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Identification of Piperazinybenzenesulfonamides as New Inhibitors of Claudin-1 Trafficking and Hepatitis C Virus Entry

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1 **IDENTIFICATION OF PIPERAZINYLBENZENESULFONAMIDES**
2 **AS NEW INHIBITORS OF CLAUDIN-1 TRAFFICKING**
3 **AND HEPATITIS C VIRUS ENTRY**

4
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28 Running Head: 5-HT6 inhibitors affect CLDN1 export and HCV entry

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38 junction protein

41 **ABSTRACT**

42 Hepatitis C virus (HCV) infection causes 500,000 deaths annually, associated with end-stage
43 liver diseases. Investigations of the HCV life cycle widened the knowledge in virology, and
44 here we discovered that two piperazinylbenzenesulfonamides inhibit HCV entry into liver
45 cells. The entry process of HCV into host cells is a complex process, not fully understood, but
46 characterized by multiple spatially and temporally regulated steps involving several known
47 host factors. Through a high-content virus-infection screening analysis with a library of 1,120
48 biologically active chemical compounds, we identified SB258585, an antagonist of the
49 serotonin receptor 6 (5-HT6), as a new inhibitor of HCV entry in liver-derived cell lines, as
50 well as in primary hepatocytes. A functional characterization suggested a role for this
51 compound, as well as for the compound SB399885 sharing a similar structure, as inhibitors of
52 a late HCV entry step, modulating the localization of the co-receptor tight junction protein
53 claudin 1 (CLDN1), in a 5-HT6 independent manner. Both chemical compounds induced an
54 intracellular accumulation of CLDN1, reflecting export impairment. This regulation
55 correlated with the modulation of protein kinase A (PKA) activity. The PKA inhibitor H89
56 fully reproduced these phenotypes. Furthermore, PKA activation resulted in increased
57 CLDN1 accumulation at the cell surface. Interestingly, increase of CLDN1 recycling did not
58 correlate with an increased interaction with CD81 or HCV entry. These findings reinforce the
59 hypothesis of a common pathway shared by several viruses, which involves G-protein
60 coupled receptor -dependent signaling in late steps of viral entry.

61 **IMPORTANCE**

62 The HCV entry process is highly complex and important details of this structured event are
63 poorly understood. By screening a library of biologically active chemical compounds, we
64 identified two piperazinylbenzenesulfonamides as inhibitors of HCV entry. The mechanism
65 of inhibition was not through previously described activity of these inhibitors as antagonists

66 of the serotonin receptor 6, but instead through modulation of PKA activity in a 5-HT6
67 independent manner, as proven by the lack of 5-HT6 in liver. We thus highlighted the
68 involvement of PKA pathway in modulating HCV post-binding step entry and in the
69 recycling of the tight junction protein claudin-1 (CLDN1) towards the cell surface. Our work
70 underscores once more the complexity of HCV entry steps and suggests a role for PKA
71 pathway as regulator of CLDN1 recycling, having an impact on both cell biology and
72 virology.

73

74 **INTRODUCTION**

75 Virus entry into specific host cells is a tightly regulated multistep process. Among
76 known viruses, the liver pathogen hepatitis C virus (HCV), which causes 500,000 deaths
77 annually, has probably one of the most complex multistep entry processes involving a
78 growing number of cellular entry factors (1). Furthermore, the regulation of trafficking of
79 these proteins provides an additional level of complexity. Although the involvement of a large
80 number of host factors has been demonstrated in HCV entry, several gaps remain in our
81 comprehension of this process. Further functional studies are therefore needed to better
82 understand the regulation of the early steps of the HCV life cycle.

83 After binding to attachment factors such as heparan sulfate proteoglycans and the low
84 density lipoprotein (LDL) receptor, HCV particles specifically bind to the entry receptors
85 scavenger receptor BI (SRB1) and CD81 tetraspanin (2). HCV binding to CD81 induces a
86 diffusion of this virion-tetraspanin complex on the plasma membrane, towards the sites of
87 viral internalization (3). Only a specific fraction of CD81, located outside of tetraspanin-
88 enriched areas, is involved in HCV entry (4) through its interaction with the tight junction
89 protein claudin 1 (CLDN1) (5–7). Importantly, CD81-CLDN1 interaction is a major event
90 driving HCV endocytosis (8). Among other identified host factors involved in HCV entry (2),

91 occludin (OCLN), another tight-junction protein, also plays a major role in a late step of HCV
92 entry through an unknown mechanism (9). Together, these observations indicate that tight
93 junction proteins play a central role in HCV entry. However, the regulation of these proteins
94 in the liver remains poorly understood and little information is available on CLDN1
95 trafficking and its interplay with CD81 and OCLN both in infected and non-infected cells.

96 In this study, we implemented a high-content screening (HCS) approach using a
97 library of chemical compounds to search for new regulators of HCV entry. We identified
98 serotonin receptor 6 (5-HT6) antagonists SB258585 and SB399885 as inhibitors of a late step
99 of HCV entry. More specifically, we showed a reduction of PKA activation upon treatment,
100 in a 5-HT6-independent manner. We confirmed the involvement of PKA pathway in HCV
101 entry and a role of its activation in CLDN1 export. Taken together, these data identify new
102 inhibitors of HCV post-binding step and provide new findings in regulation of CLDN1
103 transport and function. This work highlights once more the complexity of the HCV entry
104 process proving that the presence of CLDN1 at the cell surface is not sufficient to mediate its
105 interaction with CD81. This confirms the importance of the membrane environment and the
106 need of the synergistic action of several receptors in HCV entry.

107

108 **RESULTS**

109 **Identification of the 5-HT6 antagonist SB258585 as a new inhibitor of the HCV life** 110 **cycle.**

111 To identify new cellular factors involved in the early steps of the HCV infectious
112 cycle, we performed a HCS using a library of 1,120 biologically active chemical compounds.
113 The screen was repeated using three different concentrations (1 μ M, 10 μ M and 20 μ M) in
114 Huh-7 cells infected with cell culture derived HCV (HCVcc) strain JFH1 (genotype 2a) (10,
115 11). A re-infection experiment was performed as an additional filter to eliminate false-

116 positive compounds (Supplemental dataset S1). Compounds reducing the infection by at least
117 45% compared to the average of DMSO wells affecting cell viability for less than 35% were
118 considered as positive hits (Fig. 1A-C). Finally, we only retained compounds confirmed in the
119 re-infection experiment and inhibiting HCV infection for at least two of the three tested
120 concentrations (Fig. 1D and Table 1). According to these criteria, 20 compounds were
121 selected. Nine of them had been previously described as having an inhibitory effect on HCV
122 or targeting cellular pathways identified as important for HCV infection, thus validating our
123 screen (12–16). Among the 11 remaining hits, we identified 5 compounds targeting serotonin
124 (5-HT) receptors or transporters. Serotonin receptors constitute a class of 16 human receptors,
125 none of which have ever been identified as host factor involved in the HCV infectious cycle.
126 To identify the 5-HT receptor(s) playing a role in HCV infection, we searched the Tocriscreen
127 Total library for all the compounds targeting in a more or less specific manner each of these
128 receptors, in accordance with data available on the following databases:
129 <http://www.guidetopharmacology.org/> and <http://stitch.embl.de> (17). Interestingly, with only
130 one exception, all the compounds targeting the serotonin receptor 6 (5-HT6) showed a dose-
131 response inhibitory effect on HCV JFH1 infection (Fig. 1E), in contrast to the compounds
132 targeting other 5-HT receptors where, for most of them, only the highest concentration
133 showed an inhibitory activity. Among the inhibitors of 5-HT6, SB258585 was identified as a
134 hit according to the criteria of lack of cell toxicity and inhibition of infection chosen in the
135 screen (Fig. 1F and Table 1).

136

137 **SB258585 inhibits HCV infection, down-regulating PKA activity independently of 5-**
138 **HT6 receptor.**

139 In order to confirm the inhibitory effect of 5-HT6 antagonist SB258585 on HCV viral
140 cycle, kinetics of JFH1 infection were performed in the presence of this compound. The

141 maximum inhibitory effect of SB258585 was observed when the chemical compound was
142 added during the inoculation of the virus (Fig. 2A), suggesting an effect on virus entry. As
143 shown in Fig. 2B, the inhibition was not due to drug toxicity.

144 5-HT6 receptor, mainly studied in the central nervous system, has not been
145 characterized in the liver or hepatocyte. In order to determine its real involvement in HCV
146 infection as a target of SB258585, we quantified its expression level in the liver. To do so, we
147 compared its distribution by qRT-PCR in seventeen different human tissues. This analysis
148 showed that 5-HT6 was highly expressed in brain tissues and intestine (Fig. 2C). It was also
149 expressed in testis, while it was not detected in all the other tissues, including the liver (Fig.
150 2C). Quantification of 5-HT6 mRNA in Huh-7 cells by qRT-PCR showed a Δ Ct of around 18
151 as compared to the housekeeping gene RPLP0, confirming an almost complete absence of
152 detection of 5-HT6 in this hepatic cell line. Not surprisingly, we were unable to detect the 5-
153 HT6 protein by western blot and flow cytometry by using different antibodies (data not
154 shown). This observation implies that the effect observed on HCV infection is probably not
155 connected to 5-HT6.

156 5-HT6 is a G-protein coupled receptor (GPCR), associated with a G alpha stimulatory protein
157 ($G\alpha_s$). This $G\alpha_s$ activates the adenylyl cyclase to produce cAMP, which in turn activates
158 protein kinase A (PKA) (18). However, GPCR agonists and antagonists often show affinity
159 for additional GPCR than the one specifically targeted. Therefore, we evaluated whether the
160 presence of 5-HT6 antagonist leads to a regulation of the PKA pathway, likely through
161 modulation of other GPCRs. We thus performed a western blot with an antibody specific for
162 PKA phosphorylated substrates. The cell permeable inhibitor of cAMP-dependent protein
163 kinase (PKA) H89 was used as a positive control. This compound is described to inhibit PKA
164 by competitive binding to the ATP site on PKA catalytic subunit (19). As shown in Fig. 2D,

165 SB258585 reduced the phosphorylation level of PKA substrates in a similar manner as the
166 PKA inhibitor H89.

167 Therefore, our observations about the phosphorylation level of PKA substrates suggest
168 that the off-target effect of SB258585 targets a factor involved in PKA activation, likely
169 another GPCR coupled to a G α s protein.

170

171 **SB258585 and SB399885 inhibit a late step of HCV entry, altering cell surface**
172 **localization of CLDN1.**

173 According to the kinetics shown in Fig. 2A, SB258585 seems to inhibit HCV entry.
174 The inhibitory effect of SB258585 on HCV entry was then validated with the help of
175 retroviral pseudo-particles harboring HCV envelope glycoproteins (HCVpp) from JFH1 strain
176 (genotype 2a) (Fig. 3A). SB258585 had no effect on adenovirus infection, indicating that this
177 compound has not a global effect on viral entry (Fig. 3B). We also confirmed its inhibitory
178 effect on primary human hepatocytes (PHHs) infected by HCV strain JFH1 (genotype 2a)
179 (Fig. 3C) (20), as well as on 18 different JFH1-based Core-NS2 strain recombinants
180 representing the six HCV major genotypes and important subtypes (Fig. 3D, Table 2) (21–
181 27).

182 In order to better understand the mechanism of action of SB258585 as an inhibitor of
183 HCV infection, we decided to test another characterized 5-HT₆ antagonist, SB399885 which
184 shares a very similar structure (Fig. 3F). The fact that both antagonists inhibit a common step
185 of HCV infection (Fig. 2A, 3G-H) in the absence of their main targeted receptor, modulating
186 PKA activity (Fig. 2D) suggests that their common structure of
187 piperazinybenzenesulfonamides is likely responsible for a common off-target effect.

188 To better identify the entry step targeted by these compounds, we performed a time-
189 of-addition assay, using proteinase K and bafilomycin A respectively as controls of early and

190 late step of viral entry. Proteinase K blocks viral internalization through proteolysis of viral
191 particles exposed to the extracellular compartment, while Bafilomycin A blocks the
192 endosomal acidification resulting in the inhibition of viral fusion (28). A comparison of
193 SB258585 and SB399885 to the kinetics of proteinase K and Bafilomycin A treatment rather
194 revealed an inhibition of a late step of the entry process (Fig. 3E, 3I). It is worth noting that
195 the slight difference in the SB258585 curves obtained at 20 μ M and 100 μ M was not
196 statistically significant.

197 Knowing that CD81-CLDN1 interaction is necessary for clathrin-dependent
198 endocytosis of HCV particles (8), we analyzed the effect of these
199 piperazinylbenzenesulfonamides on CD81-CLDN1 co-localization. A dose-dependent
200 decrease of CD81-CLDN1 co-localization was observed after treatment with SB258585, as
201 confirmed by determining the Pearson correlation coefficient (Fig. 4A-B). Furthermore, we
202 observed a decrease of cell surface CLDN1 expression by flow cytometry analyses in non-
203 infected (Fig. 4C) and infected cells (data not shown) treated with SB258585. This could
204 explain the alteration in CD81-CLDN1 co-localization. The same phenotype was also
205 observed in cells treated with SB399885 (Fig. 4D). Furthermore, kinetic analyses revealed
206 that the decrease of CLDN1 cell surface expression after SB258585 treatment is quite rapid
207 and reversible (Fig. 4E-F). It is worth noting that this compound had no effect on the
208 distribution of other major HCV entry co-receptors, including CD81 (Fig. 5).

209

210 **Alteration of cell surface expression of CLDN1 mediated by SB258585 is due to CLDN1**
211 **intracellular accumulation.**

212 Decreased CLDN1 cell surface expression could be due either to CLDN1 degradation
213 or intracellular accumulation. To answer this question, we first analyzed the total level of
214 CLDN1 by western blot. Treatment of Huh-7 cells with SB258585 and SB399885 did not

215 affect the amount of CLDN1 (Fig. 4G), indicating that these compounds do not induce
216 CLDN1 degradation. Instead, immunofluorescence analyses showed an intracellular
217 accumulation of CLDN1 in the presence of SB258585 (Fig. 4H), confirmed by an increased
218 co-localization with the intracellular markers TGN46 (Fig.4I), ERGIC53 and GM130 (data
219 not shown). This intracellular accumulation could be due either to an increase in CLDN1
220 endocytosis or to an alteration in CLDN1 export. To elucidate this, we used an internalization
221 assay based on cell surface biotinylation to determine the kinetics of CLDN1 endocytosis in
222 the presence or absence of the drug. As shown in Fig. 4J, CLDN1 endocytosis was maximal
223 after 1h. The protein amount was comparable in DMSO or SB258585 treated cells, suggesting
224 no alteration of CLDN1 endocytosis. Conversely, at later time points, biotinylated CLDN1
225 disappeared in DMSO treated cells, suggesting that it had either been degraded or recycled to
226 the cell surface, while it remained stored intracellularly in the presence of the compound. This
227 is in agreement with the accumulation of CLDN1 detected by immunofluorescence (Fig. 4H).
228 Since no change in total CLDN1 level was observed between DMSO and SB258585
229 treatment (Fig. 4G), it is unlikely that endocytosed CLDN1 is degraded. Rather, CLDN1 must
230 be recycled to the plasma membrane. Accumulation of intracellular CLDN1 observed after
231 SB258585 treatment (Fig. 4H-J) suggests a defect of CLDN1 recycling and explains the
232 decrease of CLDN1 at the cell surface.

233

234 **PKA inhibition affects HCV late entry step and regulates CLDN1 trafficking.**

235 To further confirm whether SB258585 inhibitory effect on HCV could be mediated by
236 the PKA downstream pathway, we tested the effect of PKA inhibitor H89 on HCV infection.
237 As reported by Farquhar and co-workers (29), we confirmed a dose-dependent inhibitory
238 effect of this compound on both HCVpp and HCVcc of JFH1 strain (genotype 2a) at non-toxic
239 concentrations (Fig. 6A-C). We then used a time-of-addition assay with increasing

240 concentrations of H89, and our data indicated an inhibitory effect at a late step of HCV entry
241 (Fig. 6D). Furthermore, as observed for the two piperazinylbenzenesulfonamides, H89
242 treatment also induced a decrease of cell surface expression of CLDN1 (Fig. 6E) without
243 affecting its total level of expression (Fig. 4G), but rather resulting in its intracellular
244 accumulation (Fig. 4H-I). In addition, transfection of an exogenous catalytic subunit of PKA
245 (PRKACA) followed by stimulation of PKA with the activator of adenylyl cyclase forskolin,
246 also increased the phosphorylation level of PKA substrates (Fig. 7A). Interestingly, PKA
247 stimulation caused an increase in cell surface expression of CLDN1 but no increase in
248 CLDN1-CD81 co-localization (Fig. 7B-C). In agreement with this data, no increase in HCV
249 infection was observed after activation of PKA (Fig. 7D).

250 Similar experiments were performed activating the PKA pathway by overexpression
251 of a GPCR, through exogenous transfection of a plasmid encoding 5-HT6 in Huh-7 cells. The
252 increased phosphorylation level of PKA substrates (Fig. 7E), suggested that the exogenous
253 receptor is functional and able to activate the PKA downstream pathway. As observed after
254 PKA transfection, overexpression of 5-HT6 led to an increase in CLDN1 cell surface
255 expression (Fig. 7F), also observed upon SB258585 treatment (Fig. 7G). However, this
256 increase in CLDN1 cell surface localization did not correlate with an increase in CLDN1-
257 CD81 co-localization (Fig. 7H) or HCV infectivity (Fig. 7I).

258 Together, these data strongly suggest that PKA regulates CLDN1 localization at the
259 cell surface. However, its activation does not seem to be sufficient to mediate CLDN1
260 interaction with CD81, which is necessary for HCV entry. Similarly, an increase of CLDN1
261 cell surface expression without modification of CLDN1-CD81 interaction and HCV infection
262 was observed after over-expression of CLDN1 (Fig. 7J-L). The failure in enhancing HCV
263 infection upon CLDN1 overexpression is in accordance with previous data (5). Together,
264 these results suggest that decreasing CLDN1 at plasma membrane strongly impacts HCV

265 entry, while increasing CLDN1 alone has no effect, probably due to a lack of recruitment to
266 its main partner CD81.

267 One potential explanation for the failure in increasing CLDN1-CD81 co-localization
268 could be a limited availability of endogenous CD81. To address this hypothesis, we
269 overexpressed this tetraspanin concomitantly with the GPCR 5-HT6. However, no
270 enhancement of HCV infection was observed (Fig. 8A), suggesting that increasing the
271 available quantity of both co-receptors was not sufficient to enhance their interaction and
272 consequently viral entry.

273 EGFR signaling has been described as important for CLDN1-CD81 interaction (30,
274 31). To verify if our phenotype was due to an insufficient activation of this receptor and its
275 downstream pathway, we treated Huh-7 with EGF. Although the stimulation induced the
276 phosphorylation of EGFR and thus its activation (Fig. 8B), no enhancement of infectivity, but
277 rather a slight decrease, was observed in our hands after increase of cell surface CLDN1
278 induced by transfection of 5-HT6 GPCR (Fig. 8C). Therefore, an additional signal upstream
279 of EGFR is likely necessary to relocalize CLDN1 to the site of interaction with CD81.

280

281 **The C-terminal region of CLDN1 is not involved in PKA-mediated inhibition.**

282 Predicted phosphorylation sites in the cytosolic domains of CLDN1 are only present in
283 the C-terminal region of CLDN1 (32) and could be responsible for its localization. A mutant
284 where the cytosolic C-terminus of CLDN1 was deleted has previously been described to
285 remain functional for HCV entry (5). We therefore used this mutant to determine whether the
286 effect of 5-HT6 activation antagonists is maintained when CLDN1 C-terminus region is
287 deleted. For this, we generated a CRISPR/Cas9 CLDN1 KO cell line (Fig. 9A-B), and we re-
288 expressed a wild type or a C-terminal deleted CLDN1 (Δ Cter) in this cell line (Fig. 9C-D).

289 After verification that the transfection level and the surface localization of the two constructs

290 were comparable (Fig. 9C), we infected the cells with HCV in the presence or absence of
291 SB258585. As shown in Fig. 9D, the absence of the C-terminus of CLDN1 did not alter the
292 sensitivity of HCV infection to SB258585 or H89, suggesting that CLDN1 C-terminal
293 cytosolic tail is not a direct target for PKA activity and is not required for its recycling.

294

295 **DISCUSSION**

296 HCV entry is a highly complex multi-step process involving a series of cellular
297 proteins. Trafficking of these entry factors provides an additional level of complexity in the
298 regulation of HCV entry. Here, by screening a chemical compound library, we identified the
299 antagonist of 5-HT6 SB258585 as a novel inhibitor of HCV entry process. This drug, as well
300 as another 5-HT6 antagonist SB399885, with which it shares similarities in structure, inhibits
301 a post-binding step of HCV entry in a 5-HT6 independent manner. These two drugs induced a
302 decrease in recycling of HCV entry co-receptor CLDN1 and its co-localization with CD81.
303 The resulting reduction of CLDN1-CD81 interaction, known to be critical for HCV
304 internalization, likely explains the drop of HCV infectivity. Treatment with these drugs
305 showed a reduction in PKA activity and the involvement of this kinase-related pathway was
306 confirmed to regulate CLDN1 localization and HCV late entry step.

307 The attempt to detect 5-HT6 in liver biopsies and hepatocytes was unsuccessful. This
308 failure in detecting 5-HT6 mRNA is supported by other reports (33). In addition, SB258585
309 and SB399885 are supposed to inhibit 5-HT6 in a highly specific way at nanomolar
310 concentrations. Therefore, the fact that in our hands the inhibition is only observed at
311 micromolar concentrations supports the idea of an effect on another target, for which the drug
312 shows less affinity. However, although the major target of these drugs in our model is
313 unlikely to be 5-HT6, these chemical compounds inhibit PKA activity, suggesting an
314 inhibitory effect on one or several other GPCRs likely associated to a G α stimulatory protein.

315 According to Stitch 5.0 prediction (17), SB258585 shows an affinity for other 5-HT receptors,
316 including the GPCR coupled to G α s 5-HT7, recently shown to play a role in liver fibrosis
317 (33). Unfortunately no data are available concerning SB399885, although the common
318 structure could suggest similar low-affinity targets.

319 PKA likely controls the export of CLDN1. Indeed, a down-regulation of PKA activity
320 reduces the cell surface expression of CLDN1, whereas its activation shows a direct increase
321 of this tight junction protein at cell surface, confirming a direct correlation between PKA
322 pathway and CLDN1 subcellular localization. The 5-HT6 receptor is a GPCR associated with
323 a G alpha stimulatory protein, known to activate the PKA pathway (34). Therefore, it is not
324 surprising that its overexpression stimulates PKA signaling and the export of the tight
325 junction protein CLDN1, similarly to what happens upon direct PKA transfection or
326 activation. Therefore, this experiment reinforces the hypothesis of a role for GPCRs in
327 regulating PKA signaling in hepatocytes. This is in line with a previous report showing that
328 PKA inhibitors affect HCV entry (29).

329 Activation of the PKA pathway either by forskolin treatment, or by overexpression of
330 the exogenous GPCR 5-HT6, or by overexpression of PKA catalytic subunit itself, increased
331 CLDN1 export at the surface or rescued its surface localization in cells treated with
332 SB258585. However, this was not sufficient to increase the basal level or to rescue HCV
333 infection. Similarly, over-expression of CLDN1 led to an increase in CLDN1 expression at
334 the cell surface without increasing HCV entry. In our case, in addition to analyzing CLDN1
335 expression at the cell surface and HCV infection, we also monitored CD81-CLDN1 co-
336 localization, which was not rescued despite re-export of CLDN1 at the cell surface. Thus,
337 there seems to be a disconnect between the level of CLDN1 expressed at the cell surface and
338 CD81-CLDN1 interaction. These observations are in accordance with Farquhar's work (29),
339 where treatment with forskolin did not enhance HCVcc entry. Since CLDN1 interaction with

340 the tetraspanin CD81 is necessary to mediate HCV endocytosis (6, 7), this could explain the
341 lack of rescue of HCV infection. The reason why CLDN1-CD81 co-localization is not
342 restored upon PKA activation remains unclear. One possibility could be that in rescue
343 experiments, despite the restoration of cell-surface expression of CLDN1, CD81 is no longer
344 available to interact with CLDN1. The interaction between these two proteins is described to
345 be regulated by the EGF receptor through HRas signaling (30, 31). However, neither
346 overexpression of CD81 concomitantly with the GPCR 5-HT6, nor EGF stimulation restored
347 the phenotype. This suggests that an additional signal upstream of EGFR is needed to target
348 CLDN1 to the site of interaction with CD81. Further investigations are therefore required to
349 better characterize the nature and the dynamics of the interaction between CLDN1 and CD81.
350 However, we cannot exclude that this defect in HCV late entry step is not only due to the
351 decreased interaction between CLDN1 and CD81. In addition to its involvement during
352 exocytosis, there are in fact evidences of a role for cAMP/PKA pathway during endocytosis
353 (reviewed in (35)). Therefore, the defect in HCV entry could not only be associated to
354 CLDN1 localization change, but also to a general perturbation of the membrane trafficking,
355 directly impacting HCV endocytosis. Further investigations are needed in order to elucidate
356 the mechanism connecting PKA pathway activation and endocytosis, necessary to better
357 clarify its additional involvement in HCV entry.

358 Regulation of CLDN1 trafficking in hepatocytes remains poorly characterized.
359 However, the CLDN1 C-terminal cytosolic tail presents several potential sites of post-
360 translational modification, including phosphorylation and ubiquitination, which could
361 potentially play a role in its trafficking (36). Indeed, other reports indicate a role for the
362 phosphorylation of CLDN1 cytosolic tail in the regulation of its subcellular localization in
363 non-hepatic cells (37–39), or through a competition between phosphorylation and
364 ubiquitination (40), as described for other members of the CLDN family (41, 42). However,

365 in our experimental work, the absence of the C-terminus of CLDN1 did not alter the
366 sensitivity of HCV infection to 5-HT6 and PKA antagonists, excluding a role for a direct
367 phosphorylation on CLDN1. Therefore, an additional, as of yet unidentified, intermediate
368 protein likely phosphorylated by PKA is probably involved in regulation of CLDN1
369 localization in the hepatocyte. However, cAMP/PKA pathway activation has been shown to
370 stimulate exocytosis of several proteins towards both apical and basolateral surface in
371 hepatocytes (reviewed in (35)), suggesting a conserved mechanism of export.

372 Several compounds targeting other GPCRs were identified as HCV inhibitors in our
373 screen. Previous studies described antihistamines, neuroleptic drugs and other ligands of
374 GPCRs as inhibitors of HCV entry (16, 43, 44). In the future it would be interesting to test if
375 the viral resistance mutations against any of these drugs eventually confer cross-resistance to
376 piperazinylbenzenesulfonamides. Moreover, the identification of compounds targeting
377 GPCRs in HCV screens could be in accordance with data from a recent chemical compound
378 screen performed on Ebola and Marburg viruses, identifying the involvement of several
379 antagonists of GPCRs, including 5-HT receptors, as inhibitors of a post-binding step of viral
380 entry (45). In addition, connections between 5-HT receptors and viral infections have already
381 been documented for several viruses, especially post-binding steps of viral entry as for
382 reovirus, JC polyomavirus, chikungunya virus and mouse hepatitis virus (46–49). Several
383 chemical compounds targeting 5-HT receptors were identified as HCV inhibitors in other
384 HCS screens (50, 51). This suggests that a possible common mechanism, potentially affected
385 by drugs targeting 5-HT receptor, might be used by different viruses. In this context, CLDN1
386 might not be the only protein whose trafficking is altered by modulation of 5-HT pathway.
387 Interestingly, in immune cells treated with an inhibitor of 5-HT uptake, HIV entry receptor
388 and co-receptor were also observed to be down-regulated at the cell surface (52); however,
389 the mechanism was not investigated. Further studies are necessary in order to better clarify if

390 there is actually a common pathway influencing the entry of all these viruses that could
391 represent a potential drug target for wide-range antiviral development.

392 In summary, we identified SB258585 and SB399885, antagonists of 5-HT6 receptor,
393 as inhibitors of a post-binding step of HCV entry. Although we confirmed the absence of 5-
394 HT6 in liver and hepatic cells, we proved an inhibition of PKA activity as a consequence of
395 the treatment, suggesting an off-target effect of these drugs on another GPCR. We thus
396 confirmed an involvement of PKA in a post-binding step of HCV entry, showing that
397 modulation of PKA activity regulates CLDN1 localization, through regulation of CLDN1
398 recycling towards the cell surface. We thus suggest a role for PKA in the liver, as a regulator
399 of CLDN1 export, which affects the HCV entry process influencing viruses of the six major
400 genotypes. Finally, our work reinforces the hypothesis of a common pathway shared by
401 several enveloped viruses, involving PKA-dependent signaling in late steps of viral entry.

402 **MATERIAL AND METHODS**

403 **Cell culture.** Huh-7 and Huh-7.5 cells have been previously described (53, 54) and were
404 cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM)
405 supplemented with 10% fetal bovine serum (FBS). Experiments were performed in DMEM
406 supplemented with 5% FBS. Huh-7 and Huh-7.5 cells were authenticated by the company
407 Multiplexion, performing a Multiplex human cell line Authentication Test (MCA) as
408 described at www.multiplexion.de.

409 For experiments using forskolin and EGF, cells were preincubated during 2h in serum-free
410 medium before incubation with the compound. DMEM, Opti-MEM, phosphate-buffered
411 saline (PBS), goat serum, and FBS were purchased from Life Technologies. PHH were
412 purchased from Biopredic and cultured at 37°C in supplemented Hepatocyte Basal Medium
413 (Clonetics™ HCM™ BulletKit™, Lonza).

414 **Viruses.** Cell culture derived HCV (HCVcc) were produced by electroporation of Huh-7 cells
415 with *in vitro* transcribed RNA of a modified JFH1 plasmid kindly provided by T. Wakita
416 (National Institute of Infectious Diseases, Tokyo, Japan) (11). This modified JFH1 bears
417 some titer-enhancing mutations and the reconstitution of the A4 epitope in E1 (10). Sequence-
418 confirmed virus stocks of JFH1-based Core-NS2 HCV recombinants were previously
419 generated as described (21–27).

420 **Antibodies.** Anti-HCV E1 mouse monoclonal antibody (MAb) A4 was produced *in vitro*
421 using a MiniPerm apparatus (Heraeus) following the manufacturer's protocol. Anti-CD81
422 MAb (5A6) was kindly provided by S. Levy (Stanford University). Anti-SRB1 antibody
423 C1671 was kindly given by A. Nicosia (OKAIROS, Italy). Anti-CLDN1 (OM 8A9-A3) MAb
424 has been previously described (55). Anti Phospho-(Ser/Thr) PKA Substrate (9621) and anti-
425 HA tag (C29F4) and anti-Phospho-EGFR (Tyr1068)(1H12) mouse mAb (2236)
426 were supplied from Cell Signaling Technology. Anti-EGFR (cocktail R19/48), anti-OCN
427 (OC-3F10) and anti-CLDN1 (51-9000) were purchased from Life Technologies. Anti- β -
428 tubulin (T5201), anti-HA tag (16B12), anti E-cadherin (AF648), anti-TGN46 (AHP500G),
429 anti-GM130 (610822) and anti-ERGIC53 (ALX-804-602) antibodies were obtained from
430 Sigma, Covance, R&D Systems, AbD Serotec, BD Biosciences and Enzo Life Sciences,
431 respectively. Anti-rat and anti-mouse PE-coupled antibodies were supplied by BD
432 Biosciences. Anti-Mouse Cy3, anti-Mouse Alexa 488, Anti-Rabbit Alexa647, anti-Mouse
433 HRP and anti-Rabbit HRP were purchased from Jackson ImmunoResearch. Goat anti-rat and
434 anti-mouse APC-coupled antibodies as well as anti-goat Alexa488, anti-rat Alexa555, anti-
435 sheep Alexa488 and anti-human Alexa488 coupled antibody were supplied by Life
436 Technologies.

437 **Chemicals.** SB258585 hydrochloride (sc-361339), SB399885 hydrochloride (sc-204264),
438 H89 dihydrochloride (sc-3537) and forskolin (sc-3562) were purchased from Santa Cruz

439 Biotechnology. Bafilomycin A, proteinase K, DAPI and Puromycin dihydrochloride from
440 *Streptomyces alboniger* were obtained from Sigma Aldrich. Mowiol was purchased from
441 Calbiochem. Dimethyl sulfoxide (DMSO), Streptavidin–Agarose beads from *Streptomyces*
442 *avidinii* and bafilomycin A1 were obtained from Sigma Aldrich. EGF, EZ-link Sulfo-NHS-
443 SS-Biotin, Dynabeads® Sheep Anti-Rat IgG and Hoechst 33342 were purchased from Life
444 Technologies.

445 **Primer list.** CLDN1-crispR-F: 5'-CACCGCTTCATTCTCGCCTTCC-3'; CLDN1-crispR-R:
446 5'-AAACGGAAGGCGAGAATGAAGC-3'; CLDN1-R: 5'-
447 GTGGATCCTCACACGTAGTCTTTCCCGC-3'; CLDN1-F: 5'-
448 TTAAGCTTGCCACCATGGCCAACGCGGGGCTGC-3';
449 CLDN1-ΔCter-R: 5'-GTGGATCCTCAACAGGAACAGCAAAGTAGGG-3';

450 **Plasmids.** pX459-CLDN1 was generated by insertion of the described RNA guides in
451 pSpCas9(BB)-2A-Puro (PX459) (Addgene). pcDNA3.1-CD81-YFP was generated by
452 insertion of YFP sequence in pcDNA3.1-CD81 vector. pCDNA3.1-CLDN1 and pCDNA 3.1-
453 CLDN1 ΔCter were generated by amplification of CLDN1 human cDNA contained in
454 pCMV6-Ac vector (OriGene) by using CLDN1-F and CLDN1-R or CLDN1- ΔCter-R
455 primers respectively and inserted into pCDNA3.1(+) (Invitrogen) by digestion with HindIII
456 and BamH1. HTR6 N-terminus 3xHA-tagged plasmid was purchased from UMR cDNA
457 Resource Center University of Missouri-Rolla (HTR060TN00) and PRKACA ORF
458 mammalian expression plasmid C-HA tag from Sino Biological Inc. (HG11544-CY).

459 **Human biopsies.** For mRNA expression analysis of 17 normal tissue samples (Ambion),
460 total RNA (500 ng) was reverse transcribed using SuperScript™ II Reverse Transcriptase
461 (Invitrogen) and random hexamer. Using Taqman gene expression assay (Life Technologies)
462 for 5HT-6 gene (Hs00168381_m1) and RPLP0 (Hs99999902_m1) as control, real-time PCR
463 amplifications were run in duplicate using LightCycler 480 Probe Master (Roche) and were

464 performed on the LightCycler 480 Instrument II (Roche) according to the MIQE guidelines
465 (56). Samples that lacked either a template or reverse transcriptase were used as controls. The
466 relative expression of each gene was calculated according to the $2^{-\Delta\text{Ct}}$ quantification method
467 where ΔCt value was determined by subtracting the average Ct value of the target gene from
468 the average Ct value of the control gene.

469 **HCS analysis.** 1,120 biologically active compounds from Tocriscreen Total library at 10 mM
470 in pure DMSO were dispensed in 384-well μClear black plates (Greiner bio-one Cat. 781091)
471 using the nanoliter acoustic liquid handler Echo 550® (LabCyte). 16 wells containing pure
472 DMSO were used as controls. Eight hundred Huh-7 cells were seeded in 30 μl in 384-well
473 plates and incubated with each compound respectively at a final concentration of 1, 10 or 20
474 μM at 37°C and 5% CO_2 . Sixteen hours later, cells were infected with 30 μl of HCVcc at a
475 MOI of 0.4 and incubated at 37°C and 5% CO_2 . After 72 h, 40 μl the supernatant was
476 harvested on a second set of 384-well μClear black plates containing twelve hundred Huh-7
477 cells with an automated liquid handler Bravo (Agilent) and incubated at 37°C and 5% CO_2 for
478 48 h. The infected cells in the two sets of 384-well plates were fixed for 30 minutes using
479 formalin solution, neutral buffered, 10% (Sigma-Aldrich, Cat. HT5014) at room temperature.
480 Cells were then permeabilized with 0.1% Triton X-100 in PBS for 3 minutes at room
481 temperature and HCV infected cells were revealed by immunofluorescence with anti-E1
482 antibody (MAb) A4 (1:1000 dilution) for 30 minutes at room temperature. Goat anti-mouse
483 Alexa-488 was used as a secondary antibody (1:500 dilution), while nuclei were labeled with
484 DAPI (1:500 dilution) for 30 minutes at room temperature. Two fields per well were acquired
485 using the 20X objective (NA 0.45) using excitation laser (Ex) at 405 nm and emission filter
486 (Em) at 450 nm for DAPI detection and Ex at 488 nm and Em at 540 nm for virus detection.
487 on an automated confocal microscope InCell 6000 Analyzer (GE Healthcare). Images were
488 analyzed using a dedicated image analysis performed on the Columbus Software allowing

489 automated quantification of the number of cells and the percentage of infected cells per well.
490 Compounds reducing the cell number by more than 35% compared to the average of DMSO
491 control wells were considered toxic and discarded. Compounds decreasing the percentage of
492 infection of at least 45%, whose effect was confirmed upon re-infection, were identified as
493 positive hits. Image analysis parameters of Columbus software are available upon request.

494 **JFH1 infection assay.** JFH1-HCVcc infection was quantified as previously described (10).

495 **Core-NS2 HCV recombinant infection assay.** Huh7.5 cells were plated at 7000 cells/well in
496 PDL coated 96 well plates. The following day, four replicates of the described Core-NS2
497 HCV recombinants were mixed with different concentrations of SB258585 in DMEM
498 supplemented with 10% FBS, along with 8 replicates of virus only without SB258585, and
499 added to Huh7.5 cells for 3 h. Following this incubation, the cells were washed and full
500 medium was added. After a total of 48 h post virus inoculation, the numbers of focus forming
501 units were visualized by HCV-specific immunostaining using NS5A antibody, 9E10 (24).
502 Counting was automated using an ImmunoSpot Series 5 UV Analyzer as described (57) and
503 the counts were normalized to the mean count of virus only.

504 **PHH infection.** PHH (Biopredic) were inoculated for 3 h with a cell culture adapted JFH1
505 strain in the presence of increasing concentrations of SB258585 and infection was measured
506 by quantification of intracellular HCV RNA 24 h post-inoculation, as previously described
507 (20).

508 **HCVpp and infection assays.** HCVpp bearing E1E2 glycoproteins of strain JFH1 (genotype
509 2a) were produced as previously described (58). RD114pp were produced using a plasmid
510 encoding the feline endogenous virus RD114 glycoprotein (59). Huh-7 cells were incubated
511 with pseudotyped particles for 3h at 37°C, concomitantly with increasing concentrations of
512 drugs. At 48h post-infection, firefly luciferase (FLuc) assays were performed following the
513 manufacturer's protocol (Promega).

514 **HCVcc entry assay.** After 2 h of JFH1-HCVcc attachment at 4°C, Huh-7 cells were washed
515 and shifted to 37°C to induce viral internalization. Each drug was added every 15 minutes
516 during a 2 h kinetic. Proteinase K (50 µg/ml) and bafilomycin A (25 nM) were included as
517 controls respectively for an early and a late step of viral entry. When treated with proteinase
518 K, cells were washed in cold PBS and incubated with proteinase K for 1 h at 4°C under gentle
519 agitation, before washing them again and adding fresh medium at 37°C. The infection rate
520 was calculated by IF 30 h later and each time point was normalized to the corresponding
521 DMSO condition.

522 **Adenovirus infection.** A recombinant adenovirus (AdV) expressing a green fluorescent
523 protein was generated as previously described (10). Huh-7 cells were inoculated with the
524 virus for 2 h at 37°C concomitantly with increasing concentration of SB258585 and cultured
525 for 24 h at 37°C. AdV infections were scored using an Axioplan2 epifluorescence microscope
526 (Zeiss) after fixation and nuclei staining with Hoechst.

527 **Viability assay.** Huh-7 cells were incubated for 2 h with increasing concentrations of the
528 indicated compound. A MTS assay was performed 28 h post-treatment, according to the
529 manufacturer's protocol (Promega) in order to evaluate cell viability.

530 **Immunofluorescence and quantification of CLDN1 co-localization with markers of**
531 **subcellular compartments.** Huh-7 cells previously seeded on coverslips were treated with
532 the indicated compounds. After treatment cells were fixed for 30 minutes with 3 %
533 paraformaldehyde. Cells were permeabilized during 5 minutes with 0.1 % Triton X-100 and
534 saturated for 30 minutes with PBS containing 10 % goat serum. Primary antibodies were
535 incubated during 30 minutes in PBS-goat serum. After three washes, secondary antibodies
536 and DAPI were added during 30 minutes. After three more washes, coverslips were mounted
537 on glass slides using Mowiol. Images were taken using a Zeiss-LSM880 or a Zeiss-LSM780
538 confocal microscope, with a 63X oil objective. Pearson correlation coefficients were

539 calculated using the Fiji coloc2 plugin on regions of interest (ROIs) from single cells,
540 designed on the basis of cell compartment labeling. Each experiment was repeated at least
541 three times and at least 40 cells for each condition were quantified.

542 **Quantification of CD81-CLDN1 colocalization.** Huh-7 cells were seeded on coverslips and
543 treated with the indicated inhibitor. After treatment cells were fixed for 30 minutes with 3 %
544 paraformaldehyde. Cells were incubated for 30 minutes with PBS containing 10 % goat
545 serum. Primary antibodies diluted in PBS-goat serum were added during 30 minutes. After
546 three washes, secondary antibodies and Hoechst were added for another 30 minutes. After
547 three more washes, coverslips were mounted on glass slides using Mowiol. Images were
548 taken using a Zeiss-LSM880 confocal microscope, with a 63X oil objective. Pearson
549 correlation coefficients were calculated using the Fiji coloc2 plugin on regions of interest
550 (ROIs) from single cells, designed on the bases of CD81 surface labeling. Each experiment
551 was repeated at least three times and a total of at least 40 cells for each condition were
552 quantified.

553 **Flow cytometry.** Huh-7 cells treated with the indicated compound for the indicated period of
554 time or siRNA transfected Huh-7 cells were incubated for 30 minutes at 4°C with the
555 indicated primary antibody diluted in cold PBS supplemented with 2 % BSA. After three
556 washes with cold PBS-BSA, cells were incubated for 30 minutes at 4°C with secondary
557 antibodies. After three more washes, cells were incubated for another 30 minutes with cold
558 PBS supplemented with 2 mM EDTA and then resuspended and fixed with a formalin
559 solution at a final concentration of 1.5 % formalin. For PKA-HA labeling, cells were
560 permeabilized for 30 minutes with 5% saponin and each following step was performed in the
561 presence of 5% saponin. Cells were analyzed using a BD FACSCANTO II Flow Cytometer.
562 Analyses were performed using Weasel and FlowJo softwares.

563 **Surface biotinylation and endocytosis.** Biotinylation assay was performed as previously
564 described (60). A western blot was performed in order to detect CLDN1. Proteins were
565 resolved by SDS-PAGE and transferred on a nitrocellulose membrane. The membrane was
566 immunoblotted with the indicated primary antibody, followed by peroxidase-coupled
567 secondary antibodies. Proteins were detected using a LAS3000 machine (Fuji).

568 **cDNA transfection.** Huh-7 cells were seeded in 24-well plates and transfected with 250 ng of
569 DNA using TransIT®-LT1 Transfection Reagent, according to the manufacturer's protocol.
570 Either treatment followed by FACS analyses or infection experiments were performed 48 h
571 post-transfection.

572 **RNA extraction and qRT-PCR.** RNA was extracted from Huh-7 cells using the
573 NucleoSpin® RNA extraction kit (Macherey Nagel), and 2 µg were used for cDNA
574 retrotranscription using the High-Capacity cDNA Reverse Transcription Kit (Life
575 Technologies). Taqman® predesigned gene expression assay approach (Applied Biosystems)
576 using a TaqMan FAM-MGB probe (Hs00168381_m1) was used to perform a qPCR in order
577 to quantify HTR6 (50 cycles). RPLP0 (Hs00420895_gH) was used as a housekeeping gene.
578 Δ Ct values were calculated according to the formula: Ct(HTR6)-Ct(RPLP0).

579 **CLDN1-KO cell line.** Huh-7 cells were seeded in 6-well plates and transfected with 1 µg of
580 pX459-CLDN1 plasmid using the Trans-IT LT1 reagent (Mirus) according to the
581 manufacturer's protocol. Three days after transfection cells were selected using DMEM
582 supplemented with 2 µg/ml of puromycin. Selection was removed after 4 days and cells were
583 cultured in complete DMEM medium. Knocking out was checked both by Western Blotting
584 and flow cytometry analysis. In order to remove the residual population of cells still
585 expressing CLDN1, a selection was performed using Dynabeads® Sheep Anti-Rat IgG after
586 labeling of the cells with anti-CLDN1 OM 8A9-A3 antibody.

587 **Quantification and statistical analysis.** Data are presented as means \pm standard error of the
588 mean (SEM), except when specified differently. Statistical parameters and analyses are
589 reported in the Fig. legends n represents the number of independent experiments. Where
590 indicated, asterisks denote statistical significance as follows: * $p < 0.05$ and ** $p < 0.01$,
591 *** $p < 0.001$, **** $p < 0.0001$, ns=non-significant. Statistical analyses were performed using
592 GraphPad Prism 7.

593

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606

607 **AUTHOR CONTRIBUTION**

608 LR, JD, YR, LC, OS, LL, PB, JP, JB designed the experiments. LR, OS, AV, FH, JP, LF, SB
609 performed the experiments. LR, OS, LL, FH, JP analyzed the results. TFB assisted in study
610 design and contributed reagents. LR, JD wrote the manuscript and all the authors commented
611 on it.

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812

813 **FIGURE LEGENDS**

814 **Figure 1: Identification of the 5-HT6 antagonist SB258585 as a new inhibitor of the**
815 **HCV life cycle.** (A-C) A HCS screen with a library of 1120 chemical compounds was
816 performed at three different concentrations (1 μ M, 10 μ M and 20 μ M). Dot-plots show the
817 distributions of the populations on the bases of cell number and percentage of HCVcc JFH1
818 (genotype 2a) infected cells at the indicated concentrations. Each dot represents one
819 compound. Green lines represent the average values corresponding to DMSO control wells.
820 Red lines represent the selected cut-offs, determining the positive hits (light red squares). (D)
821 Summary of the positive hits selected according to the cut-offs, at each concentration. The red
822 circle indicates compounds confirmed as positive hits for at least two concentrations. (E)
823 Graph showing the percentage of HCVcc infection corresponding to the wells treated with
824 chemical compounds targeting in a more or less specific way 5-HT6, normalized to the mean
825 of the DMSO control wells at each concentration. The green line corresponds to the
826 compound SB258585 hydrochloride. (F) Wells corresponding to DMSO and SB258585 for
827 each concentration of the screen. Nuclei are shown in blue, HCV E1 staining in green.

828

829 **Figure 2: SB258585 inhibits HCV infection, modulating PKA in a 5-HT6 independent**
830 **manner.** (A) Huh-7 cells were treated with SB258585 at different concentrations for 2 h
831 prior, during or after JFH1 HCVcc incubation, following the kinetics schematized. Infection
832 was quantified at 30 h post-infection by immunofluorescence. (B) Huh-7 cells were treated

833 with the drug 2 h, followed by 28 h of rest. A MTS assay was performed in order to evaluate
834 the cell toxicity. (C) Quantification of 5-HT6 mRNA level by qRT-PCR in 17 tissues from
835 human biopsies. (D) Huh-7 cells were treated for 2 h with DMSO, H89 (10 μ M), SB258585
836 (100 μ M) or SB399885 (100 μ M). A representative western blot from n=3 and relative
837 quantification of total phosphorylation of PKA substrates normalized to the loading control β -
838 tubulin are presented. Results are presented as means \pm standard error of the mean (SEM) for
839 n=3 (A, B, D). One-way (B, D) or Two-way ANOVA (A) respectively followed by Dunnett
840 or Bonferroni post-test were performed as statistical tests. *, P < 0.05; **, P < 0.01; ***, P <
841 0.001; ****, P < 0.001.

842

843 **Figure 3: Piperazinylbenzenesulfonamides inhibit a late step of HCV entry in all the**
844 **major genotypes.** (A) Huh-7 cells were treated for 3 h with SB258585 at different
845 concentrations concomitantly with JFH1 HCVpp or RD114pp incubation. At 48 h post-
846 infection, cells were lysed and luciferase activity was measured in order to quantify the
847 infection. (B) Huh-7 cells were incubated for 2 h with SB258585 at different concentrations
848 along with GFP-Adenovirus. The percentage of infection was determined by quantifying
849 GFP-positive cells at 24 h post-infection. (C) Primary human hepatocytes (PHHs) were
850 treated for 2 h with SB258585 at different concentrations, in addition to JFH1 HCVcc.
851 Infection was quantified by RTqPCR at 24 h post-infection. (D) Huh-7.5 cells were treated for
852 3 h with SB258585 in addition to JFH1-based HCV genotype 1-6 recombinant viruses with
853 strain specific core-NS2. Infection was quantified by immunofluorescence at 48 h post-
854 inoculation. Error bars represent SD. (E) After 1 h of viral attachment at 4°C, Huh-7 cells
855 were shifted at 37°C and treated with SB258585 at the indicated concentrations, following
856 15-minute kinetics during 2 h. Proteinase K (50 μ g/ml) and bafilomycin A (25 nM) were used
857 as controls respectively for early and late entry steps. Infection was quantified at 30 h post-

858 infection by immunofluorescence and each time point was normalized to the corresponding
859 DMSO condition. (F) Chemical structure of SB258585 and SB399885. (G) Huh-7 cells were
860 treated with SB399885 at different concentrations for 2 h prior, during or after JFH1 HCVcc
861 incubation, following the kinetics schematized. Infection was quantified at 30 h post-infection
862 by immunofluorescence. (H) Huh-7 cells were treated with SB399885 2 h, followed by 28 h
863 of resting. A MTS assay was performed in order to evaluate the cell toxicity. (I) After 1 h of
864 viral attachment at 4°C, Huh-7 cells were shifted at 37°C and treated with SB399885 at the
865 indicated concentration, following 15-minute kinetics during 2 h. Proteinase K (50 µg/ml) and
866 bafilomycin A (25 nM) were used as controls respectively for early and late entry steps.
867 Infection was quantified at 30 h post-infection by immunofluorescence and each time point
868 was normalized to the corresponding DMSO condition. Results are presented as means ±
869 standard error of the mean (SEM) for n=3 (A-D, G-H) and n=2 (E, I). PHH results are
870 presented as mean of triplicates ± SEM for two independent experiments. One-way (B, C, H)
871 or Two-way ANOVA (A, G) respectively followed by Dunnett or Bonferroni post-test were
872 performed as statistical tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.001; *ns*,
873 non significant.

874

875 **Figure 4: SB258585 alters CLDN1 recycling causing its intracellular accumulation.** (A)
876 Huh-7 cells were treated for 2 h with DMSO or increasing concentrations of SB258585. Cell
877 surface expression of CD81 and CLDN1 was analyzed by immunofluorescence. Images were
878 taken using a Zeiss LSM-880 and 63X objective. (B) Pearson correlation coefficient (PCC)
879 was calculated on cell surface ROIs from at least 40 different cells in each condition. (C)
880 Huh-7 cells were treated for two hours with DMSO or increasing concentrations of SB258585
881 and CLDN1 expression was analysed by flow cytometry. Curves from a representative
882 experiment are shown. Mean fluorescent intensities relative to the DMSO-treated condition

883 are also presented. (D) Huh-7 cells were treated for two hours with DMSO or increasing
884 concentrations of SB399885 and CLDN1 expression was analyzed by flow cytometry. (E)
885 Huh-7 cells were incubated for the indicated periods with SB258585 (100 μ M). CLDN1
886 present at the cell surface was quantified by flow cytometry. (F) Huh-7 cells were treated for
887 2 h with SB258585 (100 μ M). The drug was then removed and replaced by DMEM for the
888 indicated time. Cytometry analyses were performed to quantify CLDN1 at cell surface. Mean
889 fluorescence intensities relative to the DMSO-treated condition are shown (D-F). (G) Huh-7
890 cells were treated for 2 h with DMSO, SB258585 (100 μ M), SB399885 (100 μ M) or H89 (10
891 μ M). The total quantity of CLDN1 was assessed by Western Blot. β -tubulin was used as a
892 loading control. (H) Huh-7 cells were treated for 2 h with DMSO, SB258585 (100 μ M), or
893 H89 (10 μ M). CLDN1 subcellular localization was determined by immunofluorescence after
894 membrane permeabilization. Images were taken at 63X objective. (I) TGN46 was stained
895 concomitantly with CLDN1 and PCCs were calculated for intracellular CLDN1-TGN46 co-
896 localization for $n > 35$ cells for each condition. (J) After surface biotinylation, Huh-7 cells were
897 incubated at 37°C with DMSO or SB258585 (100 μ M) for the indicated time. Biotin
898 remaining at the cell surface was cleaved using glutathione. The amount of internalized
899 CLDN1 was determined by western blotting after pull-down of biotin-labeled proteins with
900 streptavidin-agarose beads. A representative western blot from $n=3$ is presented. All results
901 are presented as means \pm standard error of the mean (SEM) for $n=3$. One-way ANOVA (B-E,
902 I) or Two-way ANOVA (F) respectively followed by Dunnett or Bonferroni post-test were
903 performed as statistical tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.001$. Scale
904 bars = 30 μ m.

905

906 **Figure 5: SB258585 does not alter the surface localization of the other main HCV entry**

907 **factors.** (A) Huh-7 cells were treated for two hours with DMSO or increasing concentrations

908 of SB258585. A surface biotinylation followed by a biotin immunoprecipitation was
909 performed and a representative western blot against OCLN is shown. The quantity of
910 biotinylated OCLN was determined using Fiji from western blots corresponding to n=3. (B-E)
911 Huh-7 cells were treated for 2 h with DMSO or increasing concentrations of SB258585.
912 CD81 (B), EGFR (C), SRB1 (D) and E-cadherin (E) expression were analyzed by flow
913 cytometry. Curves from a representative experiment are shown. Mean fluorescence intensities
914 relative to the DMSO-treated condition are presented as means \pm SEM. All the results are
915 presented as means \pm SEM for n=3. One-way ANOVA followed by Dunnett post-test were
916 performed as statistical tests. *ns*, non significant.

917

918 **Figure 6: Inhibition of PKA signaling pathway down-regulates HCV entry and CLDN1**

919 **cell surface localization.** (A) Huh-7 cells were treated 2 h with H89 at different
920 concentrations. An MTS assay was performed 30 h post treatment in order to evaluate the cell
921 toxicity of the compound. (B) Huh-7 cells were treated for 3 h with H89 at different
922 concentrations along with JFH1 HCVpp and RD114pp. At 48 h post-infection, cells were
923 lysed and luciferase activity was measured in order to quantify the infection. (C) Huh-7 cells
924 were treated with H89 at different concentrations in accordance with the kinetics schematized,
925 along with JFH1 HCVcc. Infection was quantified at 30 h post-infection by
926 immunofluorescence. (D) After 1 h of viral attachment at 4°C, Huh-7 cells were shifted at
927 37°C and treated with H89 (10 μ M), following 15-minute kinetics during 2 h. Proteinase K
928 (50 μ g/ml) and bafilomycin A (25 nM) were used as controls respectively for early and late
929 entry steps. Infection was quantified at 30 h post-infection by immunofluorescence and each
930 time point was normalized to the corresponding DMSO condition. (E) Huh-7 cells were
931 treated for 2 h with DMSO or H89 (10 μ M). Cell surface CLDN1 was analyzed by flow
932 cytometry. Curves from a representative experiment and mean fluorescence intensities

933 relative to the DMSO-treated condition are shown. Results are presented as means \pm SEM for
934 n=3 (A-C, E) and n=2 (D). Two-tailed Student's T test (E), One-way (A) or Two-way
935 ANOVA (B-C) respectively followed by Dunnett or Bonferroni post-test were performed as
936 statistical tests. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.001.

937

938 **Figure 7: PKA activation increases CLDN1 localization at cell surface, without**
939 **increasing HCV entry or CLDN1-CD81 co-localization.** (A-D) Huh-7 cells were
940 transfected with pcDNA or pcDNA-PRKACA for 48 h. Cells were treated for 2 h with
941 DMSO, forskolin (20 μ M) as indicated. (A) A representative western blot from n=3 is
942 presented, in order to analyse the phosphorylation of PKA substrates. β -tubulin was used as a
943 loading control and PKA transfection was checked by HA immuno-blotting. (B) Cytometry
944 analysis was performed for quantifying CLDN1 cell surface localization and PKA-HA
945 transfection. Mean fluorescence intensities relative to the DMSO-treated condition from n=3
946 are shown. (C) CLDN1 and CD81 were analyzed by immunofluorescence and PCCs were
947 calculated after confocal image acquisition on cell surface ROIs from at least 40 different
948 cells in each condition. (D) Transfected cells were treated for 2 h with DMSO or forskolin (20
949 μ M), together with HCVcc. Infection was quantified at 30h post-infection by
950 immunofluorescence. (E-I) Huh-7 cells were transfected for 48 h with pcDNA or pcDNA-5-
951 HT6. (E) A representative western blot from n=2 and relative quantification of total
952 phosphorylation of PKA substrates normalized to the loading control β -tubulin are presented.
953 HA-5-HT6 transfection was checked by HA immuno-blotting. (F-G). Cell surface CLDN1
954 and HA-5-HT6 were immunolabeled and quantified by flow cytometry. Mean fluorescence
955 intensities relative to the DMSO-treated condition are shown. (G) Transfected Huh-7 cells
956 were treated 2 h with SB258585 (100 μ M) before labeling for flow cytometry analyses. (H)
957 CLDN1 and CD81 immunostaining followed by confocal microscopy allowed for the

958 calculation of PCCs on cell surface ROIs from at least 40 different cells in each condition. (I)
959 Transfected Huh-7 cells were infected 2h with HCVcc. Infection was quantified at 30 h post-
960 infection by immunofluorescence. (J-L) Huh-7 cells were transfected for 48 h with pcDNA or
961 pcDNA-CLDN1. Cell surface CLDN1 was immunolabeled and quantified by flow cytometry.
962 Mean fluorescence intensities relative to the DMSO-treated condition are shown (J). CLDN1
963 and CD81 immunostaining followed by confocal microscopy allowed for the calculation of
964 PCCs on cell surface ROIs from at least 40 different cells in each condition (K). Transfected
965 cells were infected with HCVcc 48 h post-transfection. Infection was quantified at 30 h post-
966 infection by immunofluorescence (L). Results are presented as means \pm SEM for n=3 (C-D,
967 H-I) or n=4 (B, F-G, J-L) independent experiments. Two-tailed Student's T test (E-F, H-L) or
968 Two-way ANOVA (B-D, G) followed by Bonferroni post-test were performed as statistical
969 tests. *, P < 0.05; ***, P < 0.001; *ns*, non significant.

970

971 **Figure 8: Increase of CLDN1 localization at cell surface is not sufficient to increase HCV**
972 **entry, neither upon CD81 over-expression nor EGF stimulation.** (A) Huh-7 cells were
973 transfected with pcDNA, HA-5-HT6, CD81-YFP alone or in combination as indicated. 48 h
974 post-transfection cells were infected with JFH1-HCVcc. 30 hpi cells were fixed and infection
975 rate was determined by IF. (B) After 2 h of starvation, Huh-7 cells were non-stimulated or
976 stimulated 1 h with EGF (1 μ g/ml). A western blot was performed to verify the activation of
977 EGFR, through phosphorylation. β -tubulin was used as a loading control. (C) Huh-7 cells
978 transfected 48 h with pcDNA, HA-5-HT6, CD81-YFP alone or in combination, after 2 h of
979 starvation, were treated 1 h with EGF (1 μ g/ml) and infected 2 h with JFH1-HCVcc
980 concomitantly with EGF (1 μ g/ml). 30 hpi cells were fixed and infection rate was determined
981 by IF. Results are presented as means \pm SEM for n=2 (A, C). One-way (A) or Two-way

982 ANOVA (C) respectively followed by Dunnett or Bonferroni post-test were performed as
983 statistical tests. *ns*, non significant.

984

985 **Figure 9: Deletion of CLDN1 C-terminal region does not alter HCV sensitivity to**
986 **SB258585 and H89.** (A-D) Flow cytometry (A) and western blot (B) were performed on
987 CLDN1 CrispR/CAS9 and control pX459 cells, in order to evaluate CLDN1 silencing. OCLN
988 was used as a loading control for the western blot. (C-D) CLDN1 CrispR/CAS9 cells were
989 transfected respectively with pcDNA 3.1, CLDN1 WT or Δ Cter. 48 h post-transfection cells
990 were labeled for CLDN1 at the cell surface and analyzed by flow cytometry (C) or infected
991 with JFH1-HCVcc (D). 30 hpi cells were fixed and infection rate was determined by IF.
992 Results are presented as means \pm SEM for n=3. Two-way ANOVA (D) followed by
993 Bonferroni post-test was performed as statistical tests. ****, $P < 0.001$.

















