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Peroxisome proliferator-activated receptor delta antagonists inhibit hepatitis C virus RNA replication

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ABSTRACT

It has been reported that ligand-mediated transcription factor peroxisome proliferator-activated receptor alpha (hPPARα) is involved in hepatitis C virus (HCV) RNA replication, whereas hPPARγ is not, and the effect of hPPARδ is unknown. Here, we show that hPPARδ-selective antagonists effectively inhibit HCV RNA replication. We describe the design, synthesis and pharmacological evaluation of a series of biphenyl-4-carboxylic acid-type hPPARδ antagonists, including previously reported compounds, as candidate anti-HCV agents. A representative compound (**4c**) dose-dependently inhibited HCV RNA replication (EC_{50} 0.22 μM), while exhibiting relatively weak cytotoxicity to the host cells (CC_{50} 2.5 μM). It also showed an additive and dose-dependent effect on the inhibition of HCV RNA replication by pegylated interferon alpha (Peg-IFNα) alone and by both Peg-IFNα and ribavirin (currently the clinical treatment of choice for HCV infection). Thus, combination of a hPPARδ antagonist with current therapy may improve the efficacy of treatment for HCV infection.

Hepatitis C virus (HCV) is a member of the genus *Hepacivirus* of the family Flaviviridae, and approximately 180 million people worldwide were reported to be infected with it in 2007 (World Health Organization (WHO), 2007). It induces serious chronic hepatitis, leading to steatosis, cirrhosis, and ultimately hepatocellular carcinoma.[1] HCV infection is involved in about 50–70% of liver cancers, and is the underlying reason behind two–thirds of all liver transplants in the developed world.[1] The standard treatment for HCV infection is combination therapy with pegylated interferon-α (Peg-IFNα) and ribavirin, but this results in a sustained virological response (SVR) in only 40–50% of the patients infected with genotype 1 virus.[2] Therefore, there is an urgent need for new anti-HCV drugs.

HCV has a single-stranded RNA of positive polarity that encodes a polyprotein with ca. 3000 amino acid residues.[3] After maturation, this is cleaved into at least 10 proteins: structural proteins termed Core, E1, E2, and p7, and nonstructural proteins termed NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 2).[4] The viral RNA-dependent RNA polymerase is encoded by NS5b.[5] Posttranslational processing of the nonstructural proteins is catalyzed by the serine protease NS3,[6,7] together with the co-factor NS4A, which promotes anchorage on the endoplasmic reticulum membrane.[8] These molecules have been selected as targets of direct-acting antivirals (DAA),[9,10] but owing to the rapidly mutating nature of the HCV genome, drug-resistant mutants readily appear.

However, host lipid contents and host lipid homeostasis are also important factors for the assembly, budding and replication of many viruses, including HCV.[11–13] For example, exogenous administration of saturated and/or monounsaturated fatty acids enhanced HCV RNA replication, while polyunsaturated fatty acids suppressed it.[14] Therefore, we considered that receptor(s) and/or enzyme(s) associated with lipid biosynthesis might also be important target(s) for the development of anti-HCV agents. Sterol regulatory element-binding protein-1c (SREBP-1C) is a key protein for production of saturated and monounsaturated fatty acids, via up-regulation of the transcription of acyl-CoA carboxylase, fatty acid synthase and stearoyl-CoA desaturase.[15] Consequently, compounds that disrupt the production and/or function of SREBP-1c might be candidate anti-HCV agents. Here, we focused on metabolic nuclear receptors, especially peroxisome-proliferator activated receptors (PPARs).

PPARs are ligand-dependent transcription factors belonging to the nuclear receptor (NR) superfamily. The three subtypes (PPARα, PPARδ, and PPARγ) identified to date are differentially expressed in a tissue-specific manner, and play pivotal roles in lipid, lipoprotein, and glucose homeostasis.[16] However, the range of therapeutic potential for hPPAR ligands is currently considered to extend well beyond lipid, lipoprotein and glucose homeostasis, and so the biology and pharmacology of hPPARs are attracting great interest.[17–19]

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RNA interference experiments targeting hPPARα and hPPARγ have indicated that knockdown of hPPARα mRNA inhibits HCV replication, whereas knockdown of hPPARγ mRNA had no effect.[20] A perturbation study with small-molecular-weight compounds indicated that a high concentration of hPPARγ/α co-antagonist, T0070907 (**1**) (Fig. 2) reduced HCV RNA replication to a comparable extent to interferon treatment, using a subgenomic HCV replicon, via the hPPARα pathway.[20] These data clearly indicated the existence of a subtype-specific link between hPPARα and HCV replication. However, the effect of hPPARδ on HCV replication has not been established. Therefore, here we investigated the effect of our hPPARδ-selective biphenylcarboxylic acid-type ligands on HCV RNA replication.

In order to investigate the putative role of hPPARδ in HCV RNA replication, we used the OR6 assay system, which is the first replication system for genome-length HCV RNA encoding a reporter gene for simple monitoring of HCV replication levels.[21,22] A schematic representation of the replicon used in this Letter is depicted in Figure 1.

Using OR6 cells, we first performed WST-1 cell proliferation assay to determine the appropriate concentrations of compounds for the assay of anti-HCV activity (data not shown).[23] The cells were plated onto 96-well plates (1×10^3 cells per well) in triplicate and then treated with each reagent at three or four concentrations for 72 h. After treatment, the cells were subjected to WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. Based on the assay results, we chose the concentration showing a relative activity of approximately 80% as the maximum concentration for anti-HCV activity assay. For the *Renilla* luciferase (RL) assay, 2×10^4 OR6 cells were plated in 24-well plates at least in triplicate for each assay and cultured for 24 h. The cells were treated with the test compounds for 72 h, then harvested with *Renilla* lysis reagent (Promega) and subjected to the RL assay according to the manufacturer's protocol. In several cases,

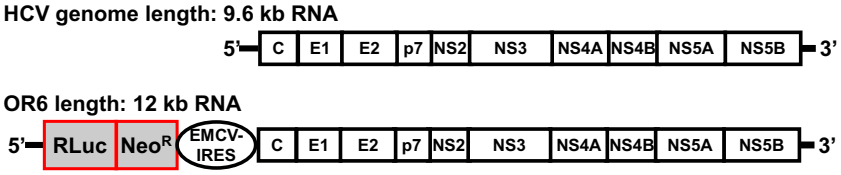


Figure 1. Schematic representation of the replicon (OR6) used in this Letter. *Abbreviations:* RLuc, *Renilla* luciferase gene; Neo^R, neomycin phosphotransferase; EMCV-IRE, Sencephalomyocarditis virus internal ribosomal entry site

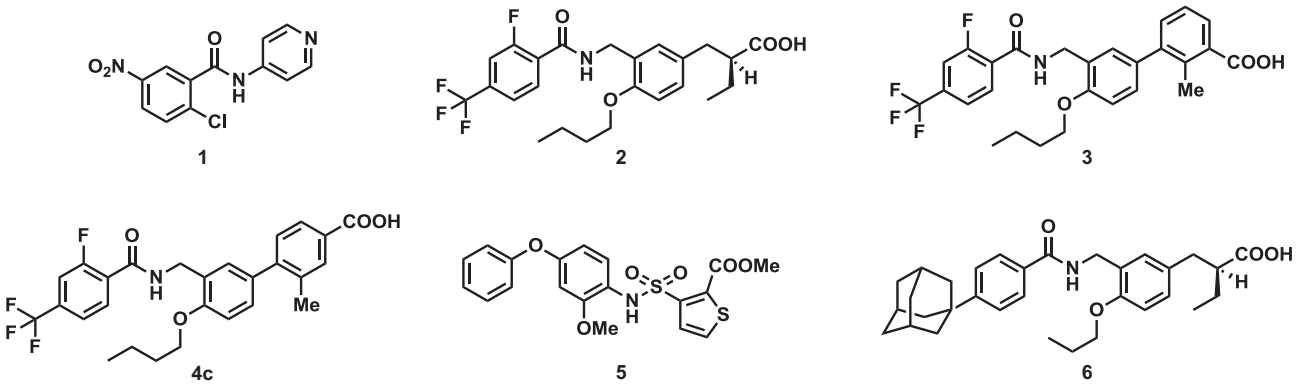


Figure 2. Structures of hPPARγ/α-co-antagonist, T0070907 (**1**), hPPARδ-selective agonist TIPP-204 (**2**), hPPARδ-partial agonist (**3**), hPPARδ-selective antagonist (**4c**), hPPARδ-selective antagonist GSK-0660 (**5**) and hPPAR-pan agonist TIPP-703 (**6**).

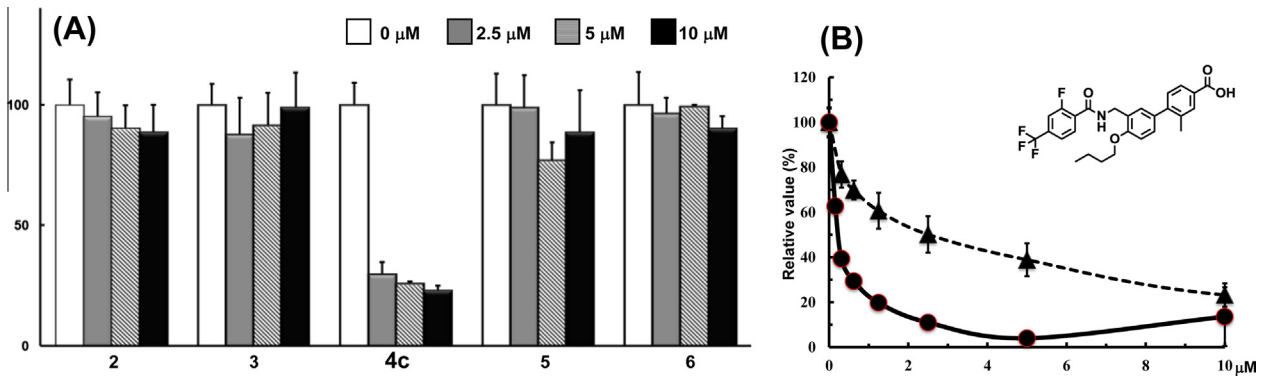
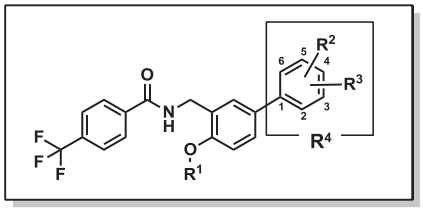


Figure 3. Effects of various hPPARs ligands in the genome-length HCV RNA replication system bearing the *Renilla* luciferase reporter. (A) Inhibition of replication of genome-length HCV RNA encoding the *Renilla* luciferase gene was monitored by luciferase reporter assay. (B) Dose–response relationship of the representative compound **4c** for inhibition of HCV RNA replication (closed circle) and for cell toxicity (closed triangle).



				HCV RNA replication inhibition			PPARδ antagonism	PPARα antagonism
compd.	R^1	R^2	R^3	ED_{50} (μM)	CC_{50} (μM)	SI	IC_{50} (μM)	% inhibition@10 μM
4a	*n*-Butyl	6-Me	3-CO_2H	ia	nt	-	ia	nt
4b	*n*-Butyl	4-Me	3-CO_2H	ia	nt	-	ia	nt
4c	*n*-Butyl	2-Me	4-CO_2H	0.28	5.0	18	0.019	12.6
4d	Et	2-Me	4-CO_2H	0.39	3.9	10	0.028	49.5
4e	*n*-Propyl	2-Me	4-CO_2H	0.22	2.2	11	0.029	49.5
4f	*n*-Pentyl	2-Me	4-CO_2H	0.53	2.7	5.0	0.092	23.3
4g	*n*-Butyl	2-F	4-CO_2H	3.9	8.1	2.1	0.1	25.4
4h	*n*-Butyl	2-Cl	4-CO_2H	1.8	3.2	1.8	0.013	30.7
RBV				5.6	100	18	nt	nt
1				4.9	2.7	0.55	> 10	31.8

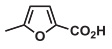
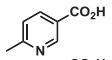
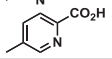
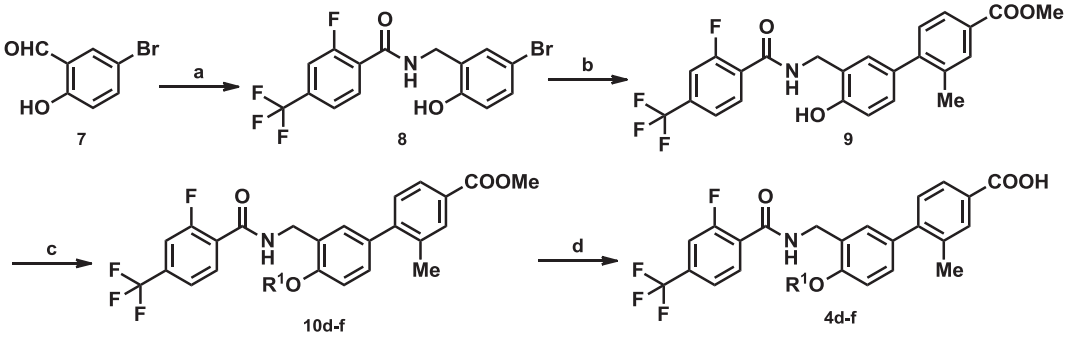
No.	R^1	R^4	ED_{50}	CC_{50}	SI	IC_{50}	% inhibition@10 μM
4i	*n*-Butyl		ia	nt	-	ia	nt
4j	*n*-Butyl		ia	nt	-	ia	nt
4k	*n*-Butyl		ia	nt	-	ia	nt

Figure 4. Structure–activity relationships of biphenyl carboxylic acid derivatives for HCV RNA replication inhibition and for hPPARδ/α-antagonistic activity. Antiviral ribavirin (RBV) and T0070907 (**1**) were evaluated as positive controls (HCV RNA replication inhibitor and hPPARγ/α-co antagonist, respectively).



Scheme 1. Synthetic routes to the present series of compounds **4d–f**. Reagents and conditions: (a) benzamide, triethylsilane, TFA, toluene, reflux, 48 h, 30%; (b) 4-methoxycarbonyl-2-methylbenzeneboronic acid pinacol ester, $(Ph_3P)_2PdCl_2$, K_2CO_3, THF, H_2O, 60 °C, 5 h, 20%; (c) R^1I, K_2CO_3, DMF, rt, overnight, 62–89%; (d) LiOH, MeOH, H_2O, THF, 100 °C, 3 h, 90–96%.

the 50% cytotoxic concentration (CC_{50}) was determined. The value of selectivity index (SI) was determined by dividing the CC_{50} value by the EC_{50} value.

The chemical structures of the hPPARs ligands used in our initial study are depicted in Figure 2, and their effect on HCV replication is shown in Figure 3.

The hPPARδ-selective agonist **2**,[24] hPPARδ-selective partial agonist **3**,[25] hPPARδ-selective antagonist **5** (GSK-0660),[26] and hPPAR-pan agonist **6**[27] all had no effect on HCV RNA replication (their chemical structures are shown in Fig. 1). On the other hand, the hPPARδ-selective antagonist **4c**[25] dose-dependently inhibited HCV RNA replication, while its cytotoxicity to the host cells was relatively weak (EC_{50}, CC_{50} and the SI were 0.22, 2.5, and 11 μM, respectively). These data indicated that the hPPARδ pathway might be involved in HCV RNA replication, in addition to the previously reported contribution of the hPPARα pathway. Although another structural class of hPPARδ-selective antagonist **5** had no apparent inhibitory activity on HCV RNA replication, the reason for this might be that it lacks in vivo bioavailability.

We then examined the effects of our previously reported hPPARδ-selective biphenyl-4-carboxylic acid-type antagonists on HCV RNA replication (Fig. 4). The alkoxy chain variants, **4d–4f**, were newly prepared according to the method illustrated in Scheme 1,[28] and the hPPARδ–antagonistic activity (IC_{50} value) was determined in the presence of 1 μM hPPAR-pan agonist **6**. The hPPARα–antagonistic activity (% inhibition) of representative compounds was also determined in the presence of 1 μM hPPAR-pan agonist **6**.

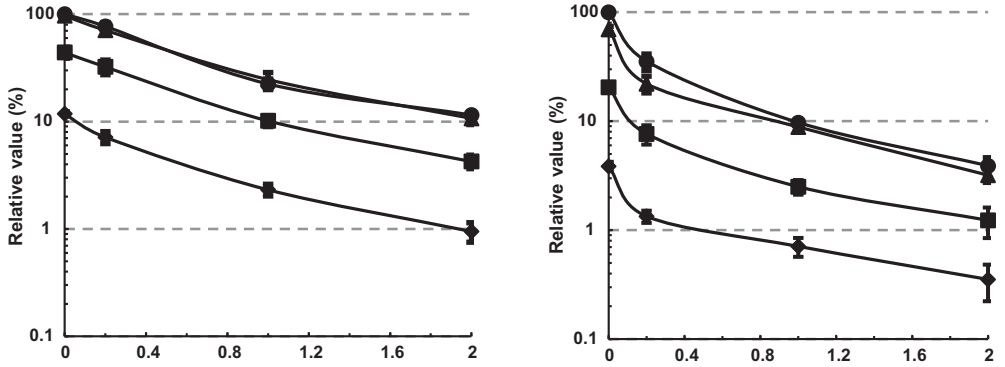


Figure 5. Additive effect on HCV RNA replication of the representative hPPARδ-antagonist **4c** (μM) in combination with IFNα alone (left) and with IFNα plus ribavirin (right). (Left) closed circle: IFN-α 0 IU/mL (IU: international unit), closed triangle: IFN-α 1 IU/mL, closed box: IFN-α 4 IU/mL), closed diamond: IFN-α 16 IU/mL. (Right) closed circle: IFN-α 0 IU/mL + RBV 0 μM, closed triangle: IFN-α 1 IU/mL + RBV 6.25 μM, closed box: IFN-α 4 IU/mL + RBV 12.5 μM, closed diamond: IFN-α 16 IU/mL + RBV 25 μM.

As previously reported, biphenyl-3-carboxylic acids (**4a** and **4b**) did not exhibit hPPARδ–antagonistic activity, but showed hPPARδ-partial agonistic activity, while biphenyl-4-carboxylic acids (**4c**–**4h**) exhibited hPPARδ–antagonistic activity.[25] Therefore, the position of the carboxylic acid functionality is critically important for the antagonistic nature of the present series of compounds, although the chain length of the central alkoxy group might not critical for activity, because all the alkoxy variants exhibited comparably potent hPPARδ–antagonistic activity (IC_{50} values reached 10 nM order). It is interesting that heteroatom-containing carboxylic acids (**4i**–**4k**) did not exhibit apparent hPPARδ–antagonistic activity, although the reason for this is not known.

In our assay system, **1** exhibited very weak hPPARδ–antagonistic activity (IC_{50} > 10 μM), and the antiviral ribavirin (RBV) did not exhibit apparent hPPARδ–antagonistic activity even at the highest concentration examined (10 μM).

We selected compounds **1**, **4c**–**4e** as representative compounds and evaluated their hPPARα–antagonistic activity. However, their activity was too weak to permit calculation of IC_{50} values. Among these four compounds, **4c** was the least effective, and it did not exhibit apparent hPPARα–antagonistic activity even at the highest examined concentration of 10 μM. Therefore, **4c** is the most hPPARδ-selective antagonist tested, and its selectivity ratio exceeded 1000 in our assay system.

As for HCV RNA replication, **1** exhibited a micromolar order ED_{50} value, but host cell toxicity appeared prior to the effect on HCV RNA replication. Therefore, the nature of the effect of **1** on HCV RNA replication is uncertain. The antiviral ribavirin (RBV) also exhibited a micromolar order ED_{50} value without concomitant host cell toxicity, and its SI value exceeded 10. hPPARδ-inactive compounds showed no response in our assay system, as expected, while all the biphenyl-4-carboxylic acid-type hPPARδ-antagonists inhibited HCV RNA replication. Indeed, the methyl derivatives at the R^2 position (**4c**–**4f**) exhibited submicromolar order EC_{50} values, being about 10 times more potent than the currently used anti-HCV agent RBV or the reported hPPARγα-co-antagonist **1**. Their SIs were more than 10 (except for compound **4f**), and the host cell toxicities were relatively weak. These data support the idea that the hPPARδ pathway might be involved in HCV RNA replication.

Since the current treatment for HCV infection is Peg-IFNα combined with ribavirin, we next evaluated the effect on HCV RNA replication of the representative hPPARδ-antagonist **4c** in combination with Peg-IFNα alone, or with both Peg-IFNα and ribavirin. As shown in Figure 5, HCV RNA was reduced by Peg-IFNα alone, and by Peg-IFNα in combination with ribavirin, in a dose-dependent manner. Furthermore, addition of **4c** dose-dependently increased the inhibition of HCV RNA replication by these agents. These results indicate that combination of **4c** with the current therapy of choice for the treatment for HCV infection has the potential to improve the outcome, at least in terms of inhibition of HCV RNA replication.

In summary, we have established that the hPPARδ pathway is involved in HCV RNA replication, in addition to the hPPARα pathway. We also found that biphenyl-4-carboxylic acid-type hPPARδ-selective antagonists, such as **4c**, effectively inhibited HCV RNA replication, with low levels of host cell toxicity. Compound **4c** showed a dose-dependent, additive effect on the inhibition of HCV RNA replication when used in combination with current HCV therapy. Further structural development studies are under way, and we are also attempting to isolate mutants resistant to OR6 in order to clarify the nature of the linkage between the hPPARδ pathway and HCV RNA replication.

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28. The physicochemical properties of the compounds **4d**–**4f** are as follows: *4-[4-ethoxy-3-({[2-fluoro-4-(trifluoromethyl)benzoyl]amino}methyl)phenyl]-3-methyl-benzoic acid (**4d**):* mp 260–261 °C. ^{1}H NMR (400 MHz, DMSO) δ 12.86 (s, 1H), 8.94 (dd, J = 6.0, 6.0 Hz, 1H), 7.83–7.74 (m, 4H), 7.65 (d, J = 8.0, 1H), 7.27–7.22 (m, 3H), 7.07 (d, J = 9.2 Hz, 1H), 4.49 (d, J = 5.6 Hz, 2H), 4.12 (q, J = 6.8 Hz, 2H), 2.27 (s, 3H), 1.38 (t, J = 6.8 Hz, 3H). HRMS (FAB) calcd for $C_{25}H_{22}F_4NO_4$ 476.1485; found 476.1466 $(M+H)^+$.
*4-[3-({[2-Fluoro-4-(trifluoromethyl)benzoyl]amino}methyl)-4-propoxyphenyl]-3-methyl-benzoic acid (**4e**):* mp 224–225 °C. ^{1}H NMR (400 MHz, DMSO) δ 12.87 (s, 1H), 8.92 (dd, J = 6.0, 6.0 Hz, 1H), 7.85–7.76 (m, 4H), 7.65 (d, J = 8.0 Hz, 1H), 7.27–7.24 (m, 3H), 7.07 (d, J = 8.8 Hz, 1H), 4.51 (d, J = 5.6 Hz, 2H), 4.03 (t, J = 6.4 Hz, 2H), 2.27 (s, 3H), 1.96–1.86 (m, 2H), 1.11 (t, J = 7.2 Hz, 3H). HRMS (FAB) calcd for $C_{26}H_{24}F_4NO_4$ 490.1642; found 490.1647 $(M+H)^+$.
*4-[3-({[2-Fluoro-4-(trifluoromethyl)benzoyl]amino}methyl)-4-pentoxyphenyl]-3-methyl-benzoic acid (**4f**):* mp 228–230 °C. ^{1}H NMR (400 MHz, DMSO) δ 12.87 (s, 1H), 8.93 (dd, J = 5.6, 5.6 Hz, 1H), 7.83–7.76 (m, 4H), 7.65 (d, J = 8.0 Hz, 1H), 7.27–7.24 (m, 3H), 7.06 (d, J = 9.2 Hz, 1H), 4.50 (d, J = 5.6 Hz, 2H), 4.05 (t, J = 6.4 Hz, 2H), 2.27 (s, 3H), 1.80–1.73 (m, 2H), 1.48–1.31 (m, 4H), 0.88 (t, J = 7.2 Hz, 3H). HRMS (FAB) calcd for $C_{28}H_{28}F_4NO_4$ 518.1956; found 518.1965 $(M+H)^+$.