units, which is in good agreement with published results. In comparison, all doses of prodrug **32** reduced HCV viral titers to a greater extent than did either of the positive controls.

Using deep sequencing technology, the circulating virus on day 7 was analyzed to identify sequence changes in the NS4B region that might lead to drug resistance.[38] Multiple mutations were identified, three of which (N56I, G60V, N99H) predominated in a number of animals dosed with prodrug **32** (Table 5.6). Similar mutations did not arise in animals treated with HCV-796 or BILN-2061. Transient replicons containing the single-point mutations were created and the antiviral activity of **31** was determined. The G60V transient replicon was not viable; however, the N56I and N99H transient replicons showed significant resistance to **31**. There was a >600-fold shift in replicon potency ($EC_{50} > 1000$ nM) between the N99H and wild-type virus.

It is noteworthy that the most prevalent mutations identified in the PXB mice study were not observed in the *in vitro* resistance passaging experiments. Discrepancies between resistance mutations generated *in vivo versus in vitro* have also been reported for HCV NS3/4A protease inhibitors. However, in many instances resistance in the replicon assay is predictive of the *in vivo* outcome, as was the case for HCV-796 in our PXB study (the major mutation was C316Y in the NS5B protein). The reason for the selection of different mutations is unclear, but may result from the presence of adaptive mutations in HCV replicon required for replication or from protein interactions unique to the full viral life cycle of a replicating live virus. Regardless, it serves as a reminder of the potential pitfalls associated with generating resistance in artificial *in vitro* systems and the continued need for developing (and utilizing) *in vivo* models for infectious diseases.

Table 5.6 Mutations in the NS4B protein identified through sequencing of the HCV virus present in PXB mice treated for 7 days with prodrug **32**. Deep sequencing allowed for quantitation and relative percentages of the mutations are reported.

		A21V	I35X	N56I	G60V	F98L	N99H	V105X	A115D	A231V
100	10									
mg kg^{-1}	102			9.60%	17%					
GSK23588	103			1.10%						
53A	104			10.10%	23.90%	3.70%		(M) 2.5%		
30	201			16.0%	11.0%	2.8%	10.3%	(M) 2.6%		
mg kg^{-1}	202	1.2%		30.6%	6.5%		6.8%	(W) 2.1%		
GSK23588	203			8.7%	6.4%		12.5%			1.2%
53A	204			3.1%	7.8%	2.1%	4.3%	(M) 1.7%	1.0%	
10	301	1.0%		1.7%			38.7%			
mg kg^{-1}	302		(V) 1.2%	1.7%			89.8%			
GSK23588	303			1.9%		1.7%	65.7%			
53A	304						81.0%			