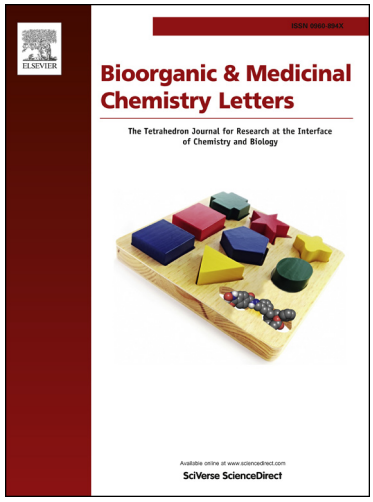

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Design and synthesis of N-alkyldeoxynojirimycin derivatives with improved metabolic stability as inhibitors of BVDV and Tacaribe virus

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Keywords: N-alkyldeoxynojirimycin; sulfonamide; urea; oxazolidinone; metabolic stability; antiviral; BVDV; Tacaribe

Abstract: Novel N-alkyldeoxynojirimycins (NADNJs) based on our previous lead **3** were designed, synthesized and tested in metabolic assays and in virus cultures. NADNJs containing terminal tertiary benzamide, sulfonamide, urea, and oxazolidinone moieties were discovered to have improved metabolic stability compared to **3**, while maintaining submicromolar EC_{50} against BVDV and Tacaribe virus; and low cytotoxicity.

The metabolic stability of a drug has significant impact on both its safety and efficacy. As a result, *in vitro* liver microsomal stability testing has been implemented in the early stages of drug discovery to evaluate the metabolic liabilities of a drug candidate in the presence of cytochrome P450 enzymes (CYPs).[1] The results can be used as a criteria to select compounds for advancement into further *in-vitro* bioactivity assays and *in vivo* animal models. For those compounds that fail in the metabolic stability test, structural modification can be executed to improve stability while maintaining attractive *in vitro* activities observed. Generally, a metabolic site, either as a labile element or functional group, needs to first be identified, and this can be achieved either by incubation with CYP isozymes followed by LC/MS[2] analysis, or by prediction[3]. Once this information is available, a metabolically more stable element[2] or surrogate group[4] can be used to block the site. Several strategies have been employed for improving metabolic stability, including reduction of lipophilicity, change of sterics and electronics, introduction of conformational constraints, and alteration of the stereochemistry of the compound.[5] These methods can be also understood as to either change the reduction potential of the labile site, or move the labile site away from the target binding position.

In our recent studies on deoxynojirimycin (**1**, DNJ) and its derivatives as glucosidase inhibitors and broad spectrum antiviral agents against hemorrhagic fever viruses,[6,7,8] compounds **2-4,** with either aromatic ether or tertiary amide terminals, were identified as having submicromolar EC_{50}s against bovine viral diarrhea virus (BVDV), Dengue virus, and Tacaribe virus, and low cellular toxicities. However, when subsequent *in vitro* ADMET studies were performed,[9] we found that compound **3** and **4** had lower microsomal stability (Table 1) as compared to compound **2**. Because these two unstable compounds share a common tertiary amide scaffold, this feature became the focus of our structural optimization.

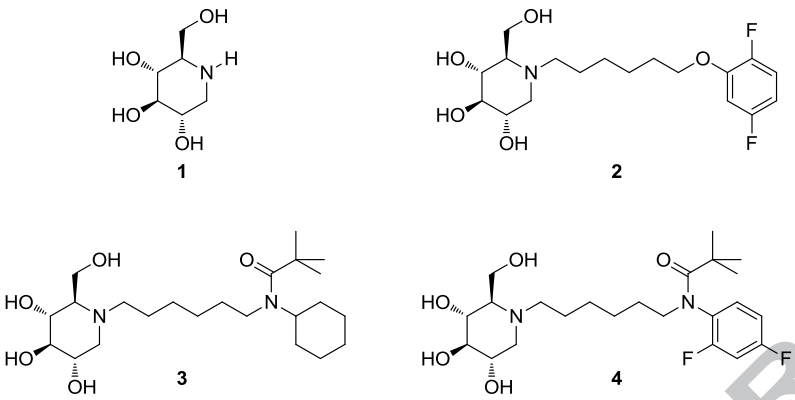


Figure 1. DNJ and active N-alkyl derivatives with aromatic ether and tertiary amide terminals

Compound	Liver Microsomal Stability Remaining @ 60 min (%)		
	Human	Rat	Mouse
2	91	79	91
3	41.56	26.87	58.57
4	36.11	21.56	55.27

Table 1. Microsomal stability tests for N-alkyl DNJ derivatives with aromatic ether and tertiary amide terminals

Compared to compound **2**, compound **4** has only one difference in its terminal structure, which consists of a nitrogen heteroatom, a pivaloyl group, and an aromatic substitution. Because the nitrogen atom is blocked by three bulky groups, we don't expect that C-N bonds are likely to be the source of liability. And arguably, the 2,4-difluorophenyl in compound **4** would have similar microsomal stability to the 2,5-difluorophenyl in compound **2**. Thus, we hypothesized that the microsomal liability for both compound **3** and **4** was due to the acetyl of the pivaloyl group. To improve the microsomal stability of this labile site, the lead compound, **3**, was selected as the starting point. We reasoned that we can change the reduction potential of the sp^3 hybridized methyl group by either replacing it with a sp^2 hybridized phenyl ring (**5** in Figure 2) or substituting the carbonyl in pivaloyl with a more electron withdrawing sulfonyl and phosphoryl group (**6, 7**); We can also change the polarity environment around the methyl group by introducing a hydrogen bonding donor NH to the carbonyl to form a urea (**8**), which is expected

to influence the interaction with the P-450 enzymes, therefore, to reduce the likelihood of leaving the methyl group at the oxidation pocket. Another potential approach is to remove the alkyl group connected to the carbonyl and introduce an oxygen atom in the form of oxazolidin-2-one, as we know that the Boc group with the same carbamate moiety could bring potency to this series of compounds.[8] Thus, compounds **5-9** were prepared as described in Schemes 1.

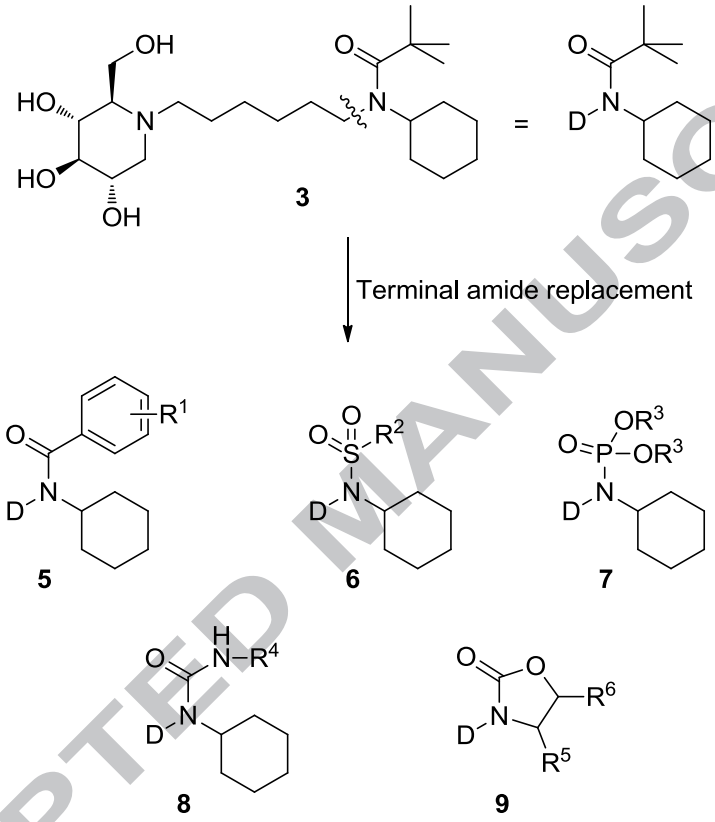
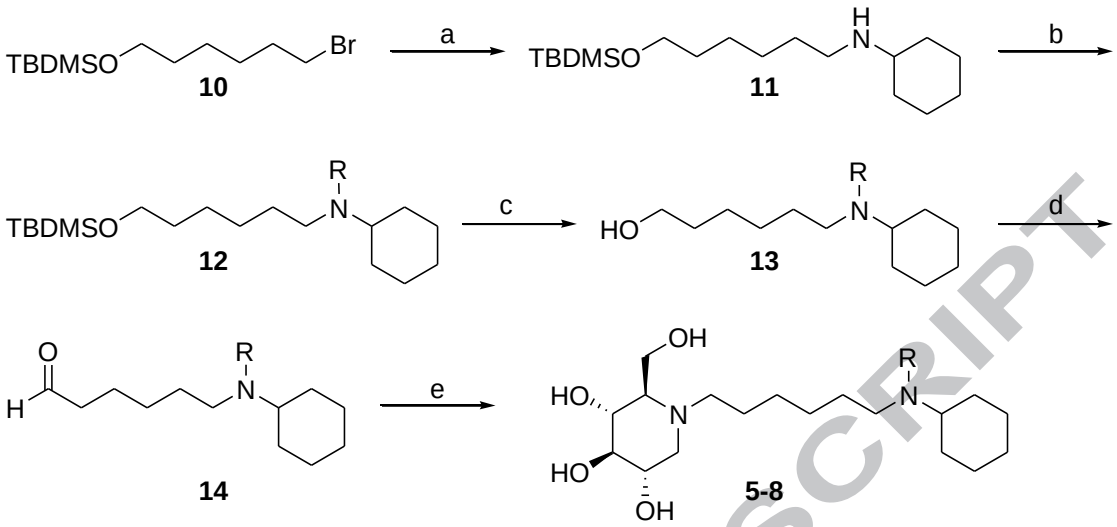


Figure 2. Proposed analog structures of compound 3 with terminal group replaced.

The compounds **5-8** were synthesized in a general procedure, which allowed us to diversify the acyl group replacement (Scheme 1). Synthesis began with the alkylation of cyclohexylamine with ((6-bromohexyl)oxy)(tert-butyl)dimethylsilane, followed by derivation with an acyl, sulfinyl, sulfonyl,[10] phosphoryl chloride or isocyanate to give a protected tertiary amine derivative **12**. Subsequent deprotection with TBAF and oxidation of the resulting alcohol **13** generated the corresponding aldehyde **14**. Reductive amination with DNJ provided the final DNJ derivatives **5-8**.



Reagents and conditions: a, $C_6H_{11}NH_2$, K_2CO_3, CH_3CN, 80 ^oC; b, Et_3N, R^7COCl or R^8SO_2Cl, R^9SOCl, $(R^{10}O)_2POCl$, or $R^{11}NCO$, CH_2Cl_2 or THF; c, TBAF, THF; d, PCC or Dess-Matin periodane; e, DNJ, AcOH, EtOH, Pd/C.

Scheme 1. General synthetic route for preparation of compound 5-8.

Compound **9** was synthesized by a similar procedure, in which compound **10** was reacted with the appropriate oxazolidinones in the presence of LiHMDS in THF. Three compounds**,** $\mathbf{9_a}$**,** $\mathbf{9_b}$**,** and $\mathbf{9_c}$**,** were prepared following this procedure.

The compounds were evaluated for their antiviral activities against BVDV (a pestivirus) in MDBK cells and tacaribe virus (an arenavirus) in Huh7.5 cells (Table 2).[6,11] The compounds that maintained the antiviral potency of lead compound **3** would be selected for metabolic stability study. The antiviral activity was measured by yield reduction assay and expressed as EC_{50} values; cytotoxicity was measured by an MTT assay and expressed as CC_{50} values. Compared to compound **3**, replacement of the *tert*-butyl group with either phenyl ring (**5a**) or 2,4,5-fluorophenyl ring (**5b**) retained the antiviral potency against both BVDV and Tacaribe virus with low cellular toxicity. When sulfonamide **6a** was introduced as a surrogate for amide in **3**, submicromolar EC_{50}s were also obtained for BVDV and Tacaribe virus. Larger hydrophobic groups contained as *tert*-butyl in **6c** and phenyl in **6b** were tolerated, but higher cellular toxicity was seen with **6b**. These results were different from the amide series in which larger hydrophobic groups afforded better potency.[8] Phosphoramide **7** reduced the activity against BVDV by more than ten-fold (EC_{50} = 3.4 μM), although it retained good potency against Tacaribe virus (EC_{50} = 0.4 μM). Of the three urea analogs tested, only compound **8c** resumed submicromolar activities against both BVDV and Tacaribe virus (EC_{50} = 0.25, 0.74 μM respectively) while exhibiting low cellular toxicity, consistent with the results in the amide series. The unsubstituted urea **8a** and n-

propyl containing compound **8b** both showed weak activity against BVDV and Tacaribe virus, suggesting that an increase in hydrophobic interaction benefits the antiviral activities of this family of compounds.

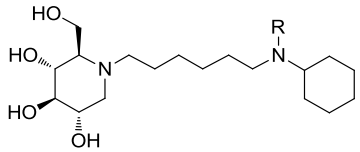
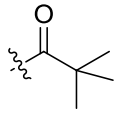
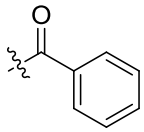
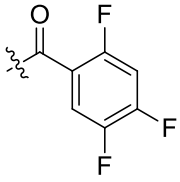
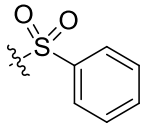
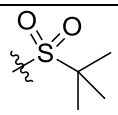
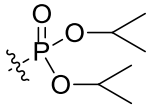
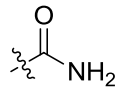
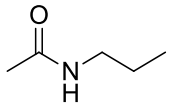
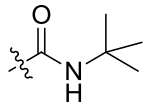


Table 2. Inhibition and toxicity evaluation of amide derivatives

		BVDV		Tacaribe	
compound	R	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
3		0.2	> 500	0.2	500
5a		0.32	> 500	0.6	> 500
5b		0.27	> 500	0.6	> 500
6a		0.2	> 500	0.7	> 500
6b		0.5	200	1.6	280
6c		0.31	> 500	0.4	> 500
7		3.4	> 500	0.3	310

8a		14.2	> 500	100	> 500
8b		15.8	> 500	25	> 500
8c		0.25	> 500	0.74	> 500

The effect of the tertiary amide to oxazolidinone conversion is illustrated in Table **3**.[11] Compound **9a** exhibited reasonable activity against BVDV (EC_{50} = 0.8 μM), but not against Tacaribe virus. It is interesting that this compound is about four-fold more potent than its diastereomer **9b**, which had weaker activity against BVDV (EC_{50} = 3.5 μM). Notably, the benzofused oxazolidinone **9c** showed improved potencies against both BVDV and Tacaribe virus (EC_{50} = 0.4, 0.4 μM respectively) and low cellular toxicity (CC_{50} > 500 μM), indicating a compact scaffold is favored in this family of the compounds.

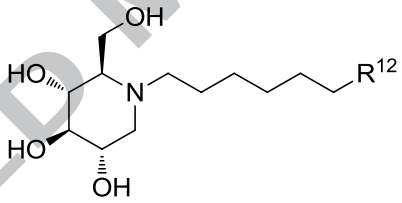
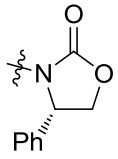
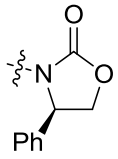
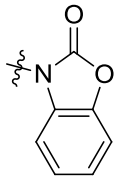


Table 3. Evaluation of DNJs with oxazolidinoe terminal

		BVDV		Tacaribe	
compound	R^{12}	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
9a		0.8	> 500	ND	ND
9b		3.5	> 500	ND	ND

9c		0.4	> 500	0.4	> 500

ND, not determined.

With some new leads with comparable activities to the early lead **3** in hand, we next selected six compounds representing benzamide, sulfonamide, urea, and oxazolidinone families and carried out metabolic stability studies.[9] We found that the benzamide **5a** exhibited more than twice the stability of **3** in human, rat, and mouse microsome assays (Table 4). The methyl sulfonamide **6a** also possessed increased metabolic stability in human liver microsome assay. However, the sulfonamide **6b** and **6c,** with bulkier phenyl or *tert*-butyl group displayed no improvement relative to compound **3**. This probably is due to the different oxidation potential or trajectory of the sulfonamide substituent from that of the amide,[12] which might deliver the phenyl or tert-butyl group into a new and better oxidation position in the pocket of P450. On the other hand, the urea **8c** exhibited excellent metabolic stability (93%, 92%, and 87% in human, rat and mouse microsome assay, respectively), even although the *tert*-butyl group was unaltered and the length of this side chain was increased by one NH group. We propose that the presence of the additional NH group changed binding conformation and physicochemical and electronic environment around the *tert*-butyl and carbonyl groups, possibly keeping the *tert*-butyl group away from the optimal oxidation position. The oxazolidinone **9c**, on the contrary, eliminated the corresponding *tert*-butyl group as in lead **3**, resulting in similar microsomal stability (100%, 87%, and 87% in human, rat and mouse microsome assay, respectively). This observation suggests that the target interacting conformation of **9c** is similar to that of the **3**.

In summary, different approaches have been used in improving the metabolic stability of the early lead **3**, with some proving to be successful. The minimalist approach was to entirely remove or trim away the labile saturated group (such as in **9c**), although structural modification at other parts of the molecule may be needed to compensate for loss of potency. Introduction of an additional polar atom also worked well (such as in **8c**), because this new polar group can change the binding conformation and electronics of the molecule itself through formation of hydrogen bonds, and this change may protect the labile group escape from the oxidative process.

Table 4. Evaluation of microsomal stability of new DNJ derivatives.

compound	Liver Microsomal Stability Remaining

	@ 60 min (%)		
	Human	Rat	Mouse
3	41.56	26.87	58.57
5a	82.56	77.42	97.37
6a	95.55	65.66	86.88
6b	12.96	3.83	42.79
6c	50.19	12.14	65.38
8c	93.56	92.76	87.58
9c	100.82	86.91	86.66

Thus, through modifying the terminal tertiary amide structure of the early lead **3**, we have identified several scaffolds, including benzamide, sulfonamide, urea, and oxazolidinone with improved metabolic stability. The newly prepared DNJ analogs, **5a**, **6a**, **8c**, and **9c,** also maintained good antiviral activity against both BVDV and Tacaribe virus.

Acknowledgements

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Rensen, W.; Coluccia, A.; Piscitelli, F.; Gatti, V.; Bolognesi, A.; Lavecchia, A.; Granata, I.; Porta, A.; Maresca, B.; Soriani, A.; Iannitto, M. L.; Mariani, M.; Santoni, A.; Brancale, A.; Ferlini, C.; Dondio, G.; Varasi, M.; Mercurio, C.; Hamel, E.; Lavia, P.; Novellino, E.; and Silvestri, R. *J. Med. Chem.*, **2011**, *54*, 8394.

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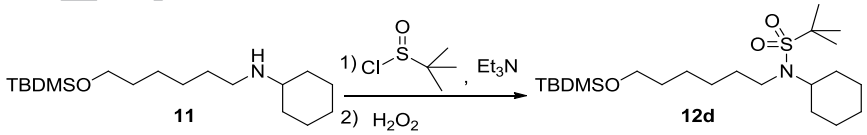
9. Microsomal stability test was conducted by Pharmaron. 0.5mg/ml of microsomes were incubated with test compounds (2μM concentration) at 37°C in presence of 1mM NADPH. Reactions without NADPH serve as negative controls.

10. Intermediate $\mathbf{12_d}$ was prepared through a two step reaction: Reaction of secondary amine **11** with 2-Methyl-propane-2-sulfinyl chloride gave a sulfinamide, which was oxidized to sulfonamide $\mathbf{12_d}$ by H_2O_2.



11. MDBK cells were infected with BVDV at MOI of 1. Huh7 cells were infected with Tacaribe virus at MOI of 0.01. Cells were treated with test compounds at concentrations ranging from 0.01 to 100 μM, immediately after incubation with virus. BVDV and Tacaribe titers were determined by standard virus yield reduction assay in MDBK and Vero cells, respectively. EC_{50} values were calculated from dose-dependent virus yield reduction curves. CC_{50} values were determined using MTT (Sigma) assay in MDBK and Huh7 cells, respectively.

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