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ScienceDirect
Bioorganic & Medicinal Chemistry Letters 17 (2007) 1843–1849
Bioorganic & Medicinal Chemistry Letters

Evaluation of the anti-hepatitis C virus effect of novel potent, selective, and orally bioavailable JNK and VEGFR kinase inhibitors

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Received 1 December 2006; revised 12 January 2007; accepted 13 January 2007
Available online 24 January 2007

Abstract—Screening of a focused library of TGF beta kinase inhibitors in the cellular HCV replicon model with luciferase read out yielded a number of low micromolar HCV inhibitors. Medicinal chemistry driven optimization resulted in the discovery of 4-[2-(5-bromo-2-fluoro-phenyl)pteridin-4-ylamino]-*N*-[3-(2- oxopyrrolidin-1-yl)propyl]nicotinamide **36** with a replicon EC_{50} of 64 nM, associated with a selective kinase inhibitory profile for human JNK kinases 2 and 3 as well as VEGFR-1, 2, and 3 kinases. Moreover, **36** showed an advantageous PK profile in mice. Experiments performed using different replicon constructs suggest that this series of kinase inhibitors might mediate their effect through the HCV non-structural protein 5A (NS5A).

Hepatitis C virus (HCV) is a positive-strand RNA virus with a genome of approximately 9600 nt belonging to the *Flaviviridae* family.[1] The World Health Organization has estimated that 120–130 million people are infected worldwide with HCV.[2] Although acute infection is often asymptomatic, around 80% of infected individuals develop persistent HCV infection which is associated with risk of severe chronic liver disease such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.[3]

At present, no vaccine is available for the prevention of HCV. Interferon (IFN)-α, a cytokine with immunomodulatory and antiviral activity,[5] has been widely used for treatment of persistent HCV infection. However, approximately 50% of patients with chronic hepatitis C caused by genotype 1 virus have no sustained virological response (SVR) even after treatment with the most effective combination therapy to date, pegylated interferon-α-2a with synthetic guanosine nucleoside ribavirin.[6] In addition, considerable side effects are associated with these treatments, resulting in limited patient compliance.[7] There is an urgent need to develop more effective and better tolerated drugs for the treatment of HCV infected patients. New antiviral agents in clinical development include inhibitors of key HCV enzymes, such as NS3 protease and NS5B RNA-dependent RNA polymerase (RdRp).[8] However, the success of these novel antiviral therapies may be hindered by the challenge of drug resistance. For this reason, the development of drugs with novel modes of action, amenable to combination therapies, is of interest.[8]

The development of the HCV replicon as a cellular surrogate model for HCV replication has enabled cellular compound screening campaigns to find novel direct and indirect antivirals active against HCV.[9]

Given the pivotal role of TGFβ in liver morphogenesis and fibrosis, as well as reports of kinase inhibitors modulating HCV replication, we investigated the antiviral potential of compounds known to alter TGFβ signaling.[10,11]

We used a Huh7-Rep cell line containing the subgenomic bicistronic replicon clone ET with a luciferase read out[12] to screen a focused library of TGFβ Receptor 1 kinase (TGFβ-R1) inhibitors.[9] We found that

Keywords: JNK; VEGFR; HCV; Hepatitis C; Kinase.
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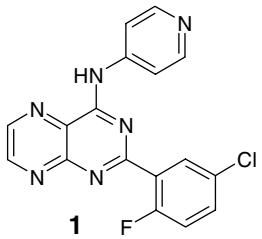
doi:10.1016/j.bmcl.2007.01.046



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(SCI-208)

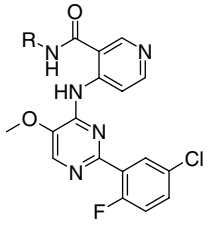
Chart 1. TGFβ-R_1 kinase inhibitor.[4]

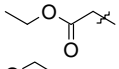
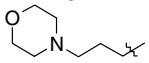
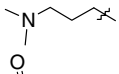
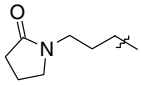
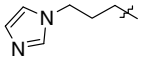
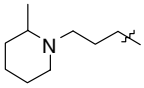
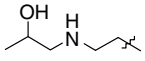
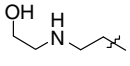
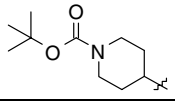
[2-(5-chloro-2-fluoro-phenyl)pteridin-4-yl]pyridin-4-yl-amine **1** (Chart 1),[4] a previously reported potent TGFβ-R1 inhibitor (IC_{50} = 53 nM), is also inhibiting HCV RNA replication with an EC_{50} of 0.89 μM with no associated cytotoxicity in MT4 and Huh7 cells engineered to constitutively express luciferase (CC_{50} > 25 μM).

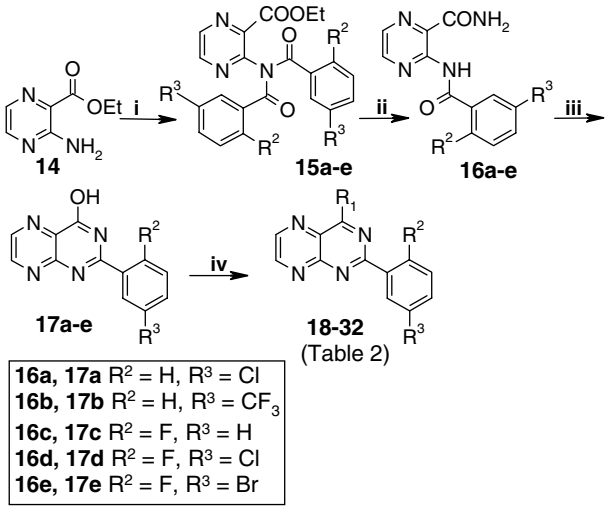
The selection of a subset of structurally related pyrimidines[13] bearing a variety of carboxamides in position 3 of the 4-(4-pyridylamino) moiety from an in-house kinase library yielded a number of low micromolar HCV inhibitors reported in Table 1. Interestingly, the nature of the amide of the pyridyl ring is known to modulate the kinase profile and potency of the pyrimidine derivatives.[4] Amongst the compounds tested (**2**–**13**), only the 2-oxopyrrolidin-1-ylpropylaminocarbonyl derivative **8** was found significantly more potent than the initial hit **1** (EC_{50} = 84 and 890 nM, respectively). It is noteworthy that a number of compounds tested (e.g., **8**, CC_{50}-(MT4-LTR-Luc) = 5.86 μM, CC_{50}-(Huh7-CMV-Luc) = 17.7 μM) exhibited micromolar cytotoxicity toward both MT4 and Huh7 cell lines. Since the pteridine **1** exhibited no cytotoxicity up to 25 μM, we decided to explore further SAR within the pteridine series.

The target pteridines **18**–**32** were synthesized as shown in Scheme 1. Attempts to acylate 3-amino-pyrazine-2-carboxylic acid ethyl ester **14** with 1 equivalent of benzoylchlorides, using different reaction conditions, failed to provide the monoacylated product in good yields.

Table 1. Inhibition of HCV replication in Huh-7-Rep cells (luciferase assay) for methoxypyrimidine **2**–**13**[13] measured by 50% effective concentration (EC_{50}) versus cytotoxicity in (a) Huh7-Luc and (b) MT4-LTR-Luc cell lines measured as CC_{50}



Compound	R	Huh7-Rep EC_{50} (μM)	ToxCC_{50}(μM)	
			MT4	Huh7
2	$NH_2CH_2CH_2$-	3.6	4.7	5.5
3	CF_3CH_2-	1.2	>32	>32
4		4.6	>32	>32
5		2.5	15.4	17.1
6		2.6	>32	>32
7		0.59	5.0	15.8
8		0.084	5.8	17.7
9		0.92	8.1	15.3
10		0.55	3.3	3.6
11		9.6	>32	>32
12		0.85	>32	>32
13		1.0	3.9	4.0



Scheme 1. Reactions and conditions: (i) 2-(R^2)-5-(R^3) benzoylchloride, pyridine; (ii) NH_3; (iii) NaOH; (iv) PyBOP, R_1-H.

Indeed, the diacylated derivatives **15a–e** were always obtained as the major products. Therefore, **14** was acylated in the presence of an excess of 2-R^2-5-R^3 benzoyl chloride affording the *N*,*N*-disubstituted intermediates **15** in almost quantitative yields.

Treatment of esters **15** with ammonia in refluxing ethanol led to the monoacetylated amides **16a–e**, which were readily cyclized to the corresponding pteridinones **17a-e** by reaction with NaOH. Eventually, intermediates **17** were activated by treatment with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), leading to a very reactive *O*-benzotriazole iminoether (structure not shown) in quantitative yield, which after subsequent treatment with the appropriate anilines resulted in the final products **18–32** (Table 2).

The synthesis of 4-[2-(5-bromo-2-fluoro-phenyl)pteridin-4-ylamino]-*N*-[3-(2-oxopyrrolidin-1-yl)propyl]nicotinamide **36** is reported in Scheme 2. The pteridinone **17e** was converted in 99% yield to the iminochloride **33** by reaction with thionylchloride and dimethylformamide. Treatment of **33** with aminonicotinic methyl ester in presence of triethylamine provided the ester **34** (75% yield), which was readily hydrolyzed to the corresponding acid by treatment with sodium hydroxide. The subsequent coupling reaction of **35** with 3-(2-oxopyrrolidin-1-yl)propylamine provided the target compound **36** in 30% yield.

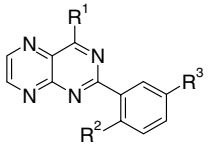
The substitution of the phenyl ring in position 2 was found to modulate the anti-HCV activity of this series. Indeed, although the removal of the chlorine or the fluoro atoms in position R^2 and R^3, respectively, led to compounds 2–10 times less potent (compare **19** and **20** to **1**), the replacement of the chlorine with a bromine resulted in a 3-fold increase of activity (compare **23** to **1** and **31** to **21**). The introduction of a methyl group in the position 3 of the pyridyl moiety decreases the activity by half (compare **21** with **1** and **31** with **23**). Surprisingly, the ethyl derivative **22** was found equally potent to **1**. Moreover, introduction of a 2-methyl group resulted in a 10-fold drop of activity (compare **32** to **23**) and a higher cytotoxicity. The endocyclic nitrogen of the pyridyl moiety was found to be important since its removal also resulted in a 10-fold dropped activity (compare **23** to **24** and **26** to **31**). Furthermore, substitution of the exocyclic nitrogen of **23** with alkyl and aralkyl led in all cases to less active compounds (**27–30**). To our satisfaction, the introduction of the 2-oxopyrrolidin-1-ylpropylaminocarbonyl moiety identified in the former 2-methoxypyrimidine series produced the same beneficial effect in the pteridine series, leading to the identification of **36**, the most active derivative of this series (EC_{50} = 64 nM).

Unlike **8**, no cytotoxicity was observed with the pteridine analog **36** even at the highest concentrations tested (CC_{50} > 32 μM). This lack of cytotoxicity was confirmed by a 4 day proliferation assay and by measurement of cellular RPL13A transcript levels. In both cases, the CC_{50} was found to be over 32 μM. In addition **36** had no effect on cell cycle and on intracellular ATP concentration up to 50 μM. Moreover, **36** was found to be inactive on BVDV up to 100 μM. Therefore, **36** was chosen as the lead compound of this series and was further profiled for its in vitro and in vivo characteristics.

To better understand the mechanism by which **36** is inhibiting the HCV replication in the replicon system, a series of in vitro experiments was conducted. Enzymatic experiments showed that **36** does not significantly inhibit either HCV NS5B polymerase or HCV NS3/4A protease (IC_{50} > 30 μM for both).

Since the initial hit **1** had been reported to inhibit the TGFβ R1 kinase with an IC_{50} of 54 nM in a biochemical assay, we evaluated a subset of pteridines against this target. In this assay, **36** was found to be a weaker inhibitor of TGFβ R1 kinase (IC_{50} = 3.2 μM, representing a 60-fold loss of activity) compared to **1** (IC_{50} = 53 nM). Moreover, as shown in Figure 1a, no overall correlation between the inhibition of TGFβ R1 kinase and inhibition of Huh7-replicon could be established for this series, suggesting that **36** is inhibiting HCV replication through another mechanism. To further assess the kinase profile of this novel series of HCV inhibitors, pteridines **1** and **36** were evaluated against a panel of 171 kinases. Pteridine **36** exhibits a selective inhibition profile against human JNK 2 and 3 as well as against human VEGFR kinases 1–3 (JNK2 IC_{50} = 37 nM; JNK3 IC_{50} = 59 nM; VEGFR-1 IC_{50} = 47 nM, VEGFR-2 IC_{50} = 140 nM and VEGFR-3 IC_{50} = 123 nM) compared to the initial hit **1**, which in addition to its TGFβ R1 activity, inhibited 15 different kinases over 85% at a concentration of 10 μM. Interestingly, **36** showed no significant inhibitory effect on closely related JNK1 (IC_{50} = 10.5 μM) and inhibits less than 70% at 10 μM all the other 165 kinases tested. To further explore if these pteridine derivatives exert their anti-HCV activity through the inhibition of JNK and/or VEGFR kinases, an additional subset of compounds was evaluated on these two kinase families. Interestingly, only VEGFR-3 inhibition seems to correlate with

Table 2. Inhibition of HCV replication in Huh-7-Rep cells (luciferase assay) for pteridines **1**, **17d**, **18**–**32**, **34**, and **36** measured by 50% effective concentration (EC_{50}) versus cytotoxicity in HUH7-CMV-Luc and MT4-LTR-Luc cell lines measured as CC_{50}



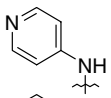
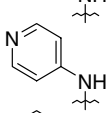
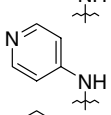
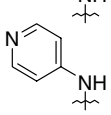
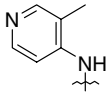
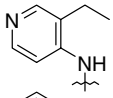
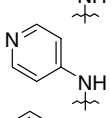
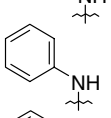
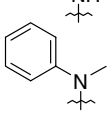
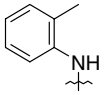
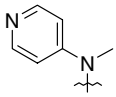
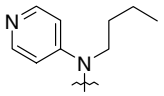
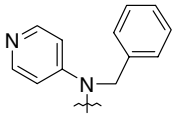
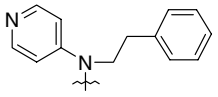
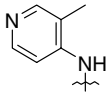
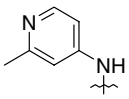
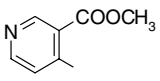
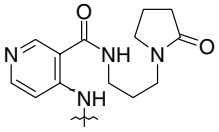
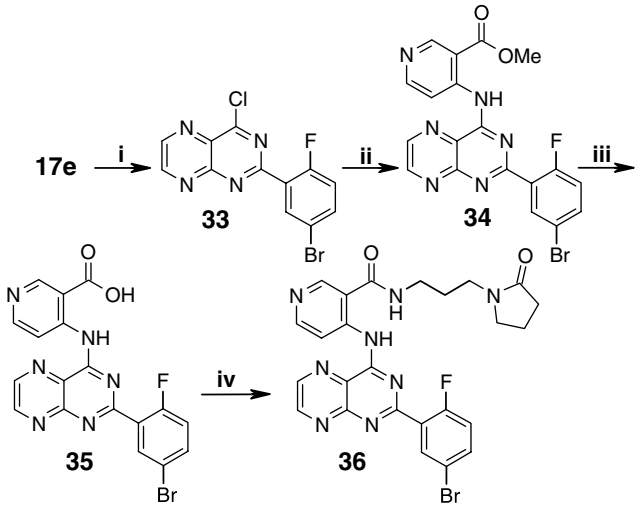
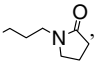
Compound	R^1	R^2	R^3	Huh7-RepEC_{50}(μM)	ToxCC_{50}(μM)	
					MT4	Huh7
17d	HO-	F	Cl	>50	>25	>25
1		F	Cl	0.89	>25	>25
18		H	CF_3	1.91	>25	>25
19		F	H	9.95	>25	>25
20		H	Cl	1.67	>32	>32
21		F	Cl	2.0	>32	>32
22		F	Cl	0.71	>32	>32
23		F	Br	0.35	>32	>32
24		F	Br	4.0	>32	>32
25		F	Br	1.98	>32	>32
26		F	Br	6.4	>32	>32
27		F	Br	3.65	>32	>32
28		F	Br	2.2	>32	>32
29		F	Br	0.99	>32	>32
30		F	Br	1.96	11.0	22.2
31		F	Br	0.80	>32	>32

Table 2 (*continued*)

Compound	R^1	R^2	R^3	Huh7-RepEC_{50}(μM)	ToxCC_{50}(μM)	
					MT4	Huh7
32		F	Br	3.60	13.2	12.9
34		F	Br	4.95	>32	>32
36		F	Br	0.064	>32	>32



Scheme 2. Reactions and Conditions: (i) $SOCl_2$, DMF; (ii) 4-amino-pyridine-3-carboxylic acid methyl ester; (iii) NaOH; (iv)  , PyBOP.

HCV-replicon inhibition, suggesting that the anti-HCV activity of 36 might be linked to VEGFR-3 inhibition (Fig. 1d) (squared correlation coefficient $r^2 = 0.480$). However, additional experiments are required to validate this hypothesis.

The antiviral activity of **36** was further analyzed in a panel of full length and subgenomic 1a and b replicons. Although **36** is a potent inhibitor of the ET replicon clone (harboring E1202G, T1280I adaptive mutations in NS3 and K1846T in NS4B), the compound was found inactive up to 32 μM in other genotype 1b and 1a derived replicons (harboring S2204I or S2197P adaptive mutation in NS5A). Since one remarkable difference between the ET clone and the other replicons tested is the lack of adaptive mutation within NS5A of the ET clone, it can be hypothesized that **36** is directly or indirectly interfering with NS5A. This hypothesis was supported by the introduction of the NS5A adaptive mutation S2204I in a replicon backbone devoid of adaptive mutation resulting in a significant loss of activity of **36** in a transient HCV

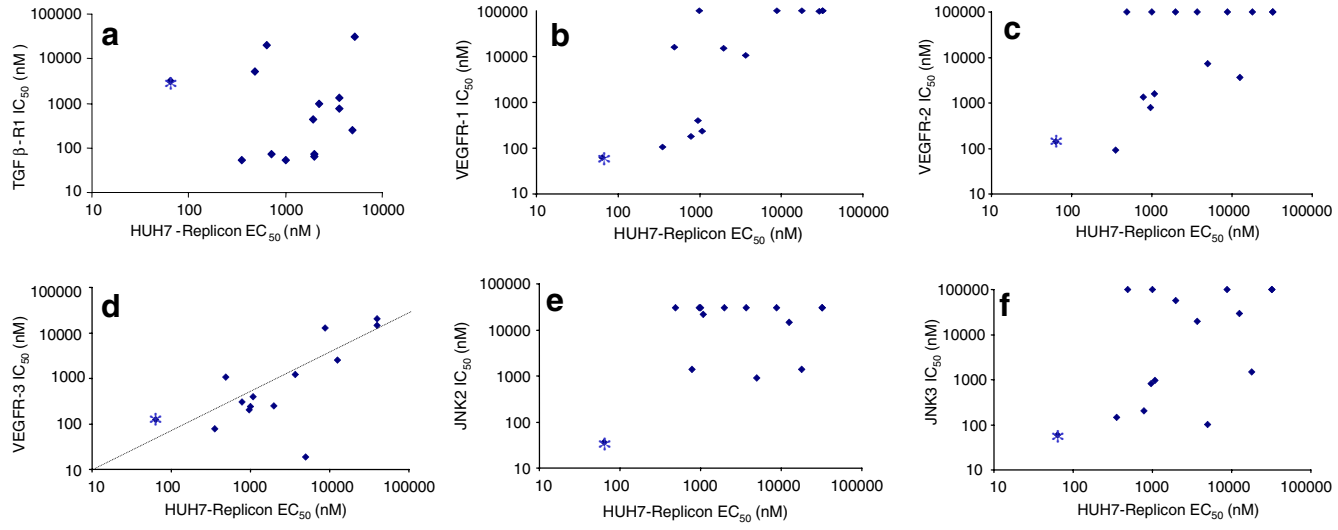


Figure 1. Inhibition of HCV replication in Huh-7 Rep cells (luciferase assay) for a subset of pteridines measured by 50% effective concentration (EC_{50}) versus inhibition of different kinases measured by 50% inhibitory concentration (IC_{50}): (a) TGFβ-R1; (b) VEGFR-1(h); (c) VEGFR-2 (h); (d) VEGFR-3 (h); (e) JNK2; (f) JNK3. Compound **36** is highlighted in the graphs with the symbol (*).

genotype 1b replicon assay.[12] It is interesting to note that the adaptive mutation S2204I is known to reduce hyperphosphorylation of NS5A. In addition, a recent report showed that kinase inhibitors, which change the hyperphosphorylation status of NS5A, modulate HCV replication.[14] Therefore it is intriguing to speculate that this series of pteridines is inhibiting HCV RNA replication by an indirect mechanism that would modulate the phosphorylation state of NS5A.

Interestingly, the introduction of Y2065H, a previously reported resistance mutation in the N-terminal domain of NS5A, had no effect on susceptibility to **36**.[15]

Before investigating further the mechanism by which these pteridines inhibit HCV replication, we determined if the lead compound **36** displayed DMPK properties which are compatible with in vivo efficacy testing.

The plasma kinetics, oral bioavailability together with heart and liver-plasma tissue distribution in male Swiss SPF (CD1)-mice were determined after a single oral administration of 20 mg/kg of **36** using 50% PEG-400 in water as vehicle. The compound levels were quantifiable up to 8 h post administration. As shown in Figure 2 and Table 3, time profile of the heart, liver, and plasma concentration is similar, indicating distribution equilibrium between plasma and these tissues. The mean maximum concentrations (C_{max}) in the plasma, heart, and liver were all achieved at 0.5 h post-dose (T_{max}), indicating a rapid absorption of **36**. Given that viral replication of HCV is reported to occur primarily in hepatocytes, achieving high drug concentrations in the liver is believed to be critical for the clinical success of HCV inhibitors. In this respect, **36** was found to be well distributed with the highest concentration observed in the liver (4057 ng/g), followed by heart (678 ng/g) and plasma (220 ng/mL). Furthermore, the mean half-life of **36** in the plasma ($t_{1/2\ (2-8\ h)}$) is 2.9 h, which is comparable with those of liver (3.5 h), and heart (3.4 h) and the AUC_{0-inf} is equal to 332 ng.h/mL. Interestingly, the tissue levels decline in a pattern similar to plasma and, no evidence of retention was found during this study.

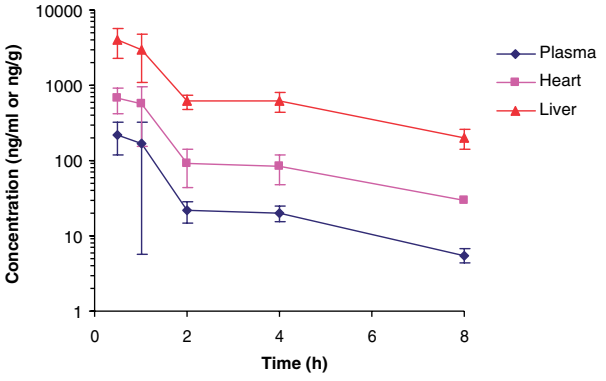


Figure 2. Mean plasma and tissue concentrations ($n = 3$) of **36** after a single oral administration at 20 mg base-equiv/kg in the male Swiss SPF (CD1)-mice.

Table 3. Mean plasma and tissue levels ($n = 3$) together with some basic pharmacokinetic parameters of pteridine **36** after a single oral administration of 20 mg base-equiv/kg in the male Swiss SPF (CD1)-mice

	Concentrations (ng/mL)		
	Plasma	Heart	Liver
0.5 h	220 (±100)	678 (±258)	4057 (±1730)
1 h	166 (±160)	557 (±403)	2937 (±1852)
2 h	21.7 (±6.7)	92.9 (±49.9)	611 (±131)
4 h	20.3 (±5.1)	83.6 (±35.9)	627 (±189)
8 h	5.52 (±1.2)	29.1 (±6.0)	201 (±59)
24 h	BQL[a] BQL[a]	BQL[a]	
C_{max} (ng/ml)	220	678	4057
T_{max} (h)	0.5	0.5	0.5
$t_{1/2(2-8\ h)}$ (h)	2.9	3.4	3.5
$AUC_{(0-8\ h)}$ (ng h/mL)	309	1119	6965
AUC_{0-inf} (ng h/mL)	332	1262	7973
Ratio tissue/plasma	—	3.8[b] 24[b]	

[a] BQL, below the limit of quantification. LLOQ was 0.500 ng/ml for plasma and ranged between 5.00 and 13.16 ng/g for tissue.
[b] Value based upon the AUC_{inf}.

In conclusion, we have reported here a novel series of potent and non-cytotoxic HCV inhibitors, which exhibits selective nanomolar activity toward both JNK and VEGFR kinase families. Among the compounds analyzed, 4-[2-(5-bromo-2-fluoro-phenyl)pteridin-4-ylamino]-*N*-[3-(2- oxopyrrolidin-1-yl)propyl]nicotinamide **36** was found to be the most potent HCV-replicon inhibitor (EC_{50} = 64 nM). Experiments performed on other replicon constructs suggest that **36** mediates its effect through NS5A. Moreover, **36** exhibits advantageous DMPK properties in mice. Additional studies are underway to further elucidate the mechanism by which this series of pteridines inhibits HCV replication.

References and notes

1. Choo, Q. L.; Kuo, G.; Weiner, A. J.; Overbv, L. R.; Bradley, D. W.; Houghton, M. *Science* **1989**, *244*, 359.
2. Shepard, C. W.; Alter, M. J. *Lancet* **2005**, *5*, 524.
3. Lauer, G. M.; Walker, B. D. *N. Engl. J. Med.* **2001**, *345*, 41.
4. Dugar, S.; Chakravarty, S.; Murphy, A.; McEnroe, G,; Conte, A.; Perumattam, J.J. WO2005032481A2.
5. (a) Tan, S.-L.; Pause, A.; Shi, Y.; Sonenberg, N. *Nat. Rev. Drug Disc.* **2002**, *1*, 867; (b) Poynard, T.; Yuen, M.-F.; Ratziu, V.; Lai, C. L. *Lancet* **2003**, *362*, 2095; (c) Manns, M. P.; McHutchison, J. G.; Gordon, S. C.; Rustgi, V. K.; Shiffman, M.; Reindollar, R.; Goodman, Z. D.; Koury, K.; Ling, M.-H.; Albrecht, J. K. *Lancet* **2001**, *358*, 958; (d) Poynard, T.; Macrellin, P.; Lee, S. S.; Niederau, C.; Minuk, G. S.; Ideo, G.; Bain, V.; Heathcote, J.; Zeuzem, S.; Trepo, C.; Albrecht, J. *Lancet* **1998**, *352*, 1426.
6. (a) Fried, M. W.; Shiffman, M. L.; Reddy, K. R.; Smith, C.; Marinos, G.; Gonçales, F. L., Jr.; Häussinger, D.; Diago, M.; Carosi, G.; Dhumeaux, D.; Craxi, A.; Lin, A.; Hoffman, J.; Yu, J. *N. Engl. J. Med.* **2002**, *347*, 975; (b) Davis, G. L.; Wong, J. B.; McHutchison, J. G.; Manns, M. P.; Harvey, J.; Albrecht, J. *Hepatology* **2003**, *38*, 645; (c) Scott, L. J.; Perry, C. M. *Drugs* **2002**, *62*, 507.

7. Fried Michael, W. *Hepatology* **2002**, *36*, S237.
8. Gordon, C. P.; Keller, P. A. *J. Med. Chem.* **2005**, *48*, 1; Beaulieu, P. L.; Tsantrizos, Y. S. *Curr. Opin. Invest. Drugs* **2004**, *5*, 838.
9. Bartenschlager, R.; Lohmann, V. *Antiviral Res.* **2001**, *52*, 1.
10. Schuppan, D.; Krebs, A.; Bauer, M. *Cell Death Differ.* **2003**, *10*, S59.
11. Neddermann, P.; Quintavalle, M.; DiPietro, C.; Clementi, A.; Cerretani, M.; Altamura, S.; Bartholomew, L.; DeFrancesco, R. *J. Virol.* **2004**, 13306.
12. Lohmann, V.; Hoffmann, S.; Herian, U.; Penin, F.; Bartenschlager, R. *J. Virol.* **2003**, *77*, 3007.
13. Axon, J.; Chakravarty, S.; Hart, B.; Mcenroe, G.; Murphy, A.; Pontius, K.; Sheng, D.; Wang, G.; Yellapregada, S. WO2006105222 A2.
14. Neddermann, P.; Quintavalle, M.; Di Pietro, C.; Clementi, A.; Cerretani, M.; Altamura, S.; Bartholomew, L.; De Francesco, R. *J. Virol.* **2004**, *78*, 13306.
15. Horscroft, N.; Lay, V. C.; Cheney, W.; Yao, N.; Wu, J. Z.; Hong, Z.; Zhong, W. *Antivir. Chem. Chemother.* **2005**, *16*, 1.