

1 **An E460D substitution in the NS5 protein of tick-borne encephalitis virus confers resistance**
2 **to the inhibitor Galidesivir (BCX4430) and also attenuates the virus for mice**

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18

19 **Abstract**

20 The adenosine analogue Galidesivir (BCX4430), a broad-spectrum RNA virus inhibitor, has
21 entered a Phase 1 clinical safety and pharmacokinetics study in healthy subjects and is under
22 clinical development for treatment of Ebola virus infection. Moreover, Galidesivir also
23 inhibits the reproduction of tick-borne encephalitis virus (TBEV) and numerous other
24 medically important flaviviruses. Until now, studies of this antiviral agent have not yielded
25 resistant viruses. Here, we demonstrate that an E460D substitution, in the active site of
26 TBEV RNA-dependent-RNA-polymerase (RdRp), confers resistance to Galidesivir in cell
27 culture. Stochastic molecular simulations indicate that the steric freedom caused by the
28 E460D substitution increases close electrostatic interactions between the inhibitor and the
29 interrogation residue of the TBEV RdRp motif F, resulting in rejection of the analogue as an
30 incorrect/modified nucleotide. Galidesivir-resistant TBEV exhibited no cross-resistance to
31 structurally different antiviral nucleoside analogues, such as 7-deaza-2'-C-methyladenosine,
32 2'-C-methyladenosine and 4'-azido-aracytidine. Although, the E460D substitution led only to
33 a subtle decrease in viral fitness in cell culture, Galidesivir-resistant TBEV was highly
34 attenuated *in vivo*, with 100% survival rate and no clinical signs observed in infected mice.
35 Our results contribute to understanding the molecular basis of Galidesivir antiviral activity,
36 flavivirus resistance to nucleoside inhibitors and the potential contribution of viral RdRp to
37 flavivirus neurovirulence.

38

39 **Keywords:** Galidesivir; BCX4430; tick-borne encephalitis virus; drug-resistance; mutation;
40 attenuation

41

42 **Importance**

43 Tick-borne encephalitis virus (TBEV) is a pathogen that causes severe human neuroinfections
44 in large areas of Europe and Asia and for which there is currently no specific therapy. We
45 have previously found that Galidesivir (BCX4430), a broad-spectrum RNA virus inhibitor,
46 which is under clinical development for treatment of Ebola virus infection, has a strong
47 antiviral effect against TBEV. For any antiviral drug, it is important to generate drug-resistant
48 mutants to understand how the drug works. Here, we produced TBEV mutants resistant to
49 Galidesivir and found that the resistance is caused by a single amino acid substitution in an

50 active site of the viral RNA-dependent RNA polymerase, an enzyme which is crucial for
51 replication of viral RNA genome. Although, this substitution led only to a subtle decrease in
52 viral fitness in cell culture, Galidesivir-resistant TBEV was highly attenuated in a mouse
53 model. Our results contribute to understanding the molecular basis of Galidesivir antiviral
54 activity.

55

56 **Introduction**

57 Flaviviruses (family *Flaviviridae*, genus *Flavivirus*) cause widespread human morbidity
58 and mortality throughout the world. These viruses are typically transmitted to humans by
59 mosquito or tick vectors. Tick-borne encephalitis virus (TBEV) is a typical flavivirus
60 transmitted by *Ixodes* spp. ticks. TBEV is a causative agent of tick-borne encephalitis (TBE), a
61 severe and potentially lethal neuroinfection in humans (Baier, 2011). The disease is
62 prevalent in the sylvatic areas of Europe and Asia with more than 13,000 cases of TBE being
63 reported annually (Dumpis, Crook, and Oksi 1999; Heinz and Mandl 1993). Clinical
64 presentation of TBE ranges from mild fever to severe encephalitis or encephalomyelitis. In
65 many cases, survivors of TBE suffer long-term or even permanent debilitating sequelae
66 (Ruzek, Dobler, and Mantke 2010). As for other flaviviral infections, there is no specific
67 treatment for TBE, other than supportive therapy. Thus, the search for antiviral agents for
68 specific chemotherapy of TBE and related viruses is urgent.

69 Among the different strategies aimed at inhibiting virus or cell components involved
70 in TBEV replication, the viral nonstructural NS5 protein, an RNA-dependent RNA-polymerase
71 (RdRp), has become an attractive target for specific and effective inhibition of viral
72 replication, with limited measurable effect on the host cells (Eyer et al., 2018). Several
73 molecules, mainly nucleoside analogues, were found to be potent inhibitors of the TBEV NS5
74 polymerase activity (Eyer et al., 2015; 2016; 2017a,b; 2018). The mechanism of action of
75 these nucleoside analogues is based on their initial metabolization to the active triphosphate
76 (nucleotide) form by cellular kinases and subsequent incorporation into the nascent genome
77 by the RdRp, leading to premature chain termination (Eyer et al., 2018). Galidesivir (also
78 known as BCX4430 or Immucillin-A, Figure 1A) is an adenosine analogue with two structural
79 modifications: (i) it is a C-nucleoside characterized by a C-glycosidic bond instead of the
80 usual N-glycosidic bond, and (ii) the furanose oxygen has been replaced by an imino group
81 (Warren et al., 2014). Galidesivir is known to have a broad-spectrum antiviral effect against
82 more than 20 different medically important RNA viruses across nine different virus families
83 (flaviviruses, togaviruses, bunyaviruses, arenaviruses, paramyxoviruses, coronaviruses,
84 filoviruses, orthomyxoviruses and picornaviruses) (Warren et al., 2014; Westover et al.,
85 2018; Julander et al., 2014, 2017; Taylor et al., 2016; De Clercq, 2016). Low micromolar levels
86 of Galidesivir were previously shown to inhibit TBEV with no or negligible cytopathic effect

87 on the host cells (Eyer et al., 2017a). A Phase 1 clinical safety and pharmacokinetics study in
88 healthy subjects has been completed, and at present, Galidesivir is under clinical
89 development as an antiviral drug for treatment of Ebola virus infection (Taylor et al., 2016).
90 The broad-spectrum antiviral activity makes this drug a promising candidate for
91 development of therapy not only for Ebola virus infection but also other important diseases
92 caused by various RNA viruses, including TBEV.

93 Although Galidesivir has been studied intensively and is known to inhibit a wide
94 range of RNA viruses, there are no published reports of resistance to this compound.
95 Experience with the treatment of other RNA virus infections shows, that resistance can
96 develop rapidly with any of the direct-acting antiviral agents (Poveda et al., 2014; Bagaglio et
97 al., 2017; Irwin et al., 2016). Due to the low fidelity of viral RdRps in general, the mutation
98 frequency is estimated to be 10^{-4} to 10^{-6} errors per nucleotide (Lauring et al., 2013). The
99 high mutation frequency and high replication rate of viral RNA copies enable the viruses
100 quickly to adapt to changes in the environment, including the introduction of antiviral drugs.
101 Identification of mutations conferring antiviral resistance provides information not only
102 about the risk of generation of drug-resistant mutants but also helps to elucidate molecular
103 mechanisms of the antiviral action. This is an integral and essential part of development and
104 testing of any new antiviral drug (Irwin et al., 2016).

105 In the present study, we identified and described a specific amino acid substitution in
106 the TBEV NS5 polymerase that confers resistance to Galidesivir. This substitution had only a
107 limited effect on viral reproduction *in vitro*, but had a cost on viral fitness when tested *in*
108 *vivo*, using mice. We also used structural modeling to link Galidesivir resistance to a
109 molecular change in the NS5 RdRP active site that affects nucleotide incorporation. Our
110 findings are important for understanding the mechanism of action of Galidesivir and for the
111 use of this molecule as an antiviral drug against TBEV and other emerging RNA viruses. In
112 addition, we highlight the discovery of a potential contribution of viral RdRp to flavivirus
113 neurovirulence.

114

115 **Results**

116 ***TBEV resistant to Galidesivir has two amino acid substitutions in the NS5 protein***

117 Studies of drug-resistant virus mutants are crucial for understanding molecular
118 interactions of antiviral drugs with target viral proteins, as well as for development of

119 efficient and specific antiviral therapies. In order to select TBEV resistant to Galidesivir, the
120 virus was serially passaged in PS cell monolayers in the presence of increasing
121 concentrations of Galidesivir up to 50 μ M (Figure 1B); this process resulted in selection of
122 two independent drug-resistant TBEV mutant strains. Whole genome sequencing of the
123 passaged viruses revealed, that both selected TBEV mutants carried a single amino acid
124 change E460D, which corresponds to the nucleotide substitution G9045T in the NS5 gene
125 (Figure 1C,D). Sequencing of viruses after each passage showed, that this mutation was
126 acquired after 5 passages. In passages 6 and 7, mixed mutated and wild-type genotypes
127 were detected; from passage 8 onwards, the mutated genotype dominated until the end of
128 the experiment (passage 9) (Figure 1D). Interestingly, in one of the selected TBEV mutants,
129 an additional amino acid change Y453H was detected; this mutation, which was acquired
130 after 8 passages, corresponds to the nucleotide substitution T9022C in the NS5 gene (Figure
131 1D). Mutations E460D and Y453H were not present in the wild-type virus passaged in the
132 absence of the selection agents. Both mutations mapped to the active site of the RdRp
133 domain of the NS5 protein. The *in vitro* selected TBEV mutants (denoted as E460D and
134 E460D/Y453H) were further evaluated for their sensitivity/resistance to Galidesivir at
135 concentrations ranging from 0 to 50 μ M and compared with the mock-selected wild-type
136 virus (Figure 2A). Whereas *in vitro* replication of wild-type was completely inhibited by
137 Galidesivir at a concentration of 12.5 μ M (EC_{50} value of 0.95 ± 0.04 μ M), both mutants were
138 approximately 7-fold less sensitive to the compound, showing EC_{50} values of 6.66 ± 0.04 and
139 7.20 ± 0.09 μ M for E460D and E460D/Y453H, respectively (Figure 2A, Table 2).

140

141 ***Site-directed mutagenesis confirms that E460D determines TBEV drug resistance***

142 In order to demonstrate the direct effect of the amino acid substitutions E460D and
143 Y453H on TBEV phenotype, the appropriate mutations were introduced into recombinant
144 TBEV strains generated by the rapid reverse genetic approach based on the use of
145 subgenomic overlapping DNA fragments (Aubry et al., 2014; Driouich et al, 2018). The entire
146 TBEV strain Hypr genome flanked at the 5' and 3' untranslated regions by the pCMV and
147 HDR/SV40pA was *de novo* synthesized in three double stranded DNA fragments of
148 approximately 4.4, 4.5 and 3.1 kb in length, overlapping by 80 to 120 pb. The substitutions
149 E460D (G9045T) and Y453H (T9022C) were introduced into the NS5 gene located on
150 fragment III using mutagenic PCR primers (Figure 4). After transfection of the sub-genomic

151 fragments into permissive BHK-21 cells, the following recombinant TBEV strains were
152 successfully rescued: E460D-Rec (E460D substitution in the NS5 gene), Y453H-Rec (Y453H
153 substitution in the NS5 gene), and recombinant wild-type (no introduced mutations). The
154 presence of the E460D and Y453H substitutions in the viral genomes was confirmed by
155 whole-genome sequencing of all recombinant viruses. Despite repeated attempts, the
156 E460D/Y453H-Rec mutant (both mutations E460D and Y453H in the NS5 gene) was not
157 rescued from the transfected BHK-21 cell culture. E460D-Rec was found to be 7.9-fold less
158 sensitive to Galidesivir than engineered wild-type, showing an EC_{50} value of $6.32 \pm 1.01 \mu\text{M}$
159 (Table 2). On the other hand, Y453H-Rec was highly sensitive to Galidesivir; the replication of
160 this mutant strain was completely inhibited at $12.5 \mu\text{M}$, showing an EC_{50} value of 0.81 ± 0.01
161 μM (Table 2). Thus, the results indicate, that the E460D (not Y453H) substitution is solely
162 responsible for the drug-resistant phenotype of TBEV (Figure 2A).

163

164 ***The E460D mutation has only a moderate effect on TBEV replication in cell culture***

165 To characterize the phenotypic properties of the drug-resistant mutant *in vitro*,
166 growth kinetics (Figure 2B) and plaque morphology (Figure 2C) of the recombinant TBEV
167 mutant E460D-Rec were assayed in cultures of PS cells and compared with the wild-type
168 virus. The wild-type virus amplified in the absence of Galidesivir (Figure 2B, red line), showed
169 a short lag-period within intervals 0 – 12 hours p.i. Starting 24 hours p.i., the wild-type TBEV
170 exerted an exponential increase in virus infectivity reaching a peak titre of 3×10^6 PFU/mL
171 within 72 hours p.i. and gradually declining thereafter. In contrast, the presence of
172 Galidesivir ($25 \mu\text{M}$) completely inhibited replication of the wild type virus (Figure 2B, violet
173 line).

174 In comparison with the wild-type virus, the E460D-Rec mutant cultured in the
175 absence of Galidesivir (Figure 2B, blue line) showed a prolonged lag-period within intervals 0
176 – 36 hours p.i. However, subsequently, the infectivity of the mutant virus increased
177 exponentially reaching a peak of 2.6×10^6 PFU/mL at 72 hours p.i. After that, the titre
178 gradually declined to 5.5×10^4 PFU/mL. The considerably longer lag-period of the E460D-Rec
179 mutant could be explained by a slightly decreased replication capacity (attenuation) of the
180 mutant, when amplified in PS cell culture. The decrease in replication capacity of E460D-Rec
181 was manifested particularly in the first few hours after cell culture infection and was no
182 longer detectable in the later stages of the infection.

183 The E460D-Rec mutant cultured in the presence of Galidesivir (25 μ M) (Figure 2B,
184 green line) also exerted an extended lag-period within intervals 0 – 36 hours p.i.; there was
185 even a moderate decrease in viral titers after 36 hours p.i. Starting 48 hours p.i., the mutant
186 showed an exponential infectivity and reached a peak titre of 3.9×10^5 PFU/mL at day 96
187 hours p.i. The results clearly indicate, that the resistance of TBEV to Galidesivir is only partial;
188 the growth of the mutant strain in the presence of Galidesivir was partially inhibited
189 compared to wild-type (Figure 2B, red line) or the E460D-Rec mutant grown in the absence
190 of Galidesivir (Figure 2B, blue line).

191 The plaque morphology of the drug-resistant TBEV mutant was similar to that of wild-
192 type virus; both viruses produced large and clear plaques which were round and regular in
193 shape and did not change in shape and size during all the consecutive passages (Figure 2C).
194 The similarity in plaque morphology of drug-resistant and wild-type TBEVs is in agreement
195 with similar growth kinetics of both viruses and supports our assumption that the mutation
196 E460D affects the viral replication in PS cell culture only to a limited extent.

197

198 ***The E460D TBEV mutant is sensitive to 7-deaza-2'-C-methyladenosine, 2'-C-*** 199 ***methyladenosine and 4'-azido-aracytidine***

200 To test whether or not E460D and Y453H substitutions affect sensitivity of TBEV to
201 structurally different nucleoside inhibitors we evaluated selected nucleoside analogues with
202 previously reported anti-TBEV activity (Eyer et al., 2016), for their capacity to inhibit *in vitro*
203 replication of Galidesivir-resistant TBEV. Inhibitory effects of 7-deaza-2'-C-methyladenosine,
204 2'-C-methyladenosine, and 4'-azido-aracytidine (RO-9187) at concentrations of 25 and 50
205 μ M were not affected in the E460D/Y453H mutant which was obtained by serial sub-culture
206 in PS cells in the presence of Galidesivir ($EC_{50} > 50 \mu$ M) (Figure 2D,E). The same drug-
207 sensitivity profile, characterized by complete inhibition of virus replication, was determined
208 for two recombinant TBEVs generated by reverse genetics, E460D-Rec and Y453H-Rec (EC_{50}
209 $> 50 \mu$ M). Wild-type virus was used as a positive control in this *in vitro* antiviral study (Figure
210 2D,E).

211

212 ***Mouse neuroinvasiveness of the E460D TBEV mutant is highly attenuated***

213 The degree of neuroinvasiveness of the E460D TBEV mutant was assessed in BALB/c
214 mice and was compared with that of wild-type virus. Adult BALB/c mice were infected

215 subcutaneously with 10^3 PFU of either virus and survival rates and clinical signs of
216 neuroinfection were monitored for 28 days. Wild-type virus produced fatal infections in all
217 mice, with mean survival times of 11 ± 2.2 days; infected mice showed severe signs of
218 disease, such as ruffled fur, hunched posture, tremor and hind leg paralysis (Figure 3A,C). In
219 contrast, all mice infected with the drug-resistant TBEV mutant E460D (obtained by serial *in*
220 *vitro* sub-culture in the presence of Galidesivir) survived ($p < 0.0001$), displaying no clinical
221 signs of TBEV infection through the entire 28-day experimental period (Figure 3A,C). The
222 same survival data (100% survival rate, $p < 0.0001$) and clinical scores (no signs of
223 neuroinfection) were obtained, when recombinant TBEV mutant (E460D-Rec) was used for
224 mouse infection using the same infectious dose and administration route (Figure 3B,D).

225

226 ***Rapid re-induction of wild-type genotype in the absence of Galidesivir***

227 In general, mutant genotypes rapidly revert to the wild-type in the absence of the
228 selection agents. Indeed, after three passages in PS cells in the absence of Galidesivir a pool
229 comprising mutated and wild-type genotypes was detected. Moreover from passage 5
230 onwards, the wild-type genotype dominated in the infected PS cell culture (Figure 1E).
231 Interestingly, the codon GAT encoding an aspartic acid in the E460D mutant had changed to
232 the codon GAA in the revertant, not to GAG as seen in mock-selected wild-type virus; both
233 codons, GAA (in the revertant) and GAG (in the wild-type), are synonymous and encode a
234 glutamic acid residue (Figure 1F). In contrast, after 6 passages in the presence of Galidesivir,
235 at concentrations up to $25 \mu\text{M}$, the E460D substitution was retained; even at low
236 concentrations of Galidesivir ($6.25 \mu\text{M}$) and did not result in reversion to the wild-type
237 genotype (data not shown).

238

239 ***Galidesivir migration towards the TBEV RdRp active site***

240 The deduced position of Galidesivir bound within the TBEV RdRp active site is shown
241 in Figure 4A with its phosphate tail forming close contacts with the manganese cofactors.
242 The E460D (and Y453H) mutation is located at motif F of the flavivirus RdRp finger domain.
243 The flavivirus RdRp motif F is highly flexible and occludes the NTP-tunnel in the nucleotide
244 bound RdRp structure (Valdés et al., 2016). Therefore, the apo-structure was used for
245 stochastic simulations to compare and contrast how Galidesivir approaches the TBEV active
246 site in the wild type and mutant RdRps. At the active site of the wild type TBEV RdRp, the

247 ribose of Galidesivir is at a 5.7 Å distance from amino acid (aa) position 460. The missing
248 carbon in Asp increases this distance (7 Å) compared to Glu. This increased distance, caused
249 by the mutation, results in more steric freedom for Galidesivir as it approaches the active
250 site (Figure 4B,C).

251 There is a ~6.7 Å distance cutoff by Galidesivir approaching the wild type TBEV RdRp
252 active site that is explored closer (<5 Å) in both mutant types (Figure 4B). The single RdRp
253 mutation, E460D, however, has lower enthalpic ligand binding values compared with the
254 double mutant, E460D/Y453H. These lower enthalpic values indicate a favorable ligand
255 binding and conformation. The distinct differences in Galidesivir exploration between the
256 wild type and mutant RdRps are shown by the average COM distance/enthalpy in Figure 4B,
257 specifically those by the single E460D mutation (wild type: 11.3 Å ± 4.6 Å/-179.9 kcal/mol ±
258 121.9 kcal/mol, E460D: 9.5 Å ± 4.9 Å/-257.3 kcal/mol ± 121.2 kcal/mol; E460D/Y453H: 10.6 Å
259 ± 4.5 Å/-235 kcal/mol ± 96.2 kcal/mol). The exploration difference between the RdRps is also
260 demonstrated by the distance of Galidesivir to aa position 453, indicated by the cutoff
261 distance ~21 Å in the wild type RdRp (Figure 4C). Average distances to aa453 are, wild type
262 24.7 Å ± 3.1 Å/7.6 Å ± 4.2 Å, E460D 21.9 Å ± 3.5 Å/8.4 Å ± 2.7 Å and E460D/Y453H 21.3 Å ±
263 2.9 Å/7.1 Å ± 2.7 Å. The Galidesivir distances to aa460 is maintained at ~7.7 Å in all three
264 RdRp types on average.

265 The Arg interrogation residue of motif F is highly conserved in flavivirus RdRps and its
266 electrostatic interaction facilitates incorporation of favorable/correct incoming nucleotides
267 (Valdés et al., 2016; Butcher et al., 2001; Bressanelli et al., 2002). Figure 4D shows that the
268 distances of both mutation sites to Arg473 of the TBEV RdRp within motif F are located 9 Å
269 and 15 Å away. The average distances of Galidesivir to Arg473 during the stochastic
270 simulations are also noted. Due to the steric freedom within the active site caused by E460D
271 (Figure 4A), both substitutions permit closer Galidesivir interactions with the interrogation
272 Arg473. Close proximity to the interrogation residue will thereby increase electrostatic
273 interactions with Galidesivir.

274

275 Discussion

276 Galidesivir is an adenosine analogue originally developed for filovirus infection
277 treatment (Ebola and Marburg) with high antiviral potency against a broad spectrum of RNA
278 viruses (Warren et al., 2014; Taylor et al., 2016), including TBEV (Eyer et al., 2017b) and

279 other medically important arthropod-borne flaviviruses (Warren et al., 2014; Julander et al.,
280 2014; Eyer et al., 2017b; Julander et al., 2017). Currently, this compound entered first-in-
281 human clinical studies that focused on intramuscular administration in healthy volunteers
282 showing promising pharmacokinetics properties and good tolerability (Taylor et al., 2016).
283 This makes Galidesivir a promising candidate drug to treat patients with TBE or with other
284 flaviviral infections. However, antiviral therapy based on small molecule inhibitors of viral
285 replication can be accompanied by rapid evolution of drug-resistance which can abolish the
286 progress of infection treatment and finally lead to the failure of the therapy. Therefore, for
287 each new antiviral agent, the risks of resistance are important to assess in terms of (i)
288 identification of key mutations conferring virus drug-resistance and (ii) phenotype
289 characterization of drug-resistant mutants.

290 Serial *in vitro* passaging of TBEV in the presence of increasing concentrations of
291 Galidesivir (up to 50 μ M) resulted in generation of two drug-resistant TBEV mutants which
292 were approximately 7-fold less sensitive to Galidesivir than the mock-selected wild-type
293 virus. The first TBEV mutant was characterized by a single amino acid change E460D; the
294 other one carried two amino acid changes, E460D and Y453H. Both mutations mapped to
295 the active site of the viral RdRp. Location of the resistance-associated mutations within the
296 viral RdRp active site is essential to understand the mechanism of action of Galidesivir; this
297 compound prevents the binding of subsequent nucleotides to the RdRp active site, being
298 considered a non-obligate chain terminator of viral RNA synthesis (Warren et al., 2014; De
299 Clercq and Neyts, 2009). Single amino acid changes within the RdRp were previously
300 identified in flaviviruses resistant to structurally different nucleoside analogues, as
301 exemplified by the mutations S603T, S604T and S282T conferring a high-level resistance to
302 2'-C-methylated nucleosides in TBEV, Zika virus and hepatitis C virus, respectively (Eyer et al.,
303 2017a; Hercik et al., 2017; Migliaccio et al., 2003). In Alkhurma haemorrhagic fever virus, the
304 mutation S603T was associated with additional amino acid substitutions located in the NS5
305 RdRp active site, particularly with C666S and M644V (Flint et al., 2014).

306 Using a previously described reverse genetics system (Aubry et al., 2014; Driouich et
307 al., 2018) we have demonstrated, that the E460D mutation alone is crucial for resistance of
308 TBEV to Galidesivir; the recombinant E460D-Rec mutant was approximately 7-fold less
309 sensitive to Galidesivir compared with wild-type. On the other hand, the growth kinetics of
310 the Y453H-Rec mutant was almost indistinguishable from that of wild-type virus; it is likely

311 that Y453H can represent a compensation mutation or was acquired randomly. Because of
312 the unique structural features of Galidesivir (C-glycosidic bond and furanose oxygen on the
313 ribose ring replaced by nitrogen) (De Clercq, 2016), no cross-resistance was seen to
314 structurally different nucleoside analogues, such as 7-deaza-2'-C-methyladenosine, 2'-C-
315 methyladenosine and 4'-azido-aracytidine.

316 The E460D TBEV mutant showed similar growth kinetics to the wild-type virus, when
317 cultured *in vitro* on PS cell monolayers. Although, a decreased replication capacity of the
318 E460D mutant was observed in the first few hours after cell culture infection (0 – 12 hours
319 p.i.), both viruses reached a peak titre of about 10^5 – 10^6 PFU/mL at days 2 – 4 after
320 infection. Similarly, the plaque morphology of the E460D mutant and wild-type virus were
321 almost identical to each other; large, clear and round plaques reflected rapid and aggressive
322 spread of both mutant and wild-type viruses in PS cell cultures. Thus, the E460D TBEV
323 mutant differs from the recently isolated S603T TBEV mutant resistant to 2'-C-methylated
324 nucleosides; the S603T mutant exerted significantly decreased replication capacity in PS cells
325 and completely different plaque morphology (small, turbid plaques) compared with the wild-
326 type virus (Eyer et al., 2017a). Our results demonstrate that antiviral resistance developed
327 against two structurally different nucleoside analogues having the same mechanism of
328 action can result in different effects on viral replication capacity in cell culture. Interestingly,
329 in some drug-resistant virus mutants the cell-type dependent replication fitness was
330 observed, as seen in chikungunya virus resistant to T-705 showing the attenuated phenotype
331 in mosquito cell culture, whereas the replication fitness in Vero cells was similar to that of
332 the wild type (Delang et al., 2018).

333 Although, the introduction of the E460D mutation affects amplification of the virus in
334 PS cell culture (i.e., *in vitro*) only slightly, the E460D substitution resulted in a total loss of
335 neuroinvasiveness for mice, *in vivo*; the E460D-infected animals all survived and displayed no
336 clinical signs of neuroinfection during the 28-day experimental period. In contrast, infection
337 with wild-type virus resulted in fatal infections for all animals. We propose that the E460D
338 substitution could affect viral capacity to cross host barriers or responses that restrict the
339 virus infection and translocation to the target tissues/organs *in vivo* but such possibilities do
340 not occur during virus replication in PS cell culture, i.e. *in vitro*. Nevertheless, similar levels
341 of attenuation *in vivo* have previously been reported for drug-resistant RNA or DNA viral
342 mutants, i.e. for TBEV resistance to 2'-C-methylated nucleosides (Eyer et al., 2017a),

343 chikungunya virus resistance to T-705 (Delang et al., 2018), Ribavirin-resistance to porcine
344 reproductive and respiratory syndrome virus (Khatun et al., 2016), vaccinia virus resistance
345 to acyclic nucleoside phosphonates (Gammon et al., 2008), and pleconaril-resistance to
346 coxsackievirus (Groarke and Pevear, 1999).

347 The mutant genotype rapidly reverted to wild-type, when the virus was cultured in PS
348 cell monolayers in the absence of selection agents. However, reversion was not observed,
349 when the virus was cultured in the presence of Galidesivir in concentrations ranging from
350 6.25 to 25 μ M. Thus, under the selection pressure of Galidesivir, the mutation provides a
351 replicative advantage over wild-type variants in the virus quasispecies population resulting in
352 predominance of the mutant in the infected cell culture, despite the fact that the replication
353 characteristics of both variants in cell culture are similar.

354 We conclude that the resistance of TBEV to the nucleoside analogue Galidesivir is
355 conferred by the single amino acid substitution E460D in the NS5 protein. Although this
356 subtle mutation in the active site of the viral RdRp occurs after a few *in vitro* passages of
357 TBEV in the presence of Galidesivir, the E460D TBEV mutant displays dramatically attenuated
358 phenotype in mice showing high survival rates and reduction of clinical signs of
359 neuroinfection. The stochastic molecular simulations indicate that the steric freedom caused
360 by the E460D mutation increases close electrostatic interactions between Galidesivir and the
361 interrogation residue of the TBEV RdRp motif F. Such close electrostatic interactions will
362 reject the analogue as an incorrect nucleotide. The E460D substitution did not confer cross-
363 resistance to unrelated antiviral nucleoside analogues, such as 7-deaza-2'-C-
364 methyladenosine, 2'-C-methyladenosine and 4'-azido-aracytidine. This suggests that a
365 combination treatment based on two or more inhibitors could be a possible strategy in order
366 to minimize the risk of the emergence of viral drug resistance following therapeutic
367 treatment with Galidesivir.

368

369 **Materials and methods**

370 ***Ethics statement***

371 This study was carried out in strict accordance with the Czech national law and
372 guidelines on the use of experimental animals and protection of animals against cruelty (the
373 Animal Welfare Act Number 246/1992 Coll.). The protocol was approved by the Committee
374 on the Ethics of Animal Experiments of the Institute of Parasitology and of the Departmental

375 Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of
376 Sciences of the Czech Republic (Permit Number: 29/2016).

377

378 ***Virus, cells, and antiviral compounds***

379 A well-characterized, low-passage TBEV strain Hypr (Pospisil et al., 1954), a member
380 of the European TBEV subtype, was used in this study. Before use, the virus was sub-cultured
381 intracerebrally six times in suckling mice. Porcine kidney stable (PS) cells (Kozuch and Mayer,
382 1975) were used for viral subculture, selection of drug-resistant viruses, viral growth kinetics
383 studies, and plaque assays. The cells were cultured at 37 °C in Leibovitz (L-15) medium
384 supplemented with 3% newborn calf serum and a 1% mixture of Penicillin and glutamine
385 (Sigma-Aldrich, Prague, Czech Republic). BHK-21 cells (obtained from the American Type
386 Culture Collection [ATCC]), used for transfection of Hypr-derived subgenomic fragments,
387 were cultured at 37 °C with 5% CO₂ in Minimal Essential Medium (MEM) containing 7%
388 bovine serum, 1% Penicillin/Streptomycin and glutamine. Galidesivir (BCX4430) and 4'-azido-
389 aracytidine (RO-9187) were purchased from Medchemexpress (Stockholm, Sweden); 2'-C-
390 methyladenosine and 7-deaza-2'-C-methyladenosine were from Carbosynth (Compton, UK).
391 For *in vitro* studies, the test compounds were solubilized in 100% DMSO to yield 10 mM
392 stock solutions.

393

394 ***Selection of drug-resistant viruses***

395 The *in vitro* selection of drug-resistant TBEV clones was performed, as described
396 previously (Eyer et al., 2017a). Briefly, PS cells seeded in 96-well plates (2×10⁴ cells per well)
397 and incubated to form a confluent monolayer were infected with TBEV at a multiplicity of
398 infection (MOI) of 0.1 and cultivated in the presence of 5 μM of Galidesivir. After 3 to 5 days,
399 the culture medium was harvested and used for infection of fresh cell monolayers. Individual
400 passages were performed with gradually increasing concentrations of Galidesivir as follows:
401 passage 1 at 5 μM, passage 2 to 4 at 10 μM, passages 5 and 6 at 20 μM, passage 7 at 40 μM
402 and passages 8 and 9 at 50 μM (Figure 2B). In parallel, control TBEV was also passaged in the
403 absence of Galidesivir (with 0.5% (v/v) DMSO) as a mock-selected wild-type virus. After
404 passage 9, the drug-resistant and control TBEVs were subjected to an additional subculture
405 to prepare virus stocks for further testing (average titres were between 10⁵ - 10⁶ PFU/mL).
406 The *in vitro* selection protocol was carried out in duplicate, resulting in two independent

407 TBEV mutants, denoted as E460D and E490D/Y453H. In order to recover the revertant wild-
408 type virus from the E460D population, the E460D virus pool was repeatedly sub-cultured in
409 PS cells in the absence of Galidesivir; after 6 serial sub-cultures, the obtained revertant was
410 amplified in PS cells to prepare a virus stock for further testing. Each of these viruses was
411 subjected to full-length sequence analysis, sensitivity/resistance assessment to Galidesivir
412 and other nucleoside analogues, and virulence characterization in mice.

413

414 ***RNA isolation, PCR and whole-genome sequencing***

415 RNA was isolated from growth media using QIAmp Viral RNA Mini Kit (Qiagen).
416 Reverse transcription was performed using ProtoScript First Strand cDNA Kit (New England
417 Biolabs) according to the manufacturer's instructions for the synthesis of first strand cDNA,
418 which was subsequently used for PCR amplification. To cover the whole genome of TBEV, 35
419 overlapping DNA fragments were produced by PCR as described previously (Růžek et al.,
420 2008). DNA was purified using High Pure PCR Product Purification Kit (Roche), according to
421 the recommendations of the manufacturer. The PCR products were directly sequenced by
422 commercial service (SEQme, Czech Republic) using the Sanger sequencing method. Both
423 nucleotide and deduced amino acid sequences were analyzed using BioEdit Sequence
424 Alignment Editor, version 7.2.0.

425

426 ***Reverse genetics system for TBEV Hypr***

427 Reverse genetics system used in this study was based on the generation of infectious
428 subgenomic overlapping DNA fragments that encompass the entire viral genome as
429 previously described (Aubry et al., 2014; Driouich et al., 2018). Three *de novo* synthesized
430 DNA fragments cloned into a pUC57 vector were used in this study (GenScript, Piscataway,
431 NJ, USA): fragment I (nucleotide position 1 to 3662), fragment II (nucleotide position 3545 to
432 8043), and fragment III (nucleotide position 7961 to 11100). The first and last fragment were
433 flanked respectively in 5' and 3' with the human cytomegalovirus promoter (pCMV) and the
434 hepatitis delta ribozyme followed by the simian virus 40 polyadenylation signal
435 (HDR/SV40pA) (Figure 5).

436 Fragments I and II were generated using the SuPREMe method (Driouich et al., 2018).
437 Briefly, plasmids that contained the DNA fragments I and II were digested using respectively
438 AgeI/FseI and SmaI/DraI restriction enzymes (New England BioLabs, Ipswich, MA, USA)

439 (Figure 4). Fragment III was used as template to generate by PCR two overlapping amplicons
440 following the original ISA method (Aubry et al., 2014). Unmodified primers were used to
441 generate two unmodified amplicons (*i.e.* production of wild-type virus). Mutated primers
442 located on the targeted region were used to generate two mutated amplicons (*i.e.*
443 production of mutated viruses, denoted as E460D-Rec, Y453H-Rec, and E460D/Y453H-Rec)
444 (Table 1, Figure 4).

445 The PCR was performed using the Platinum SuperFI PCR Master Mix (Thermo Fisher
446 Scientific, Prague, Czech Republic). The mixture (final volume, 50 μ l) contained 45 μ l of
447 SuperMix, 2 μ l of DNA template (fragment III) at 1 ng/ μ l. Assays were performed on a
448 Biometra TProfessional Standard Gradient thermocycler with the following conditions: 94 °C
449 for 2 min followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s, 68 °C for 5 min and a final
450 elongation step of 68 °C for 10 min. Size of the PCR products was verified by gel
451 electrophoresis and purified using an Amicon Ultra 0.5 ml kit (Millipore).

452 An equimolar mixture of these four DNA fragments was used for cell transfection.
453 DNA-lipid complex was prepared as follows: 12 μ l of Lipofectamine 3000 (Life Technologies)
454 was diluted in 250 μ l Opti-MEM medium (Life Technologies) and then mixed with a master
455 solution of DNA which contained 3 μ g of DNA and 6 μ l of P3000 reagent diluted in 250 μ l
456 Opti-MEM medium. After 45-min incubation at room temperature BHK-21 cells were
457 transfected, as described previously (Aubry et al., 2015) and incubated for 5-7 days. Cell
458 supernatant media were then harvested and serially passaged twice in fresh BHK-21 culture.

459

460 ***Growth kinetics, dose-response studies and viral inhibition assays***

461 To evaluate growth kinetics of drug-resistant TBEV mutants, PS cell monolayers
462 incubated for 24 h in 96-well plates were treated with 200 μ l of medium containing
463 Galidesivir at concentrations of 25 μ M (compound-treated cells) or 0.5% (v/v) DMSO (mock-
464 treated cells) and simultaneously infected with TBEV; the MOI of 0.1 was used for all TBEV
465 mutant/wild-type tested. The medium was collected from the wells daily at days 1 to 7 p.i.
466 (three wells per interval); viral titres (expressed as PFU/mL) were determined by plaque
467 assay as described previously (De Madrid and Porterfield, 1969; Eyer et al., 2015) and used
468 to construct TBEV growth curves.

469 For dose-response studies, 200 μ l of fresh medium containing Galidesivir at
470 concentrations ranging from 0 to 50 μ M was added to PS cell monolayers, infected with

471 TBEV at an MOI of 0.1 and incubated for 3-4 days p.i. Then, the medium was collected from
472 the wells and the viral titres were determined by plaque assay. The obtained viral titre
473 values were used for the construction of TBEV dose-response/inhibition curves and for
474 estimation of the 50% effective concentration (EC₅₀) of the drug.

475 To measure the sensitivity/resistance of the obtained TBEV mutants to Galidesivir
476 and several structurally unrelated nucleoside analogues in cell culture by viral titre inhibition
477 assay, confluent PS cell monolayers cultured for 24 h at 37 °C in 96-well plates were treated
478 with Galidesivir, 7-deaza-2'-C-methyladenosine, 2'-C-methyladenosine, or 4'-azido-
479 aracytidine (RO-9187) at concentrations of 25 or 50 µM and simultaneously infected with
480 TBEV at an MOI of 0.1 (3 wells per compound). As a mock-treated control, DMSO was added
481 to virus- and mock-infected cells at a final concentration of 0.5% (v/v). The formation of
482 cytopathic effect (CPE) was monitored visually using the Olympus BX-5 microscope to yield
483 70%–90% CPE in virus-infected cultures and viral titres were determined by plaque assays
484 from cell culture supernatants.

485

486 ***Mouse infections***

487 To evaluate the virulence of the Hypr E460D mutant in mice, four groups of six-week
488 old BALB/c female mice (purchased from AnLab, Prague, Czech Republic) were infected
489 subcutaneously with TBEV (1,000 PFU/mouse) as follows: group 1 (n = 10), infected with *in*
490 *vitro* mock-selected wild-type; group 2 (n = 10), infected with *in vitro* selected mutant
491 (E460D); group 3 (n = 10), infected with recombinant wild-type TBEV; and group 4 (n = 10),
492 infected with recombinant mutant (E460D-Rec). Survival rates of TBEV-infected mice were
493 monitored daily over the 28-day experimental period. At the same time, controlling of illness
494 symptoms and evaluation of clinical scores were performed in infected animals. Signs of
495 sickness were evaluated as follows: 0 for no symptoms; 1 for ruffled fur; 2 for slowing of
496 activity or hunched posture; 3 for asthenia or mild paralysis; 4 for lethargy, tremor, or
497 complete paralysis of the limbs; 5 for death. All mice exhibiting disease consistent with
498 clinical score 4 were terminated humanely (cervical dislocation) immediately upon
499 detection.

500

501 ***Stochastic molecular simulations***

502 The unbound, apo-form of the TBEV RdRp used in this study is a homology-based
503 predicted structure previously prepared for simulations in other published studies (Valdés et
504 al., 2016, 2017). The stochastic molecular simulations were conducted using the online
505 software, Protein Energy Landscape Exploration (PELE) that employs a Metropolis Monte
506 Carlo algorithm which accepts or rejects a protein-ligand conformation based on its
507 enthalpy. The PELE method and its applications are thoroughly explained online
508 (<https://pele.bsc.es/>) and elsewhere (Madadkar-Sobhani et al., 2013; Borrelli et al., 2005)
509 and therefore are briefly described here. The PELE software comprises three steps: a local
510 protein and ligand perturbation, amino acid sidechain sampling, and a global minimization.
511 These steps are repeated for a few thousand iterations resulting in a trajectory of a ligand
512 (i.e., Galidesivir) approaching the active site of the target protein (i.e., TBEV RdRp). The first
513 25 snapshots were removed from the final trajectories prior to analysis since the stochastic
514 simulations reached a relatively stable total enthalpy. Geometric and enthalpy
515 measurements recoded via the PELE script were used for data analysis. The stochastic
516 migration simulations were performed in triplicates, each with separate positioning of
517 Galidesivir 20 Å ~ 25 Å away from the centre of mass (COM) within the TBEV RdRp active
518 site.

519

520 ***Statistical analyses***

521 Data are expressed as means ± SD, and the significance of differences between
522 groups was evaluated using the Mann-Whitney *U* test or ANOVA. Survival rates were
523 analysed by log-rank Mantel-Cox test. All tests were performed using GraphPad Prism 5.04
524 (GraphPad Software, Inc., San Diego, CA, USA). *P*-values < 0.05 were considered to be
525 statistically significant.

526

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534

535 **References**

- 536 Aubry F, Nougairède A, de Fabritus L, Piorkowski G, Gould EA, de Lamballerie X. "ISA-Lation"
537 of Single-Stranded Positive-Sense RNA Viruses from Non-Infectious Clinical/Animal
538 Samples. *PLoS One*. 2015 Sep 25;10(9):e0138703. doi:
539 10.1371/journal.pone.0138703. eCollection 2015.
- 540 Aubry F, Nougairède A, de Fabritus L, Querat G, Gould EA, de Lamballerie X. Single-stranded
541 positive-sense RNA viruses generated in days using infectious subgenomic amplicons.
542 *J Gen Virol*. 2014 Nov;95(Pt 11):2462-7. doi: 10.1099/vir.0.068023-0. Epub 2014 Jul
543 22.
- 544 Bagaglio S, Uberti-Foppa C, Morsica G. Resistance Mechanisms in Hepatitis C Virus:
545 implications for Direct-Acting Antiviral Use. *Drugs*. 2017 Jul;77(10):1043-1055. doi:
546 10.1007/s40265-017-0753-x.
- 547 Baier A. 2011. Flaviviral infections and potential targets for antiviral therapy. p.89-104. In:
548 Ruzek D (ed), *Flavivirus Encephalitis*. InTech, Rijeka, Croatia.
- 549 Borrelli KW, Vitalis A, Alcantara R, Guallar V, PELE: protein energy landscape exploration. a
550 novel Monte Carlo based technique, *J. Chem. Theory Comput*. 1 (2005) 1304e1311.
- 551 Bressanelli S, Tomei L, Rey FA, De Francesco R. Structural analysis of the hepatitis C virus
552 RNA polymerase in complex with ribonucleotides. *J Virol*. 2002 Apr;76(7):3482-92.
- 553 Butcher SJ, Grimes JM, Makeyev EV, Bamford DH, Stuart DI. A mechanism for initiating RNA-
554 dependent RNA polymerization. *Nature*. 2001 Mar 8;410(6825):235-40.
- 555 De Clercq E and Neyts J. Antiviral Agents Acting as DNA or RNA Chain Terminators. *Handb*
556 *Exp Pharmacol* 2009; 189: 53-84.
- 557 De Clercq E. C-Nucleosides To Be Revisited. *J Med Chem* 2016; 59: 2301-2311.
- 558 De Madrid AT, Porterfield JS. 1969. A Simple Micro-Culture Method for Study of Group B
559 Arboviruses. *Bull World Health Organ*. 1969;40(1):113-21.
- 560 Delang L, Yen PS, Vallet T, Vazeille M, Vignuzzi M, Failloux AB. Differential Transmission of
561 Antiviral Drug-Resistant Chikungunya Viruses by Aedes Mosquitoes. *mSphere*. 2018
562 Aug 22;3(4). pii: e00230-18. doi: 10.1128/mSphere.00230-18.
- 563 Driouich JS, Ali SM, Amroun A, Aubry F, de Lamballerie X, Nougairède A. SuPReMe: a rapid
564 reverse genetics method to generate clonal populations of recombinant RNA viruses.
565 *Emerg Microbes Infect*. 2018 Mar 21;7(1):40. doi: 10.1038/s41426-018-0040-2.

- 566 Dumpis, U., D. Crook, and J. Oksi. 1999. "Tick-borne encephalitis." *Clinical Infectious Diseases*
567 28:882-890. doi: 10.1086/515195.
- 568 Eyer L, Kondo H, Zouharova D, et al. Escape of tick-borne flavivirus from 2' -C-methylated
569 nucleoside antivirals is mediated by a single conservative mutation in NS5 that has a
570 dramatic effect on viral fitness. *J Virol*. Epub ahead of print 16 August 2017. DOI:
571 10.1128/JVI.01028-17.
- 572 Eyer L, Nencka R, de Clercq E, Seley-Radtke K, Růžek D. Nucleoside analogs as a rich source of
573 antiviral agents active against arthropod-borne flaviviruses. *Antivir Chem Chemother*.
574 2018 Jan-Dec;26:2040206618761299.
- 575 Eyer L, Smidkova M, Nencka R, et al. Structure-activity relationships of nucleoside analogues
576 for inhibition of tick-borne encephalitis virus. *Antivir Res* 2016; 133: 119-129.
- 577 Eyer L, Valdes JJ, Gil VA, et al. Nucleoside Inhibitors of Tick-Borne Encephalitis Virus.
578 *Antimicrob Agents Chemother* 2015; 59: 5483-5493.
- 579 Eyer L, Zouharová D, Širmarová J, Fojtíková M, Štefánik M, Haviernik J, Nencka R, de Clercq E,
580 Růžek D. Antiviral activity of the adenosine analogue BCX4430 against West Nile virus
581 and tick-borne flaviviruses. *Antiviral Res*. 2017 Jun;142:63-67.
- 582 Flint M, McMullan LK, Dodd KA, et al. Inhibitors of the Tick-Borne, Hemorrhagic Fever-
583 Associated Flaviviruses. *Antimicrob Agents Chemother* 2014; 58: 3206-3216.
- 584 Gammon DB, Snoeck R, Fiten P, Krecmerová M, Holý A, De Clercq E, Opendakker G, Evans
585 DH, Andrei G. Mechanism of antiviral drug resistance of vaccinia virus: identification
586 of residues in the viral DNA polymerase conferring differential resistance to
587 antipoxvirus drugs. *J Virol*. 2008 Dec;82(24):12520-34. doi: 10.1128/JVI.01528-08.
- 588 Groarke JM, Pevear DC. Attenuated virulence of pleconaril-resistant coxsackievirus B3
589 variants. *J Infect Dis*. 1999 Jun;179(6):1538-41.
- 590 Heinz FX, Mandl CW. The Molecular-Biology of Tick-Borne Encephalitis-Virus. *APMIS* 1993;
591 101: 735-45.
- 592 Hercik K, Brynda J, Nencka R, et al. Structural basis of Zika virus methyltransferase inhibition
593 by sinefungin. *Arch Virol* 2017; 162: 2091-2096.
- 594 Irwin KK, Renzette N, Kowalik TF, Jensen JD. Antiviral drug resistance as an adaptive process.
595 *Virus Evol*. 2016 Jun 10;2(1):vew014. doi: 10.1093/ve/vew014.

- 596 Julander JG, Bantia S, Taubenheim BR, et al. BCX4430, a Novel Nucleoside Analog, Effectively
597 Treats Yellow Fever in a Hamster Model. *Antimicrob Agents Chemother* 2014; 58:
598 6607-6614.
- 599 Julander JG, Siddharthan V, Evans J, et al. Efficacy of the broad-spectrum antiviral compound
600 BCX4430 against Zika virus in cell culture and in a mouse model. *Antivir Res* 2017;
601 137: 14-22.
- 602 Khatun A, Shabir N, Seo BJ, Kim BS, Yoon KJ, Kim WI. The Attenuation Phenotype of a
603 Ribavirin-Resistant Porcine Reproductive and Respiratory Syndrome Virus Is
604 Maintained during Sequential Passages in Pigs. *J Virol*. 2016 Apr 14;90(9):4454-4468.
605 doi: 10.1128/JVI.02836-15.
- 606 Kozuch O, Mayer V. 1975. Pig Kidney Epithelial (Ps) Cells - Perfect Tool for Study of Flavi-
607 Viruses and Some Other Arboviruses. *Acta Virol* 19:498.
- 608 Lauring AS, Frydman J, Andino R. The role of mutational robustness in RNA virus evolution.
609 *Nat Rev Microbiol*. 2013 May;11(5):327-36. doi: 10.1038/nrmicro3003.
- 610 Madadkar-Sobhani A, Guallar V. PELE web server: atomistic study of bio-molecular systems
611 at your fingertips. *Nucleic Acids Res*. 2013 Jul;41(Web Server issue):W322-8. doi:
612 10.1093/nar/gkt454.
- 613 Migliaccio G, Tomassini JE, Carroll SS, Tomei L, Altamura S, Bhat B, Bartholomew L,
614 Bosserman MR, Ceccacci A, Colwell LF, Cortese R, De Francesco R, Eldrup AB, Getty
615 KL, Hou XS, LaFemina RL, Ludmerer SW, MacCoss M, McMasters DR, Stahlhut MW,
616 Olsen DB, Hazuda DJ, Flores OA. Characterization of resistance to non-obligate chain-
617 terminating ribonucleoside analogs that inhibit hepatitis C virus replication in vitro. *J*
618 *Biol Chem* 2003; 278: 49164-49170.
- 619 Pospisil L, Jandasek L, Pesek J. 1954. Isolation of new strains of meningoencephalitis virus in
620 the Brno region during the summer of 1953. *Lek List* 9:3-5.
- 621 Poveda E, Wyles DL, Mena A, Pedreira JD, Castro-Iglesias A, Cachay E. Update on hepatitis C
622 virus resistance to direct-acting antiviral agents. *Antiviral Res*. 2014 Aug;108:181-91.
623 doi: 10.1016/j.antiviral.2014.05.015.
- 624 Růžek D, Gritsun TS, Forrester NL, Gould EA, Kopecký J, Golovchenko M, Rudenko N,
625 Grubhoffer L. Mutations in the NS2B and NS3 genes affect mouse neuroinvasiveness
626 of a Western European field strain of tick-borne encephalitis virus. *Virology*. 2008
627 May 10;374(2):249-55. doi: 10.1016/j.virol.2008.01.010. Epub 2008 Mar 14.

628 Ruzek, D., G. Dobler, and O. Donoso Mantke. 2010. "Tick-borne encephalitis: Pathogenesis
629 and clinical implications." *Travel Medicine and Infectious Disease* 8:223-232. doi:
630 10.1016/j.tmaid.2010.06.004.

631 Taylor R, Kotian P, Warren T, et al. BCX4430-A broad-spectrum antiviral adenosine
632 nucleoside analog under development for the treatment of Ebola virus disease. *J*
633 *Infect Public Heal* 2016; 9: 220-226.

634 Valdés, J.J., Butterill, P.T., Růžek, D. (2017) Flaviviridae viruses use a common molecular
635 mechanism to escape nucleoside analogues. *Biochemical and Biophysical Research*
636 *Communications*. 492(4):652-658.

637 Valdés, J.J., Gil, V.A., Butterill, P.T., Ruzek, D. (2016) An all-atom, active site exploration of
638 antiviral drugs that target flaviviridae polymerases, *J. Gen. Virol.* 97:2552-2565.

639 Warren TK, Wells J, Panchal RG, et al. Protection against filovirus diseases by a novel broad-
640 spectrum nucleoside analogue BCX4430. *Nature* 2014; 508: 402-405.

641 Westover JB, Mathis A, Taylor R, Wandersee L, Bailey KW, Sefing EJ, Hickerson BT, Jung KH,
642 Sheridan WP, Gowen BB. Galidesivir limits Rift Valley fever virus infection and disease
643 in Syrian golden hamsters. *Antiviral Res.* 2018 Aug;156:38-45. doi:
644 10.1016/j.antiviral.2018.05.013.

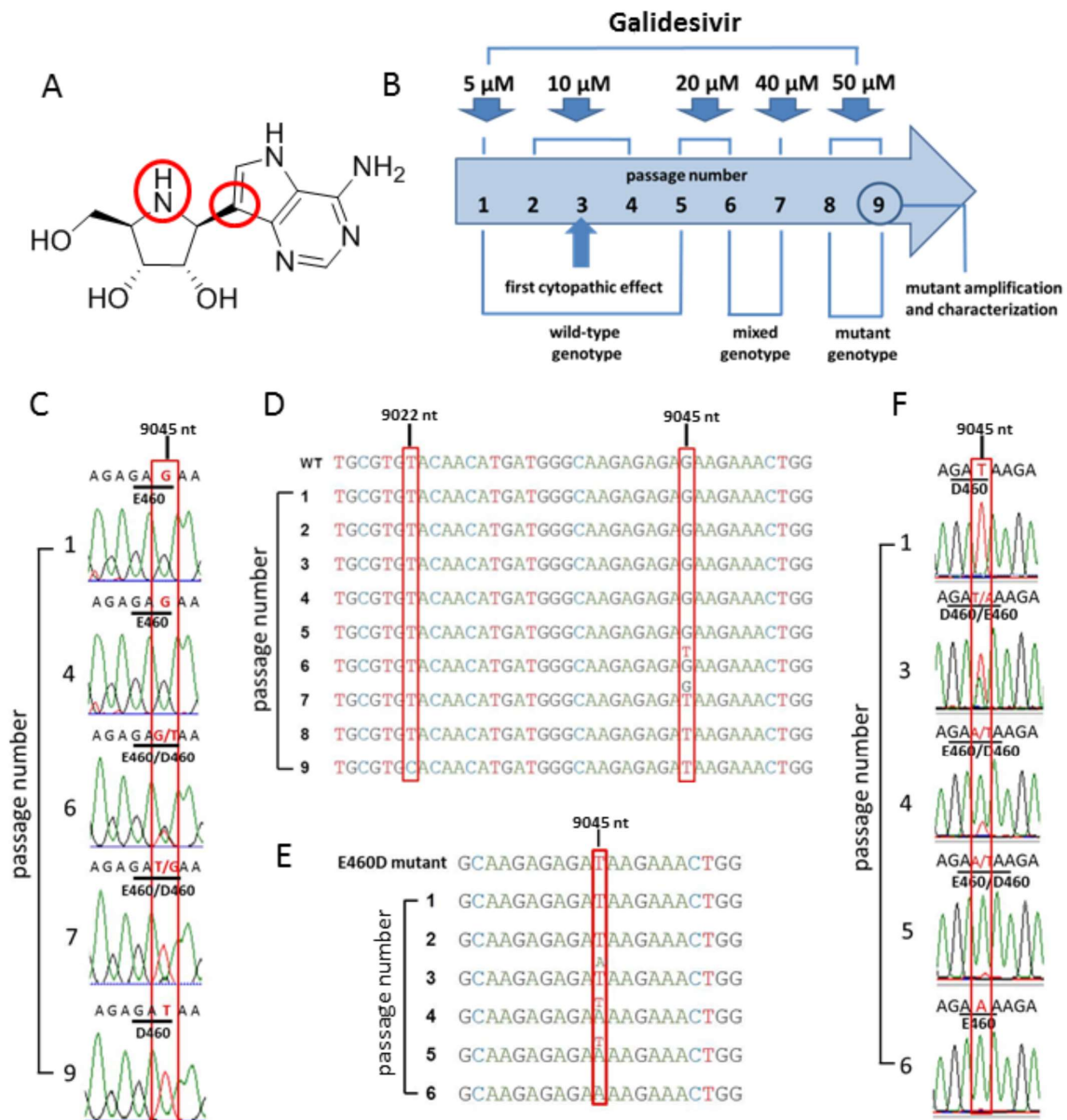
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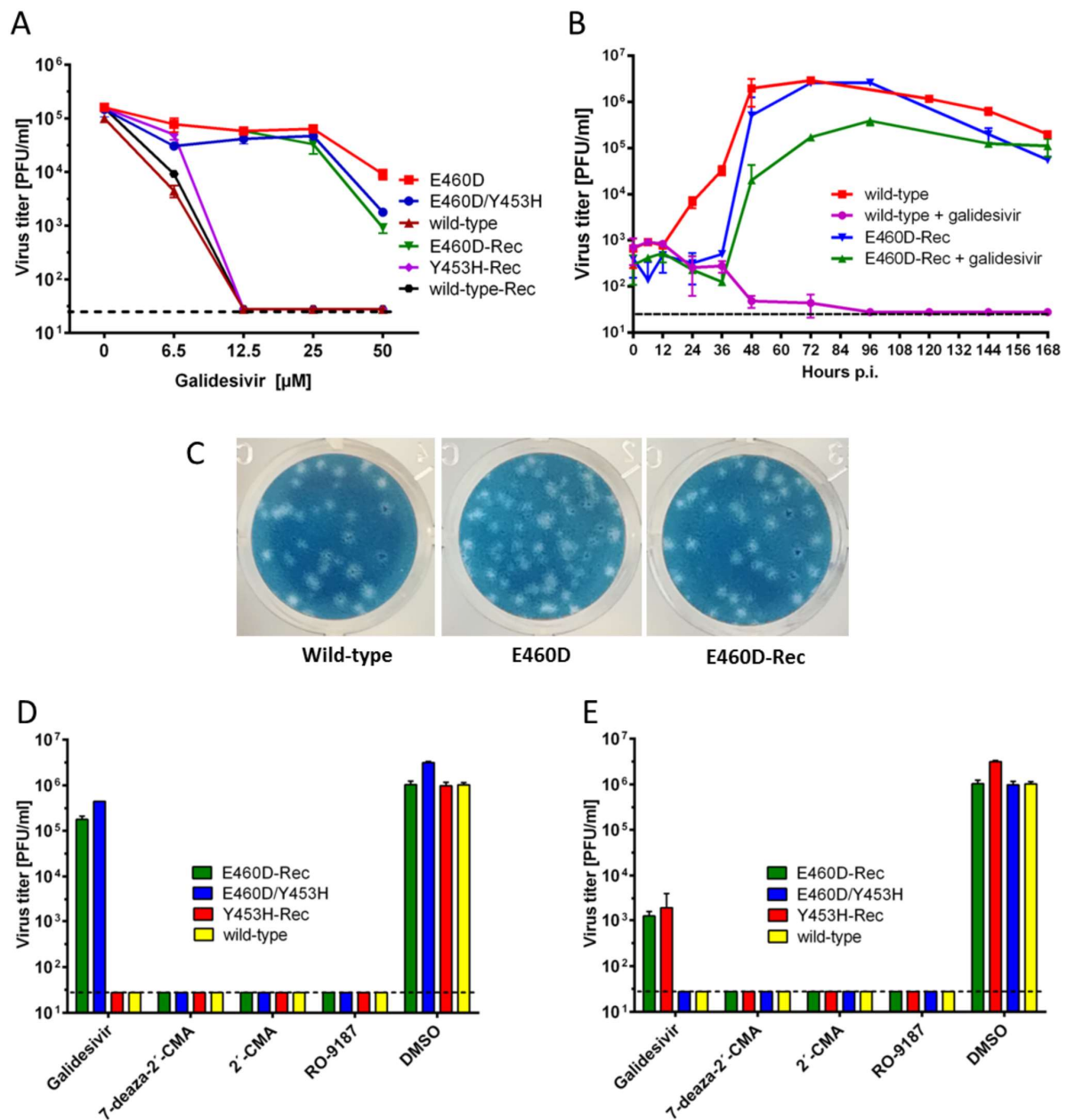
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649 **Figures**



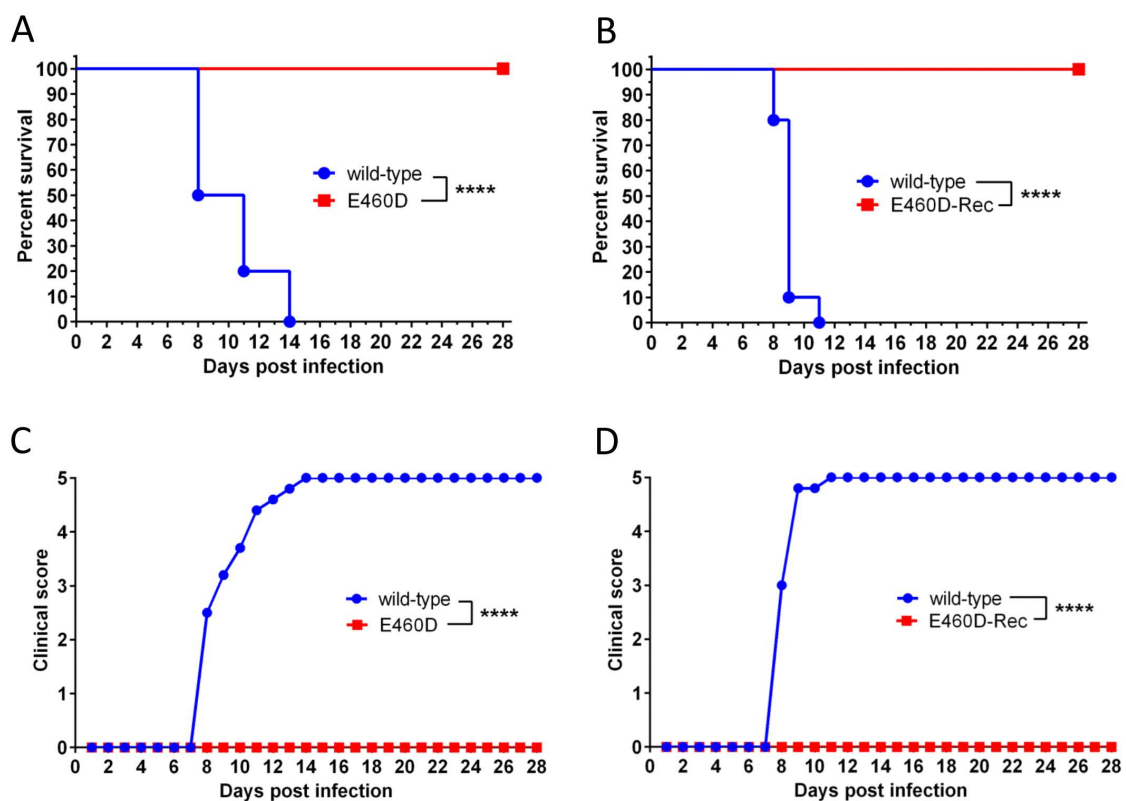
650
 651 **Fig 1. TBEV resistance to Galidesivir is associated with a single mutation in the NS5 gene.**
 652 (A) The structure of the nucleoside analogue Galidesivir. (B) Scheme of the selection process
 653 for generation of TBEV resistance to Galidesivir. TBEV was serially passaged in PS cells in the
 654 presence of increasing Galidesivir concentrations. (C) Whole-genome sequence analysis of
 655 the passaged viruses revealed a mutation at the amino acid position 460 in the NS5 protein,
 656 changing the glutamic acid residue to aspartic acid. (D) In the E460D/Y453H mutant, both
 657 amino acid changes E460D (nucleotide position G9045T) and Y453H (nucleotide position and
 658 T9022C) were detected in the NS5 protein after 5 and 8 passages in the presence of
 659 Galidesivir, respectively. (E) Rapid re-induction of the wild-type genotype in PS cells

660 following serial passage of TBEV in PS cells in the absence of Galidesivir. (F) During serial
661 passage of the E460D mutant virus in PS cells in the absence of galidesivir, the amino acid
662 codon GAT (in the mutant) was changed to the codon GAA (in the revertant).
663

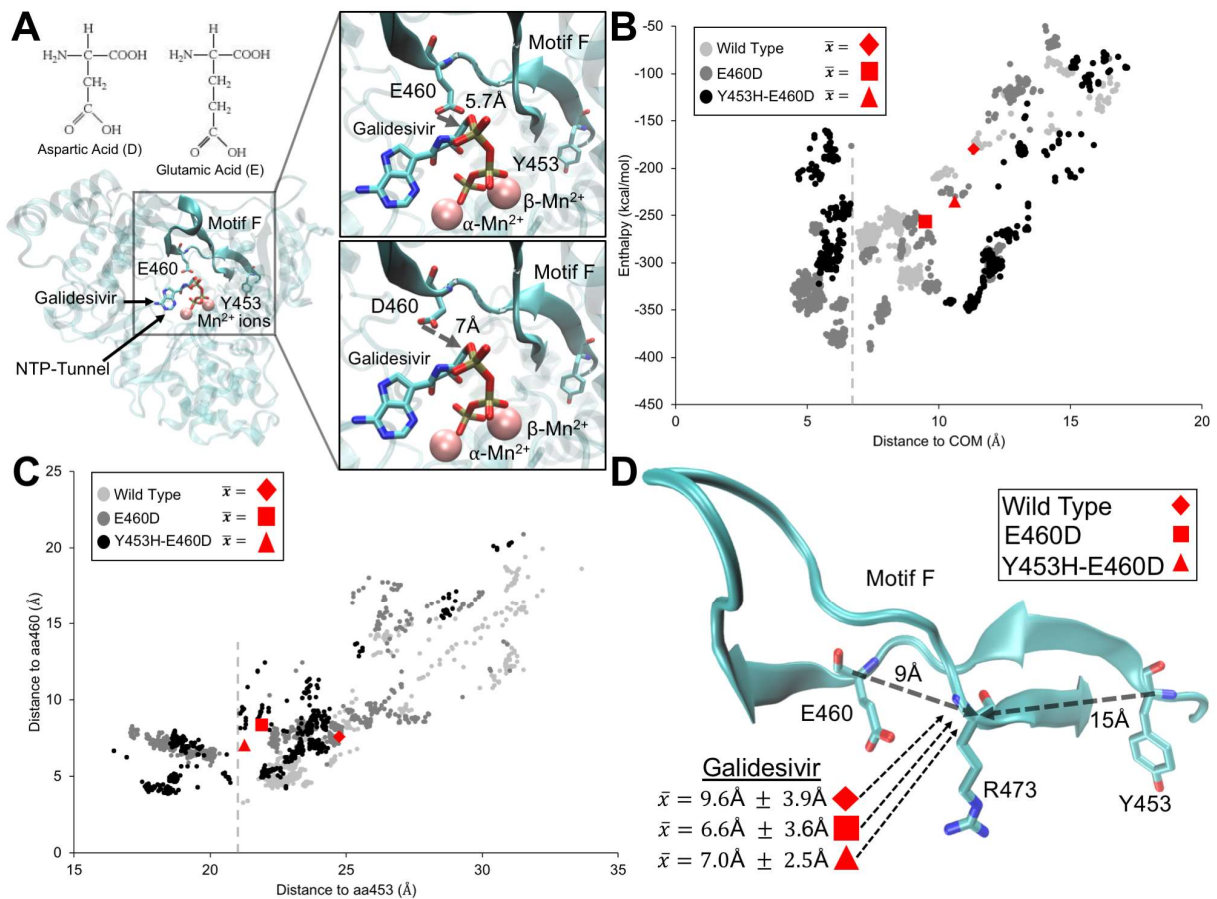


664
665 **Fig 2. Phenotypic properties of TBEV mutant resistant to Galidesivir *in vitro*.** (A) The dose-
666 response curves for TBEV mutants E460D, E460D/E453H, E460D-Rec, Y453H-Rec, and
667 corresponding wild-types grown in PS cells in the presence of Galidesivir at indicated
668 compound concentrations. Only TBEVs bearing the E460D mutation (i.e., E460D,
669 E460D/E453H, and E460D-Rec) were resistant to Galidesivir, indicating that this mutation is

670 responsible for the resistance phenotype. (B) Growth kinetics of the E460D-Rec mutant and
671 wild-type TBEV in the presence (25 μ M) or absence (0 μ M) of Galidesivir within the 7-day
672 experimental period to assess the replication efficacy of the mutant TBEV in PS cells. (C)
673 Plaque morphology of the E460D and E460D-Rec mutants was assessed in PS cell
674 monolayers and compared with the wild-type virus. (D-E) The sensitivity/resistance profiles
675 of the E460D/Y453H, E460D-Rec, and Y453H-Rec to diverse nucleoside inhibitors
676 (concentrations of 25 μ M (D) and 50 μ M (E)) were evaluated in PS cells and compared with
677 the corresponding wild-type TBEV. The mean titers from two independent experiments,
678 each performed in triplicate are shown and error bars indicate standard errors of the mean.
679 The horizontal dashed line indicates the minimum detectable threshold of 1.44 log₁₀
680 PFU/mL.
681



682
683 **Fig 3. Phenotypic properties of the TBEV mutant resistant to Galidesivir in mice.** The extent
684 of neuroinvasiveness of the E460D and E460D-Rec TBEV mutants was investigated in BALB/c
685 mice and compared with that of the wild-type virus. Adult BALB/c mice were infected
686 subcutaneously with 10³ PFU of either virus and survival (A-B) and clinical scores of the
687 neuroinfection (C-D) were monitored for 28 days. ****, *p* < 0.0001.



688

689 **Fig 4. Proposed positioning of Galidesivir and its migration in the wild type and mutant**

690 **TBEV polymerase.** (A) The TBEV polymerase structure shown at 180° from its canonical

691 right-hand conformation. Motif F and the residues subject to mutation are highlighted with

692 Galidesivir and two Mn^{2+} ions (box). The NTP-tunnel is indicated by the arrow. The insets

693 show the distance from the oxygen of E460 to the oxygen 5' of Galidesivir and the distance

694 to the mutant, D460 - shown at a 90° orientation. The Lewis structures show the missing

695 carbon in Asp compared to Glu. (B) The x-y scatter plot is the Galidesivir binding enthalpy in

696 kcal/mol (y-axis) during its migration to the hypothetical center of mass (COM) position

697 within the TBEV polymerase active site (x-axis). The averages of the three replicates are

698 shown as red geometric shapes (legend). (C) The x-y scatter plot is the Galidesivir distance to

699 amino acid (aa) residue positions 453 (x-axis) and 460 (y-axis) that are subject to mutation in

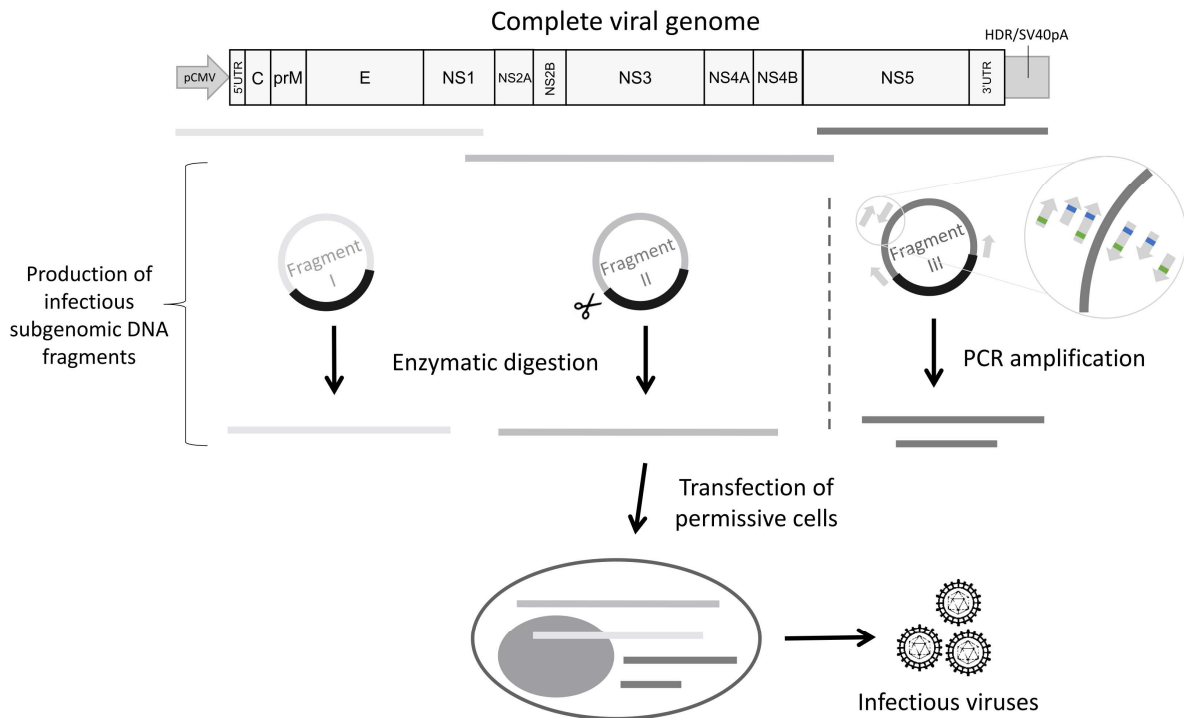
700 the TBEV polymerase. (D) Structural representation of motif F in a similar orientation as in

701 (A) showing the average distance of Galidesivir, as red geometric shapes, to the interrogating

702 residue R473. The distances of Y453 and E460 to the interrogating residue R473 (carbon

703 backbone) are indicated by arrows.

704



705

706 **Fig 5. General overview of the reverse genetics method presented in this study**

707 The reverse genetics method used in this study was based on the generation of infectious
708 subgenomic overlapping DNA fragments that encompass the entire viral genome. Three *de*
709 *novo* synthesized DNA fragments cloned into a pUC57 vector were used. The first and last
710 fragment were flanked respectively in 5' and 3' with the human cytomegalovirus promoter
711 (pCMV) and the hepatitis delta ribozyme followed by the simian virus 40 polyadenylation
712 signal (HDR/SV40pA). Fragments I and II were generated using the SuPREMe method:
713 plasmids were digested using restriction enzymes. Fragment III was used as template to
714 generate by PCR two overlapping amplicons following the original ISA method. Unmodified
715 primers were used to generate two unmodified amplicons (*i.e.* production of wild-type
716 virus). Mutated primers located on the targeted region were used to generate two mutated
717 amplicons (green and blue squares represent respectively mutations G9045T and T9022C).
718 An equimolar mix of these four DNA fragments was used to transfect BHK-21 cells.

719

720 Table 1. Unmodified and mutated primers used to generate two overlapping amplicons of
 721 fragment III (fragment IIIa and IIIb), i.e. to produce recombinant wild-type and mutated
 722 viruses
 723

Primer type	Fragment III position	Forward/reverse	Generation of wild-type/mutated virus	Primer sequence (the T9022C and G9045T mutations and their complementary counterparts are marked in green)
Primers to amplify fragment IIIa	14-36	forward	-	ATACACCATTGGTGGAAAGAGGGC
	975-1034	reverse	wild-type-Rec	CTTTCTCTCTTCATCCACGAGGTGCCAGAATGCAGG ATCCTCTACAGCCTCTCTTGC
	1070-1097	reverse	E460D-Rec	CAGTTTCTT A TCTCTCTTGCCCATCATG
	1059-1090	reverse	Y453H-Rec	TCTCTCTCTTGCCCATCATGTTGT G CACGCA
	1048-1096	reverse	E460D/Y453H-Rec	AGTTTCTT A TCTCTCTTGCCCATCATGTTGT G CACGCAG TGCGCGCATC
Primers to amplify fragment IIIb	1009-1072	forward	wild-type-Rec	ACCTCGTGGATGAAGAGAGAGAAAGGCACCTCATGGG GAGATGCGCGCACTGCGTGTAC
	1079-1100	forward	E460D-Rec	CAAGAGAGA T AAGAAACTGGGA
	1049-1074	forward	Y453H-Rec	ATGCGCGCACTGCGTG G ACAACATGA
	1059-1107	forward	E460D/Y453H-Rec	TGCGTG G ACAACATGATGGGCAAGAGAGA T AAGAAAC TGGGAGATTTCG
	3050-3071	reverse	-	CTCAGGGTCAATGCCAGCGCTT

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 725

726 Table 2. Inhibitory properties of Galidesivir for the obtained TBEVs

Virus	Method to generate wild-type/mutated virus	EC₅₀ [μM]* (fold increase compared to wild-type value)
E460D	<i>In vitro</i> selection (passaging in PS cells)	6.66 ± 0.04 (7.01)
E460D/ Y453H		7.20 ± 0.09 (7.57)
wild type		0.95 ± 0.04
E460D-Rec	Reverse genetics	6.32 ± 1.01 (7.90)
Y453H-Rec		0.81 ± 0.01 (1.01)
E460D/ Y453H-Rec		not rescued
wild-type-Rec		0.80 ± 0.01

727 *Determined from three independent experiments. Expressed as a 50% reduction of viral titers and
 728 calculated according to the Reed-Muench method. PS cells were infected with the virus (MOI of 0.1)
 729 and cultivated at Galidesivir concentrations ranging from 0 to 50 μM for three days.

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