there was a limit to the size of the ring that could be accommodated as the cycloheptyl derivative was ~10-fold less active relative to the cyclohexyl analog. Both the cyclopentyl and cyclohexyl derivatives tested positive for the formation of reactive metabolites (rat and human microsomes) due to apparent oxidation of the C5-furan followed by the addition of glutathione. Replacing the furan with cyclopropyl (**26**, Table 5.1) eliminated this liability and yielded a potent inhibitor of HCV replication.

Although the potency of the piperazinone **26** was attractive, the high rat clearance (Cl_{rat} > hepatic blood flow) precluded further development. An analysis of the metabolites generated *in vitro* from rat and human hepatocytes showed rapid oxidation of the terminal cyclohexyl substituent. Efforts to block the metabolism *via* methylation or fluorination of the ring were unsuccessful. Realizing that the cyclohexyl substituent was cleared *via* an oxidative pathway, we became interested in introducing hydroxyl groups at various positions of the ring as a means to alter metabolism and reduce lipophilicity. The racemic 2-hydroxy (*trans*-**27**) and 3-hydroxy (*cis*-**28** and *trans*-**29**) cyclohexyl analogs were significantly less active than the unsubstituted cyclohexyl analog (**26**) in the replicon and NS4B binding assays (Table 5.1). The 4-hydroxy derivatives proved far more interesting with the *trans*-4-OH analog **31** exhibiting low nanomolar potency against the 1a and 1b replicons. The spatial orientation of the hydroxyl group was important as the corresponding *cis*-isomer **30** was 20–100-fold less potent. As expected, the addition of the hydroxyl group reduced the cLog*P* relative to the unsubstituted analog.

An important benefit of adding the 4-*trans*-OH group to the cyclohexyl substituent (*i.e.* **31**) was the substantial improvement in PK relative to the unsubstituted derivative **26**.[33] The clearance of **31** was low (<10% hepatic blood flow) in mouse, dog and cynomolgus monkey and moderate in rat (Table 5.2). Low oral doses of **31** (5 mg kg^{-1} in a solution formulation) achieved high oral bioavailability (F > 80%) in three of the four species. Furthermore, **31** did not inhibit any human cytochrome P450 isozymes (IC_{50} > 30 μM *versus* 3A4, 1A2, 2C9, 2C19 or 2D6) and was negative in the glutathione-trapping reactive metabolite assay (rat and human microsomes). Based on experience with the oxazolidinone compounds (**17**–**19**, Figure 5.7), the potential for **31** to induce metabolism was also evaluated. Compound **31** did not activate rat or human PXR and did not upregulate the mRNA of the major P450 isozymes in rat, dog, or human hepatocytes.

The high binding affinity to NS4B, attractive antiviral profile, and favorable low-dose PK supported the further preclinical development of **31**. Despite the relatively low cLog*P* (3.3), the FaSSIF solubility of **31** was poor (11 μg mL^{-1}), which negatively impacted the ability to achieve the higher plasma drug exposures needed to support preclinical safety studies. For example, a 300 mg kg^{-1} suspension dose of crystalline **31**, wet-bead milled to maximize the surface area for faster dissolution, achieved low levels of exposure in rat ($AUC_{0-24\,h}$ = 11.3 μg h mL^{-1}, Figure 5.10). Ultimately, we examined the use of prodrugs to overcome this liability. Two common prodrug classes were selected (phosphate **32** and esters **33**–**36**) and appended to the 4-hydroxyl group.