived from a series previously described by Sparatore *et al.* [10,11,14,15] of which we designed homologues of 2-phenylbenzimidazoles by inserting, at position 2, "spacers" such as a 4'-substituted-diphenyle, a -styryl substituent or, simply, an α -naphthyl group. On the other hand, we extended the series of 5(6)-substituted-(1*H*) or 1-alkyl-2-benzyl and/or 2-phenylbenzimidazoles developed at the Genova University by Boido *et al.* [16], by synthesizing the compounds of structure B (Fig. 5).

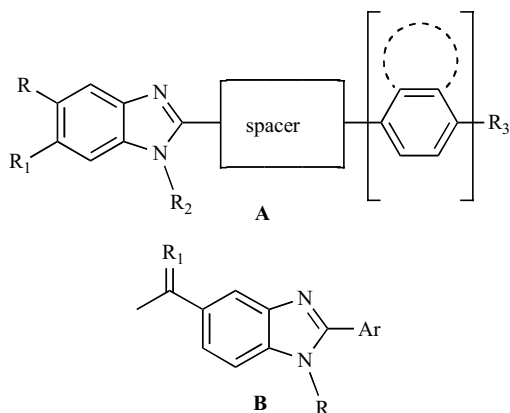


Fig. (5). General Structure of the designed drugs.

In this paper we report the preparation and the biological activity of compounds 13-36 of Fig. (6), as part of the above

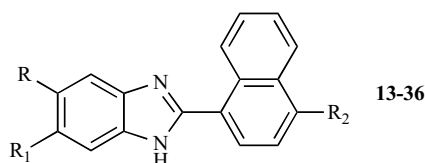
described program, in order to select novel potential leads against the cited targets and discover a possible antiproliferative activity. The naphthylbenzimidazoles are new compounds and represent the simplest case of homologation of 2-phenylbenzimidazoles. The effect of a bulkier substituent at position 2 of heteroring has not been so far considered in this class of compounds and the extra phenyl ring might result oriented preferentially towards or against the most prototropic stable imidazole form. Conformational analysis of compound 13, using montecarlo method, confirms that three minima were obtained and the two rings are not coplanar. At the lowest energies the naphthyl group is almost planar as shown in Fig. (7).

It is worth noting that compound 13 has been recently described as an example of a small-molecule switch for zinc finger transcription factor and its inhibitory activity has been reported [17]. Because of the above indication, we deemed important to check whether any antiproliferative activity might be shown by derivatives with this particular structure.

CHEMISTRY

The preparation of 2-[naphthalen-1-yl]-1*H*-benzimidazoles (13-36) has been accomplished according to Scheme (1).

The diamines a-f were condensed with bisulphite compounds g and h according to the procedure described by Ridley *et al.* [18] to give the desired derivatives 13-24 in good yield (Table 1). The nitro derivatives 19-24 were in turn converted into amines by reduction with hydrazine in the presence of palladium as catalyst. The primary amines 25-30 were then acetylated with acetic anhydride. The yields recorded in the last steps were from very high to moderate. Elucidation of the structures of prepared compounds were achieved by the whole of the elemental analyses and spectro-



Compd.	R	R ₁	R ₂
13	H	H	H
14	CH ₃	H	H
15	CH ₃	CH ₃	H
16	Cl	H	H
17	Cl	Cl	H
18	CF ₃	H	H
19	H	H	NO ₂
20	CH ₃	H	NO ₂
21	CH ₃	CH ₃	NO ₂
22	Cl	H	NO ₂
23	Cl	Cl	NO ₂
24	CF ₃	H	NO ₂
25	H	H	NH ₂
26	CH ₃	H	NH ₂
27	CH ₃	CH ₃	NH ₂
28	Cl	H	NH ₂
29	Cl	Cl	NH ₂
30	CF ₃	H	NH ₂
31	H	H	NHCOCH ₃
32	CH ₃	H	NHCOCH ₃
33	CH ₃	CH ₃	NHCOCH ₃
34	Cl	H	NHCOCH ₃
35	Cl	Cl	NHCOCH ₃
36	CF ₃	H	NHCOCH ₃

Fig. (6). General formula of the compounds synthesized.

scopic data (Table 1). From the spectroscopic point of view it is interesting to note the differences existing in the UV spectra between the analogues 2-phenyl-1*H*-benzimidazoles described by some of us [13] and the 2-naphtyl derivatives of Table (1). 2-Phenylbenzimidazoles possess fine structure whereas the presence of a naphtyl ring at the same position causes the appearance of a broad intense band in the range of 309-289 nm for **13-18** and 327-306 nm for **30-36**. In the case of 2-[4-nitronaphtalen-1-yl]-1*H*-benzimidazoles (**19-24**) the maxima descend to a flat band at higher wavelength 358-323 nm because of conjugation with the nitro group. Compounds (**25-28**) exhibited three set of maxima around 332-325, 284-279 and 234-232 nm, while in compounds **29** and **30** the

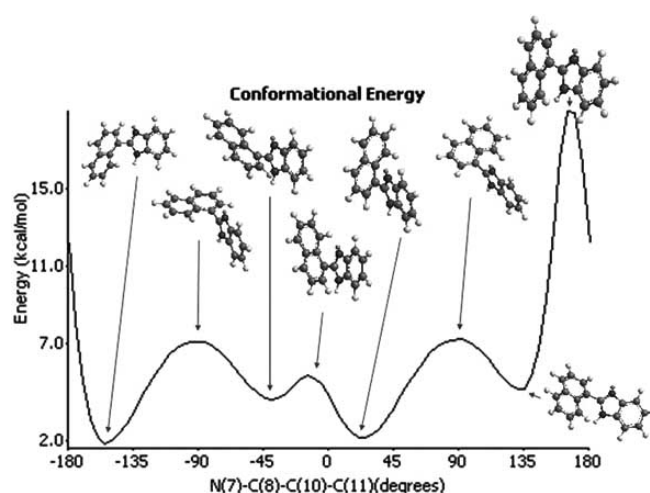


Fig. (7). Energy minimization profile of compound 13.

band around 280 nm decreases and the most intense band is bathochromically shifted at 342 nm.

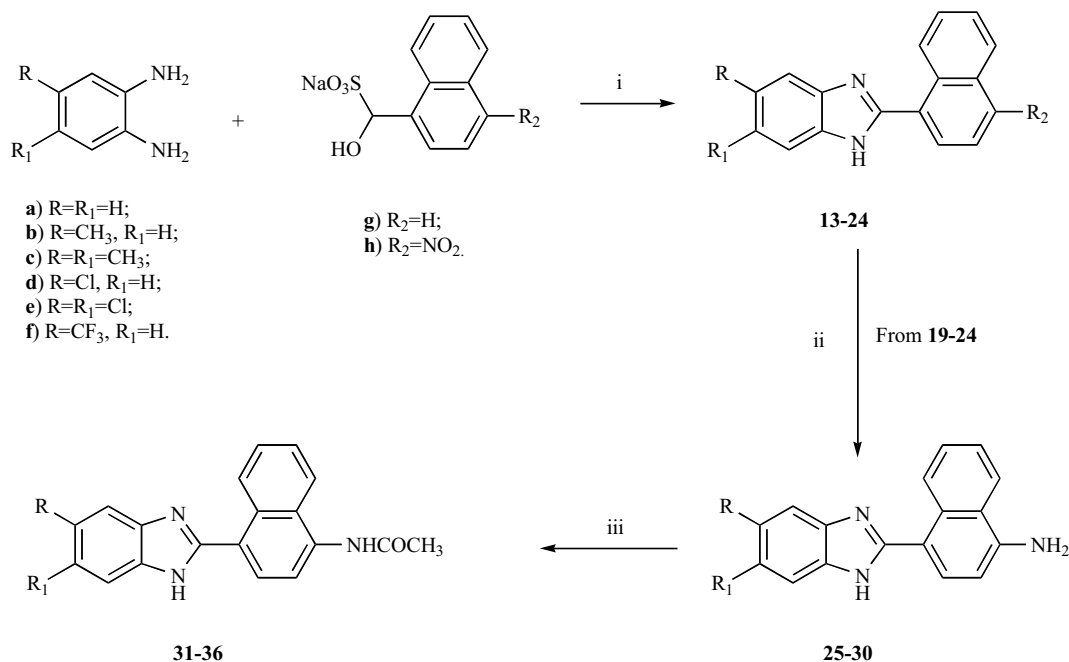
RESULTS AND DISCUSSION

As reported in Table (2), title compounds were evaluated *in vitro* against ssRNA⁺ viruses representative of the *Flaviviridae* and *Picornaviridae* families. In particular, viruses representative of two of the three genera of the *Flaviviridae* family, [i.e., YFV (*Flaviviruses*) and BVDV (*Pestiviruses*), as HCV (*Hepacivirus*) can hardly be used in routine cell based assays], and of one genus of the *Picornaviridae* family [CVB-2 and Polio 1 (*Enteroviruses*)] were tested. Compounds were also tested against an additional ssRNA⁺ virus [Human immunodeficiency virus (HIV-1)], and representatives of dsRNA viruses [*Reoviridae* (Reo-1)] and ssRNA⁻ viruses, *Paramyxo* [respiratory syncytial virus (RSV)] and *Rhabdo* [vesicular stomatitis virus (VSV)]. Two representatives of DNA virus families were also included: HSV-1 (*Herpesviridae*) and VV (*Poxviridae*).

As far as the antiviral activity is concerned, compounds **15**, **28** and **29** resulted moderately active against the sole YFV (range 6-27 μM). In particular, against this virus **15** resulted only 6-fold less potent than the reference nucleoside analogue 2'-C-Me-guanosine (NM108). The other benzimidazole derivatives showed activity neither against HIV-1, nor against representatives of dsRNA (Reo) and ssRNA⁻ viruses (RSV or VSV) (data not shown) at not cytotoxic concentrations.

The benzimidazole derivatives (**15**, **16**, **18**, **21**, **22**, **24**, **27**, **28**, **30**, **33**, **34**, **36**) which showed some cytotoxicity for resting cell monolayers were evaluated against a panel of exponentially growing human cell lines derived from haematological (CCRF-CEM, WIL-2NS and CCRF-SB) (Table 3) or solid tumours (SK-MEL-28, MCF7, SK-MES-1, HepG2 and DU145) (Table 4). They were also evaluated against cell lines (MRC-5 and CRL7065) derived from normal human tissues (Table 5). In these assays, etoposide and 6-mercaptopurine (6-MP) were used as reference drugs.

The data reported in Tables (3-5) show that compound **27** was the most active against both haematological and solid



Scheme (1). Synthesis of 5-mono- or 5,6-di- substituted -2-(4- R_2 -naphthyl-1-yl)-1H-benzimidazoles (13-36).

tumour cells, resulting as potent as etoposide and more potent than 6-MP against all the exponentially growing cell lines tested.

In the series of compounds examined it seems evident that substituents in both benzimidazole and naphthalene rings affect cytotoxicity. In the set of compounds which are unsubstituted at the 4' position of the naphthalene moiety, the presence of two methyl electron-releasing groups at positions 5 and 6 of the heterocycle (compound **15**) strongly influence the activity (0.06 μ M), which is equivalent to that of compound **27**, bearing an amino group at 4' position. The nitro-precursor **21** is 5-fold less potent, while its acetyl derivative (**32**) is 26-fold less potent. Among the other substituents at the benzimidazole ring, the dichloro and trifluoromethyl groups are the least cytotoxic. This behaviour was also observed in the antiproliferative activity (Tables 3 and 4).

EXPERIMENTAL SECTION

General Remarks

Melting points were carried out with a K f ler hot stage or Digital Electrothermal melting point apparatus and are uncorrected. Infrared spectra were recorded as nujol mulls on NaCl plates with a Perkin-Elmer 781 IR spectrophotometer and are expressed in ν (cm^{-1}). UV spectra are qualitative and were recorded in nm for solutions in EtOH with a Perkin-Elmer Lambda 5 spectrophotometer. Nuclear magnetic resonance (^1H -NMR) spectra were determined in CDCl_3 , $\text{DMSO}-d_6$, $\text{CDCl}_3/\text{DMSO}-d_6$ (in the ratio 1:3) and were recorded in a Varian XL-200 (200 MHz). Chemical shifts (δ scale) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) as internal standard. Splitting patterns are designated, as follows: s, singlet; d, doublet; t, trip-

let; q, quadruplet; m, multiplet; br s, broad singlet; dd, double doublets.

The assignment of exchangeable protons (OH and NH) was confirmed by the addition of D_2O . Analytical thin-layer chromatography (TLC) was performed on Merck silica gel F-254. Pure compounds showed a single spot in TLC. For flash chromatography, Merck silica gel 60 was used with a particle size 0.040-0.063 mm (230-400 mesh ASTM). Elemental analyses were performed on a Perkin-Elmer 2400 instrument at Laboratorio di Microanalisi, Dipartimento di Chimica, Universit  di Sassari, Italy, and results were within $\pm 0.4\%$ of theoretical values.

Chemistry

Intermediates

The diamines (**a-e**) were commercially available. The diamine (**f**) was prepared according to Walley [19] starting from the commercially available parent nitro compound (Aldrich). The bisulphite compounds (**h** and **g**) were obtained in high yields from the commercially available 1-naphthaldehyde and the purposely prepared 4-nitro-naphthalene-1-carbaldehyde following the procedure described by Katritzky and Xie [20].

General Procedure for Preparation of Compounds (13-24)

To a solution of 0.45 g (2.54-4.16 mmol) of the appropriate diamine (**a-f**) in ethanol (60 mL) was added in 1:1 molar ratio the sodium hydroxy(4- R_2 -naphth-1-yl)methanesulfonate (**h,g**). Then, the mixture was heated under reflux for 8 h. On cooling, a small amount of inorganic compound was filtered off and the ethanol mother liquors were evaporated to dryness in vacuo. The solid residues, coloured from white to

Table 1. Yields, Physical and Spectroscopic Data of Compounds of Scheme 1

Compd.	Yield %	M.p. °C	Analysis for	UV $\lambda_{\max}(\text{EtOH})$ nm	IR $\nu_{\max}(\text{Nujol})$ cm^{-1}	$^1\text{H-NMR}$ (CDCl_3 -DMSO- d_6), $J = \text{Hz}$, δ :
13 ²²	79	220-222	$\text{C}_{17}\text{H}_{12}\text{N}_2$	303, 224, 207	3344	8.79-8.75 (1H, m, H-8'), 8.04-7.94 (3H, m, arom.), 7.74 (5H, m, arom.), 7.34-7.28 (2H, m, arom.).
14	78	223-225	$\text{C}_{18}\text{H}_{14}\text{N}_2$	309, 222, 206	3346	8.83-8.78 (1H, m, H-8'), 8.01-7.91 (3H, m, arom.), 7.69-7.46 (5H, m, arom.), 7.11 (1H, d, $J = 8.4$; H-7), 2.51 (3H, s, 5-CH ₃).
15	66	245-250	$\text{C}_{19}\text{H}_{16}\text{N}_2$	298, 205	3362	8.86-8.81 (1H, m, H-8'), 8.00-7.90 (3H, m, arom.), 7.78 (1H, s, H-4), 7.64-7.59 (3H, m, arom.), 7.45 (1H, s, H-7), 2.40 (6H, s, 5,6-CH ₃).
16	81	138-140	$\text{C}_{17}\text{H}_{11}\text{ClN}_2$	(386, 377), 289, 248, 208	3351	8.75-8.72 (1H, m, H-8'), 8.00-7.89 (4H, m, arom.); 7.67-7.57 (4H, m, arom.); 7.24 (1H, dd, $J_{6,7} = 8.8$, $J_{6,4} = 2.0$, H-6), 6.63 (1H, m, arom.).
17	85	>300	$\text{C}_{17}\text{H}_{10}\text{Cl}_2\text{N}_2$	300, 208	3360	13.10 (1H, s, NH), 8.98-8.94 (1H, m, H-8'), 8.08 (1H, s, H-4), 8.04-7.92 (3H, m, arom.), 7.74 (1H, s, H-7), 7.69-7.50 (2H, m, arom.).
18	92	215-218	$\text{C}_{18}\text{H}_{11}\text{F}_3\text{N}_2$	290, 209	3361	8.88-8.84 (1H, m, H-8'), 8.07-7.97 (4H, m, arom.), 7.82-7.78 (1H, m, arom.), 7.68-7.51 (4H, m, arom.).
19	76	289-291	$\text{C}_{17}\text{H}_{11}\text{N}_3\text{O}_2$	334, 222sh	1515	12.90 (1H, s, NH), 9.21-9.16 (1H, m, H-8'), 8.51-8.48 (1H, m, H-5'), 8.30 (1H, d, $J = 7.9$, H-2'), 8.08 (1H, d, $J = 7.9$, H-3'), 7.85-7.62 (4H, m, arom.), 7.33-7.29 (2H, m, arom.).
20	87	124-127	$\text{C}_{18}\text{H}_{13}\text{N}_3\text{O}_2$	349, 232sh	3319, 1506, 1329	8.21 (1H, d, $J = 7.9$, H-8'), 8.49 (1H, d, $J = 7.9$, H-5'), 8.29 (1H, d, $J = 7.9$, H-2'), 8.06 (1H, d, $J = 8.0$, H-3'), 7.83 (2H, m, H-6',7'), 6.51 (1H, d, $J = 8.4$, H-6), 7.49 (1H, s, H-4), 7.13 (1H, d, $J = 8.2$, H-7), 2.52 (3H, s, CH ₃).
21	60	250-254	$\text{C}_{19}\text{H}_{15}\text{N}_3\text{O}_2$	358, 224sh	1516, 1337	9.03-8.99 (1H, m, H-8'), 8.53-8.49 (1H, m, H-5'), 8.19 (1H, d, $J = 8.0$, H-2'), 7.92 (1H, d, $J = 8.0$, H-3'), 7.78-7.42 (4H, m, arom.), 2.42 (6H, s, 5,6-CH ₃).
22	73	122-125	$\text{C}_{17}\text{H}_{10}\text{ClN}_3\text{O}_2$	334, 224sh	1508, 1329	9.15-9.11 (1H, m, H-8'), 8.52-8.47 (1H, m, H-5'), 8.29 (1H, d, $J = 8.2$, H-2'), 8.07 (1H, d, $J = 8.2$, H-3'), 7.85-7.64 (4H, m, H-4,7,6',4'), 7.27 (1H, dd, $J_{6,7} = 8.6$, $J_{6,4} = 2.0$, H-6).
23	50	285-290	$\text{C}_{17}\text{H}_9\text{Cl}_2\text{N}_3\text{O}_2$	336, 232	3359, 1516, 1337	13.30 (1H, s, NH), 9.18-9.14 (1H, m, H-8'), 8.46-8.42 (1H, m, H-5'), 8.34 (1H, d, $J = 7.6$, H-2'), 8.13 (1H, s, H-4), 8.10 (1H, d, $J = 7.6$, H-3'), 8.02 (1H, s, H-7), 7.92-7.70 (2H, m, H-6',7').
24	66	121-123	$\text{C}_{18}\text{H}_{10}\text{F}_3\text{N}_3\text{O}_2$	330, 242sh, 205	1513, 1335	9.12-9.07 (1H, m, H-8'), 8.50 (1H, dd, $J_{6,7} = 8.6$, $J_{4,6} = 1.6$, H-6), 8.30 (1H, d, $J = 8.6$, H-2'), 8.09 (1H, d, $J = 8.6$, H-3'), 8.04 (1H, s, H-4), 7.85 (4H, m, arom.), 7.52 (1H, d, $J = 8.4$, H-7).

(Table 1. Contd....)

Compd.	Yield %	M.p. °C	Analysis for	UV λ_{max} (EtOH) nm	IR ν_{max} (Nujol) cm^{-1}	$^1\text{H-NMR}$ (CDCl_3 -DMSO- d_6), $J = \text{Hz}$, δ :
25	89	277-279	$\text{C}_{17}\text{H}_{13}\text{N}_3$	327, 280, 233	3443, 3366	8.98-8.94 (1H, m, H-8'), 8.10-8.03 (1H, m, H-5), 7.73-7.49 (5H, m, arom.), 7.30-7.20 (2H, m, arom.), 6.85-6.82 (1H, m, arom), 5.41 (2H, s, NH_2).
26	92	250-255	$\text{C}_{18}\text{H}_{15}\text{N}_3$	325, 280, 233	3453, 3343, 3160	8.62-8.56 (1H, m, H-8'), 7.66-7.62 (1H, m, H-5'), 7.50 (1H, d, $J = 7.8$, H-2'), 7.32-7.26 (4H, m, H-4,6,6',7'), 7.05 (1H, d, $J = 7.9$, H-3'), 6.49 (1H, d, $J = 7.6$, H-7), 4.57 (2H, s, NH_2), 2.45 (3H, s, CH_3).
27	83	224-226	$\text{C}_{19}\text{H}_{17}\text{N}_3$	327, 285, 235	3376	8.90-8.86 (1H, m, H-8'), 8.13-8.09 (1H, m, H-5'), 7.71 (1H, d, $J = 8.0$, H-2'), 7.60-7.44 (2H, m, arom.), 7.37 (2H, s, H-4,7), 6.83 (1H, d, $J = 8.0$, H-3'), 5.75 (2H, s, NH_2), 2.38 (6H, s, 5,6- CH_3).
28	83	222-225	$\text{C}_{17}\text{H}_{12}\text{ClN}_3$	332, 280, 233	3404, 3383, 3334	8.75-8.71 (1H, m, H-8'), 7.84-7.80 (1H, m, H-5'), 7.68-7.48 (3H, m, arom.), 7.30-7.18 (2H, m, arom.) 6.74-6.68 (2H, m, arom.), 4.41 (2H, s, NH_2).
29	90	295-300	$\text{C}_{17}\text{H}_{11}\text{Cl}_2\text{N}_3$	342, 234	3402, 3234	12.60 (1H, s, NH), 9.07 (1H, d, $J = 8.4$, H-8'), 8.13 (1H, d, $J = 8.0$, H-5'), 7.76 (1H, d, $J = 8.0$, H-2'), 7.63-7.43 (4H, m, arom.), 6.83 (1H, d, $J = 8.0$, H-3'), 5.92 (2H, s, NH_2).
30	90	228-230	$\text{C}_{18}\text{H}_{12}\text{F}_3\text{N}_3$	342, 248, 205	3460, 3374	8.75-8.71 (1H, m, H-8'), 7.84-7.80 (2H, m, arom.), 7.59-7.46 (6H, m, arom.), 6.70 (1H, d, $J = 8.6$, H-7), 4.44 (2H, s, NH_2).
31	75	>300	$\text{C}_{20}\text{H}_{14}\text{N}_2\text{O}$	307, 216	3263, 1660	10.04 (1H, s, NHCO), 9.09-9.04 (1H, m, H-8'), 8.27-8.22 (1H, m, H-5'), 8.00-7.95 (2H, m, arom.), 7.68-7.59 (4H, m, arom.), 7.27-7.22 (2H, m, arom.), 2.29 (3H, s, CH_3).
32	78	>300	$\text{C}_{21}\text{H}_{16}\text{N}_2\text{O}$	306, 214	3234, 1658, 1529	10.03 (1H, s, NHCO), 9.07-9.02 (1H, m, H-8'), 8.25-8.22 (1H, m, H-5'), 7.99-7.93 (2H, m, arom.), 7.62-7.52 (4H, m, arom.), 7.43 (1H, s, H-4), 7.07 (1H, d, $J = 8.6$, H-7), 2.50 (3H, s, CH_3), 2.29 (3H, s, CO- CH_3).
33	65	>300	$\text{C}_{22}\text{H}_{18}\text{N}_2\text{O}$	(388), 309, 211	3268, 1658, 1528	10.02 (1H, s, NHCO), 9.10-9.05 (1H, m, H-8'), 8.25-8.20 (1H, m, H-5'), 8.02 (1H, s, H-4), 7.92 (1H, s, H-7), 7.61-7.32 (4H, m, arom.), 2.39 (6H, s, 5,6- CH_3), 2.28 (3H, s, CO- CH_3).
34	70	>300	$\text{C}_{20}\text{H}_{13}\text{ClN}_2\text{O}$	(386), 308, 216	3239, 1660, 1529	10.02 (1H, s, NHCO), 9.10-9.02 (1H, m, H-8'), 8.28-8.20 (1H, m, H-5'), 7.99-7.95 (2H, m, arom.), 7.63-7.58 (3H, m, arom.), 7.26-7.18 (2H, m, arom.), 2.29 (3H, s, CH_3).
35	53	>300	$\text{C}_{20}\text{H}_{12}\text{Cl}_2\text{N}_2\text{O}$	328, 233, 209	1660, 1528	9.97 (1H, s, NHCO), 8.90-8.85 (1H, m, H-8'), 8.28-8.23 (1H, m, H-5'), 8.00 (1H, d, $J = 8.2$, H-2'), 7.93 (1H, d, $J = 8.0$, H-3'), 7.81 (2H, s, H-4,7), 7.64-7.59 (2H, m, H-6,7), 2.33 (3H, s, CH_3).
36	50	>300	$\text{C}_{21}\text{H}_{13}\text{F}_3\text{N}_2\text{O}$	326, 232, 205	1658, 1529	9.99 (1H, s, NHCO), 9.01-8.98 (1H, m, H-8'), 8.28-8.25 (1H, m, H-5'), 7.97-7.60 (6H, m, arom.), 7.50 (1H, d, $J = 8.4$, H-7), 2.32 (3H, s, CH_3).

dark brown, were purified by recrystallization from ethanol or methanol. Melting points, yields, analytical and spectroscopic data are reported in Table (1).

General Procedure for Preparation of Compounds (25-30)

To a solution of 0.36 g (1.0-1.24 mmol) of compounds (19-24) in ethanol (20 mL) was added an excess of hydrazine

Table 2. Antiviral Activity Against ssRNA⁺ Viruses

Compd.	R	R ₁	R ₂	EC ₅₀ [μM]				
				^a HIV-1	^b BVDV	^c YFV	^d CVB-2	^d Sb-1
13	H	H	H	>0.7	>4	>80	>100	>100
14	CH ₃	H	H	>0.2	>0.4	>15	>60	>60
15	CH ₃	CH ₃	H	>0.06	>0.5	6	>2	>2
16	Cl	H	H	>0.3	>0.7	>13	>2	>2
17	Cl	Cl	H	>0.9	>2	>100	>100	>100
18	CF ₃	H	H	>0.5	>1	>6	>75	>75
19	H	H	NO ₂	>6	>20	>58	>100	>100
20	CH ₃	H	NO ₂	>6	>8	>18	>33	>33
21	CH ₃	CH ₃	NO ₂	>0.3	>4	>9	>5	>4
22	Cl	H	NO ₂	>1.7	>2.5	>14	>4	>4
23	Cl	Cl	NO ₂	>5	>4	>6	>18	>18
24	CF ₃	H	NO ₂	>3	>6	>2.3	>22	>22
25	H	H	NH ₂	>0.7	>2.4	>6	>20	>20
26	CH ₃	H	NH ₂	>0.2	>0.4	>26	>4	>4
27	CH ₃	CH ₃	NH ₂	>0.06	>0.3	>3	>0.6	>0.6
28	Cl	H	NH ₂	>0.3	>0.7	27	>1	>1
29	Cl	Cl	NH ₂	>0.6	>0.7	16	>33	>33
30	CF ₃	H	NH ₂	>0.5	>0.8	>6	>20	>20
31	H	H	NHCOCH ₃	>2	>100	>100	>100	>100
32	CH ₃	H	NHCOCH ₃	>2	>17	>53	>100	>100
33	CH ₃	CH ₃	NHCOCH ₃	>1.6	>16	>21	>65	>65
34	Cl	H	NHCOCH ₃	>1.8	>6	>17	>11	>11
35	Cl	Cl	NHCOCH ₃	>2	>5	>54	>100	>100
36	CF ₃	H	NHCOCH ₃	>5	>7	>8	>40	>40
^o AZT				0.01	§ND	ND	ND	ND
[*] NM108				>100	4	1	30	35
[^] NM176				≥100	40	>100	25	23

^aCompound concn. (μM) required to achieve 50% protection of MT-4 cells (CD4+ Human T-cells containing an integrated HTLV-I genome) from the HIV-1-induced cytopathogenicity, as determined by the MTT method.

^bCompound concn. (μM) required to achieve of MDBK cells from the BVDV-induced cytopathogenicity, as determined by the MTT method.

^cCompound concn. (μM) required to achieve 50% protection of BHK (Kidney fibroblast) cells from the YFV- and Reo-induced cytopathogenicity, as determined by the MTT method.

^dCompound concn. (μM) required to reduce the plaque number of CVB-2 and Sb-1 by 50% in Vero 76 monolayers.

^oAZT. 3'-azido-thymidine

§ND. Not Determined

^{*}2'-C-Me-guanosine

[^]2'-Ethynyl-cytidine

Variation among duplicate samples was less than 15%.

Table 3. Antiproliferative Activity Against Human Cells from Haemathologic Tumours

Compd.	^a CC ₅₀ [μM]			
	MT-4	CCRF-CEM	WILS-2NS	CCRF-SB
15	0.06±0.04	0.6±0.3	1.2±0.2	0.7±0.05
16	0.3±0.01	0.5±0.1	0.05±0.7	0.7±0.05
18	0.5±0.1	1.2±0.1	1.8±0.7	1.0±0.01
21	0.3±0.02	2.4±0.05	2.8±0.4	2.0±0.1
22	1.7±0.2	2.7±0.4	2.4±0.2	2.2±0.05
24	3.0±0.1	4.7±0.7	5.9±0.6	3.9±0.3
27	0.06±0.05	0.2±0.05	0.4±0.03	0.2±0.05
28	0.3±0.01	0.5±0.1	0.9±0.2	0.6±0.1
30	0.5±0.1	0.9±0.05	0.8±0.05	0.8±0.05
33	1.6±0.4	4.8±1	8.7±2	5.0±0.9
34	1.8±0.6	3.7±0.3	7.0±0.3	3.9±0.3
36	5.0±0.1	15±1	15±0.2	12±0.5
Etoposide	0.09±0.01	0.09±0.01	0.2±0.05	0.08±0.03

^aCompound concn. (μM) ±SD required to reduce the viable cell number of MT-4 cells (CD4⁺ Human T-cells containing an integrated HTLV-1 genome), CCRF-CEM (CD4⁺ human acute T-lymphoblastic leukaemia), WIL-2NS (human splenic B-lymphoblastoid cells) and CCRF-SB (human acute B-lymphoblastic leukaemia) by 50%, as determined by the MTT method.

hydrate (98%) (1.5 ml) in 1:1.25 molar ratio and the mixture was refluxed in the presence of 45 mg of 10% Pd charcoal for 1 h. The solution was then allowed to reach room temperature and the catalyst was removed by filtration through filter paper washed with ethanol. The mother liquors were evaporated in rotavapor under reduced pressure. Compounds **25-30** were obtained as coloured solids, which were purified by triturating with methanol.

Melting points, yields, analytical and spectroscopic data are reported in Table (1).

General Procedure for Preparation of Compounds (31-36)

A mixture formed by 0.1 g (3.00-3.9 mmol) of the amines (**25-30**) and acetic anhydride (3-4 mL) was kept under stirring at 80-100 °C for about 15 min. On cooling, the precipitate formed was collected and washed with diethyl ether. The acetyl derivatives (**31-36**) were obtained pure. Melting points, yields, analytical and spectroscopic data are reported in Table (1).

Biology

Compounds

Compounds were dissolved in DMSO at 100 mM and then diluted in cell culture medium so that the highest compound concentration used (100 μM) contains a DMSO concentration of 0.1% which, when used alone, is not cytotoxic for any of the cell lines used in this study.

Cells and Viruses

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. Cell lines supporting the multiplication of RNA viruses were the following: CD4⁺ human T-cells containing an integrated HTLV-1 genome (MT-4); Madin-Darby Bovine Kidney (MDBK); Baby Hamster Kidney (BHK-21); Monkey kidney (Vero 76) cells.

Cytotoxic / Antiproliferative Assays

For cytotoxicity tests, run in parallel with antiviral assays, MDBK, BHK and Vero 76 cells were resuspended in 96 multiwell plates at an initial density of 6x10⁵, 1x10⁶ and 5x10⁵ cells/mL, respectively, in maintenance medium, without or with serial dilutions of test compounds. Cell viability was determined after 48-96 hrs at 37 °C in a humidified CO₂ (5%) atmosphere by the MTT method. The cytotoxicity of test compounds for Vero 76 monolayers was determined by staining with the crystal violet dye and, after extensive washing, comparing the amount of dye (recovered with a 0.1% SDS solution) to that of untreated monolayers.

It is worth noting that, due to the high cell density needed for virus growth, all the above cell monolayers contained resting cells.

For antiproliferative evaluations, exponentially growing cells derived from human haematological tumors [CD4⁺ human T-cells containing an integrated HTLV-1 genome (MT-

Table 4. Antiproliferative Activity Against Human Cells from Solid Tumors

Compd.	^a CC ₅₀ [μM]				
	SK-MEL-28	MCF7	SK-MES-1	HepG2	DU145
15	8.7±1	17±3	7.4±2	11±1	7.5±0.5
16	51±2	37±2	2.3±0.3	7.7±0.5	2.8±0.5
18	3.1±0.1	4.2±0.5	3.2±0.1	6.5±2	4.3±0.2
21	10±3	7.2±0.6	9.7±0.9	8.3±0.8	7.2±2
22	11±1	7.2±0.4	11±0.2	8.6±0.5	6.1±0.9
24	8.8±0.1	9.5±0.6	8.8±0.7	10±1	7.7±0.5
27	1.0±0.05	0.7±0.1	0.9±0.04	1.1±0.1	0.9±0.05
28	2.9±0.5	2.8±0.3	2.6±0.8	3.3±0.05	2.2±0.3
30	1.9±0.2	2.1±0.2	1.9±0.1	2.8±0.3	1.9±0.2
33	34±4	56±7	34±4	>100	33±2
34	33±5	10±0.01	7.5±0.3	23±1	2.8±0.1
36	11±0.1	6.0±0.8	4.6±0.9	25±1	3.5±0.2
6-MP	15±2	3.2±1.2	>100	8.0±2.6	2.0±0.2
Etoposide	1.2±0.5	1.0±0.7	0.3±0.2	0.7±0.1	0.4±0.2

^aCompound concn. (μM) ±SD required to reduce the viable cell number of SK-MEL-28 (skin melanoma), MCF-7 (breast adenocarcinoma), SK-MES-1 (lung squamous carcinoma), HepG2 (hepatocellular carcinoma) and DU-145 (prostate carcinoma) by 50%, as determined by the MTT method.

4); CD4⁺ human acute T-lymphoblastic leukaemia (CCRF-CEM); Human splenic B-lymphoblastoid cells (WIL-2NS); Human acute B-lymphoblastic leukemia (CCRF-SB)] were seeded at an initial density of 1x10⁵ cells/ml in 96 well plates in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin G and 100 μg/mL streptomycin. Human cell lines derived from solid tumours [skin melanoma (SK-MEL-28); breast adenocarcinoma (MCF-7); lung squamous carcinoma (SK-MES-1); hepatocellular carcinoma (HepG-2); prostate carcinoma (DU-145)] or normal tissues [lung fibroblasts (MRC-5); foreskin fibroblasts CRL-7065]] were also seeded at 1x10⁵ cells/ml in 96 well plates in the same specific media supplemented with 10% FCS and antibiotics as above. Cell cultures were then incubated at 37 °C in a humidified, 5% CO₂ atmosphere in the absence or presence of serial dilutions of test compounds. The extent of viable cell growth was determined after 96 hrs at 37 °C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method [21]. It is worth noting that the effect of compounds on these cell lines was determined under experimental conditions allowing cells to undergo exponential growth for at least three cell cycles.

In conclusion, we will refer to the effect of compounds on the various cell lines as “cytotoxic” or “antiproliferative”, according to whether the cells are resting or proliferating, respectively.

Antiviral Assays

Activity of compounds against Human Immunodeficiency virus type-1 (HIV-1) was based on inhibition of virus-

induced cytopathogenicity in MT-4 cells acutely infected with a multiplicity of infection (m.o.i.) of 0.01. Briefly, 50 μL of RPMI containing 1x10⁵ MT-4 were added to each well of flat-bottom microtitre trays containing 50 μL of RPMI, without or with serial dilutions of test compounds. Then, 20 μL of an HIV-1 suspension containing 100 CCID₅₀ were added. After a 4-day incubation, cell viability was determined by the MTT method.

Activity of compounds against Yellow fever virus (YFV), 17D vaccine strain and Reo virus type-1 (Reo-1) was based on inhibition of virus-induced cytopathogenicity in acutely infected BHK-21 cells. Activities against Bovine viral diarrhoea virus (BVDV), in infected MDBK cells were also based on inhibition of virus-induced cytopathogenicity.

BHK and MDBK cells were seeded in 96-well plates at a density of 5x10⁴ and 3x10⁴ cells/well, respectively, and were allowed to form confluent monolayers by incubating overnight in growth medium at 37 °C in a humidified CO₂ (5%) atmosphere. Cell monolayers were then infected with 50 μL of a proper virus dilution (in serum-free medium) to give an m.o.i = 0.01. One hr later, 50 μL of MEM Earle's medium, supplemented with inactivated foetal calf serum (FCS), 1% final concentration, without or with serial dilutions of test compounds, were added. After a 3 day incubation at 37 °C, cell viability was determined by the MTT method.

Activity of compounds against Coxsackie virus, B-2 strain (CVB-2), Polio virus type-1 (Sb-1), Sabin strain, Vesicular Stomatitis Virus (VSV) and against Respiratory syncytial virus (RSV), A-2 strain, in infected Vero 76 cells, was

Table 5. Antiproliferative Activity Against Human Cells from Normal Tissues

Compd.	^a CC ₅₀ [μM]	
	MRC-5	CRL7065
15	9.0±3	30±0.5
16	40±6	56±9
18	5.8±1	6.0±0.2
21	10±1	93±7
22	10±0.7	40±6
24	8.6±0.7	15±4
27	1.1±0.1	44±6
28	2.6±0.4	>100
30	2.7±0.7	5.0±2
33	41±5	83±5
34	32±1	>100
36	21±3	>100
6-MP	>100	>100
Etoposide	>100	>100

^aCompound concn. (μM) ±SD required to reduce the viable cell number of MRC-5 (lung fibroblast) and CRL7065 (foreskin fibroblast) by 50%, as determined by the MTT method.

determined by plaque reduction assays in Vero 76 cell monolayers. To this end, Vero 76 cells were seeded in 24-well plates at a density of 2.5×10^5 cells/well and were allowed to form confluent monolayers by incubating overnight in growth medium at 37 °C in a humidified CO₂ (5%) atmosphere. Then, monolayers were infected with 250 μL of proper virus dilutions to give 50-100 PFU/well. Following removal of unadsorbed virus, 500 μL of Dulbecco's modified Eagle's medium supplemented with 1% inactivated FCS and 0.75% methyl cellulose, without or with serial dilutions of test compounds, were added. Cultures were incubated at 37 °C for 2 (Sb-1 and VSV), 3 (CVB-2) or 5 days (RSV) and then fixed with PBS containing 50% ethanol and 0.8% crystal violet, washed and air-dried. Plaques were then counted. 50% effective concentrations (EC₅₀) were calculated by linear regression technique.

Linear Regression Analysis

Viral and cell growth at each drug concentration was expressed as percentage of untreated controls and concentrations resulting in 50% (EC₅₀ and CC₅₀) growth inhibition were determined by linear regression analysis.

ACKNOWLEDGEMENT

This work was supported by grants from Italian FIRB [(RBNE01J3SK 001)] and Cybersar (MIUR, PON) Projects.

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