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Deoxynojirimycin and its hexosaminyl derivatives bind to natural killer cell receptors rNKR-P1A and hCD69

Giorgio Catelani [a,*], Felicia D'Andrea [a], Alessio Griselli [a], Lorenzo Guazzelli [a], Petra Němcová [c], Karel Bezouška [b,c], Karel Křenek [b,c], Vladimír Křen [b,*]

[a] Dipartimento di Scienze Farmaceutiche, Università di Pisa, Via Bonanno, 33, I-56126 Pisa, Italy
[b] Institute of Microbiology, Academy of Sciences of the Czech Republic, Centre of Biocatalysis and Biotransformation, CZ 142 20 Praha 4, Czech Republic
[c] Department of Biochemistry, Faculty of Science, Charles University Prague, Hlavova 8, CZ-12840 Praha 2, Czech Republic

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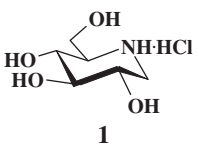
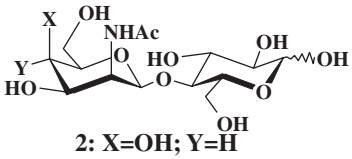
ABSTRACT

Deoxynojirimycin (**1**) and two new related 4-*O*-hexosaminyl-containing disaccharide mimics, β-D-TalNAc-(1→4)-DNJ (**4**) and β-D-ManNAc-(1→4)-DNJ (**5**), have been studied as agonists of natural killer (NK) cell receptors. As a positive and unexpected result, DNJ (**1**) displayed a remarkable activation effect towards both NKR-P1A (rat) and CD69 (human) receptors, and a quite similar activity was found for **4** and **5**. The synthesis of the two disaccharide mimics is based on an approach that avoids the glycosylation step using known intermediates arising from lactose. The key stage of the synthesis involves the construction of the DNJ unit through an initial C-5 oxidation of the reducing D-glucopyranosyl unit followed by a stereoselective double-reductive aminocyclization of the 1,5-dicarbonyl disaccharide intermediates.

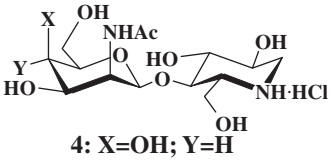
Natural killer (NK) cells are a distinct subset of lymphoid cells that have innate immune functions, providing an early protection against viruses, parasites, bacteria, and tumors. Recent studies have revealed that NK cells are also involved in T-cell-mediated adaptive immune responses via indirect mechanisms (secretion of cytokines or chemokines, DC maturation/activation) or by direct cell-to-cell interactions.[1] Detailed studies aimed at determining the structural requirements of optimal carbohydrate ligands for NKR-P1, the major NK rat receptor, have established that:[2] (i) the presence of a 2-deoxy-2-acetamido group is crucial for binding; (ii) the agonist activity is related to the relative C-2, C-4 group orientation, determining the following activity order: ManNAc > GalNAc > GlcNAc ≫ TalNAc; (iii) a group with hydrogen bond accepting properties (–OH, –COOH, –COOR) is needed for a strong interaction with the receptor; (iv) a β-oriented anomeric substituent, and β-(1→4) interglycosidic connection for di- and oligosaccharides are required. Recently, β-D-TalNAc-(1→4)-D-Glc (**2**) and β-D-ManNAc-(1→4)-D-Glc (**3**) disaccharides have been synthesized and tested for their affinity towards natural killer cell NKR-P1A and CD69 receptors,[3] exhibiting interesting binding activities. This prompted us to synthesize some structural analogues and/or mimetics of **2** and **3**, such as compounds **4** and **5** by replacing



1



2: X=OH; Y=H
3: X=H; Y=OH



4: X=OH; Y=H
5: X=H; Y=OH

* Corresponding authors. Tel.: +39 0502219678; fax: +39 0502219660 (G.C.).
E-mail addresses: giocate@farm.unipi.it, g.catelani@tin.it (G. Catelani).

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the D-glucose unit with a 1-deoxynojirimycin (DNJ, **1**), and evaluate them as NK cell receptor agonists.

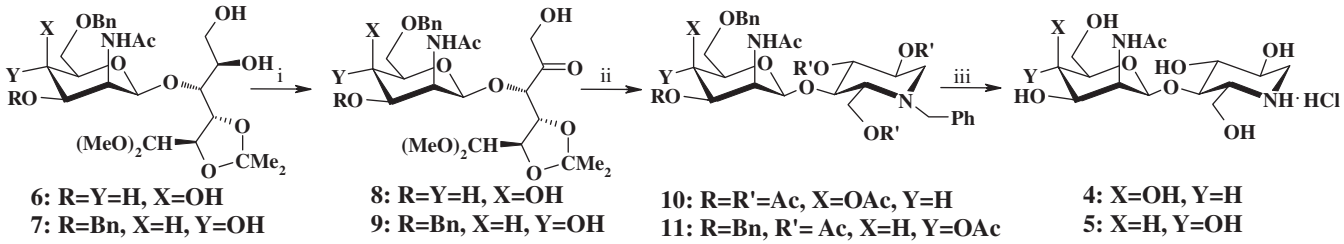
DNJ is by far the most investigated iminosugar, a class of carbohydrate mimetics, which have been the focus of increasing attention over the last two decades because of their glycosidase inhibitory properties.[4] As a result, *N*-hydroxyethyl-DNJ (Glyset™) and *N*-butyl-DNJ (Zavesca™) have been approved as drugs for type II diabetes and Gaucher disease treatment, respectively. More recently, DNJ and a large number of structurally related iminosugar analogues have been synthesized and extensively studied for their biological activities, exhibiting therapeutic potential towards a range of diseases, including cancer, viral infections, and lysosomal diseases.[5] However, the immunomodulation properties of iminosugars have received little attention and their effect on the immune system has only been reported in a limited number of papers.[6,7] Van den Broek et al. reported in 1996 that DNJ and *N*-7-oxadecyl-DNJ inhibit the in vitro proliferation of lymphocytes.[6] More recently, a contribution from a Chinese group claims that *N*-pentafluorobenzyl-DNJ displays a specific inhibitory effect on cytokine secretion from human peripheral blood mononuclear cells (PMBC) and human CD4+ T cells.[7a] Further studies by the same group reported remarkably immunosuppressant activities of *N*-alkylated 1,6-dideoxy-DNJ derivatives[7d] and of D-*galacto*- and L-*altro*-configured 1-hydroxyethyl[7b] and 1-acylamidoethyl[7d] DNJ derivatives. Unexpectedly, the interferon-γ (IFN-γ) secretion from mice splenocytes was found to be strongly enhanced by some compounds of the latter type, thus for the first time suggesting immunostimulating properties for specific iminosugars.[7e]

Several glycosylated DNJ derivatives have been isolated from natural products exhibiting interesting biological activities.[8] However, to the best of our knowledge, no DNJ derivative glycosylated with a hexosaminyl unit has so far been isolated from natural sources or synthesized. Taking advantage of the availability[9] of intermediates **6** and **7** from lactose, we considered an approach for the synthesis of **4** and **5** based on the intramolecular double-reductive amination of β-D-hexosaminyl-(1→4)-D-*xylo*-aldohexos-5-ulose derivatives. This approach, leading to disaccharide azamimics avoiding the glycosylation step of a DNJ acceptor, was proposed by ourselves some years ago[10] and further used by Stütz for the synthesis of biologically relevant azadisaccharide mimics.[11]

The selective oxidation at C-5 of polyols **6** and **7** was performed using the well known stannylidene acetal mediated method.[12] Thus, the 5,6-*O*-stannylidene acetal obtained from the triol **7** by reaction with Bu_2SnO (1.0 equiv) in refluxing toluene under azeotropic water removal conditions, followed by treatment with NBS after changing the solvent from toluene to chloroform produced the C-1 aldehydo protected ketone **9**[13] (Scheme 1) with an excellent 94% yield. A similar result was obtained for the oxidation of the tetraol **6**, leading to the corresponding ketone **8**[13] as the sole oxidation product isolated with a 67% yield. It is noteworthy that also in this case the oxidation takes place with complete regioselectivity, despite the possible involvement of a 3′,4′-*O*-stannylidene acetal. This is at variance with the result reported for the analogous tetraol derivative of lactose,[10] in which the corresponding 4′-keto derivative was isolated as a by-product. This difference could be attributed to a destabilization of the 3′,4′-stannylidene acetal ring by the steric hindrance of the axial C-2′ acetamido group. The aldehydo group of **8** and **9** was deprotected by treatment with 90% aq CF_3COOH, also causing the removal of the 2,3-*O*-isopropylidene acetal protection. 1,5-Dicarbonyl disaccharide intermediates, comprised of a mixture of tautomeric forms (NMR), were submitted without further purification to a double-reductive aminocyclization by treatment with $NaBH_3CN$ (2.2 equiv) and $BnNH_2{\cdot}HCl$ (1 equiv) in MeOH at 60 °C for 12 h, followed by direct acetylation (Ac_2O/Py, 24 h) of the crude reaction mixtures. Completely protected aza-disaccharides **10**[13] and **11**[13] were isolated with acceptable overall yields (42% and 40%, respectively). The structure and stereochemistry of **10** and **11** were firmly established by ^1H, ^{13}C NMR and 2D NMR experiments. In particular, diagnostic signals for the DNJ unit were the H-1*ax*, H-1*eq* and H-5 protons located, for example in **11**, at δ 2.16 (dd, $J_{1ax,1eq}$ 11.8 Hz, $J_{1ax,2}$ 10.5 Hz), at δ 2.90 (dd, $J_{1eq,2}$ 5.2 Hz) and at δ 2.64 (dd, $J_{4,5}$ 9.6 Hz, $J_{5,6a}$ 3.1 Hz, $J_{5,6b}$ 2.3 Hz). As for some reported aminocyclizations of *xylo* configured aldohexos-5-uloses,[7d,10,14] also here the intramolecular reductive amination takes place with complete stereoselectivity, leading to the isolation of the sole iminosugar with a *gluco* configuration, probably because of the presence of stereoelectronic and conformational factors involving an axial hydride attack during the stereodifferentiating step on the more stable (all equatorial) conformer of the cyclic iminium ion intermediate.[15] Finally, compounds **10** and **11** were routinely deprotected via an initial de-*O*-acetylation (MeONa/MeOH), followed by catalytic hydrogenolysis (H_2, Pd/C in 1.3 M methanolic HCl) leading to the target disaccharide mimics β-D-TalNAc- and β-D-ManNAc-(1→4)-DNJ as hydrochlorides (**4** and **5**).[16]

New compounds **4** and **5** were tested for binding affinity towards the activation receptors of NK cells, rat NKR-P1A[17], and human CD69.[18] As controls for assessing the structure–activity relationship, the respective building units were also included in the plate inhibition assays as well as the standard positive control GlcNAc.[19] Surprisingly, DNJ gave comparable results to GlcNAc, used in these tests as a benchmark. Coupling the two units increases the binding, but with NKR-P1A it only has an additive and not multiplicative effect. A different situation, however, was found with CD69 in compound **5**, where the attachment of ManNAc (that is per se inactive), to DNJ (**1**) increases the binding by more than one order of magnitude. Nevertheless, the most interesting result from a practical point of view seems to be the binding of DNJ to the activation receptors of NK cells.

In a series of previous studies,[2] it was clearly demonstrated that NKR-P1 receptor interacts strongly with *N*-acetylhexosamines. The binding groove optimally binds linear oligosaccharides (typically chitoteraose); GalNAc and ManNAc being stronger ligands than GlcNAc. In contrast, CD69 only binds well to GlcNAc, but linked to the branched, multiantennary structures, where multivalency plays a crucial role.[20] This is also the reason why the binding



Scheme 1. Reagents and conditions: (i) Bu_2SnO, CH_3Ph, reflux, 20 h, then NBS, $CHCl_3$, rt, 0.5–1.5 h (**8**: 67%; **9**: 94%); (ii) (a) 90% aq TFA, rt, 2–3 h; (b) $PhCH_2NH_2{\cdot}HCl$, $NaBH_3CN$, MeOH, 60 °C, 12 h; (c) Ac_2O, Py, rt, 24 h (**10**: 42% from **8**; **11**: 44% from **9**); (iii) (a) MeONa, MeOH, 0 °C, 4–5 h; (b) H_2, Pd/C 10%, HCl, MeOH, rt, 2.5–5 h (**4**: 92%; **5**: 81%).

Table 1
Affinity of DNJ and its glycosyl derivatives towards two NK cell activation receptors, NKR-P1A (rat), and CD69 (human), expressed on a logarithmic scale ($-\log IC_{50}$)

Compound	NKR-P1A	CD69
DNJ·HCl (**1**)	5.0	3.2
TalNAc-DNJ·HCl (**4**)	5.5	3.3
ManNAc-DNJ·HCl (**5**)	6.5	4.5
TalNAc	4.6	0
ManNAc	6.7	0
GlcNAc	5.6	3.5

Data are presented as average values from three independent experiments.

affinities with simple oligosaccharidic ligands (see Table 1) are much weaker in CD69, where the presence of multivalent ligands improves the binding by several orders of magnitude. Typically, binding affinity correlates well with the killing activity of the respective NK cells towards tumor or virally infected somatic cells.

In summary, probably the most interesting result is the binding of DNJ itself to NK cell activation receptors. DNJ is an iminosugar active as a selective α-glucosidase inhibitor and is used for the treatment of various viral infections—typically HIV infections. During such treatment, the level of activity of the immune system is of utmost importance and compromising it can lead to the failure of the treatment. The immunomodulatory activity of these iminosugars has rarely been studied and only a few reports can be found in the literature, mostly describing the immunosuppressive activity of such compounds.[6,7] Van den Broeck[6] tested a series of DNJ-*N*-alkyl derivatives and found that an *N*-7-octadecyl derivative inhibited PBMC (lymphocyte)-induced proliferation. Unfortunately DNJ was not tested. Ye et al.[7b] tested a series of newly prepared iminosugar derivatives for their effects on the secretion of IL-4 and IFN-γ from mouse splenocytes and found that some compounds are strong immunosuppressants with potential for use in the treatment of autoimmune diseases. Recently, Zhou et al.[7d] prepared a series of 1,6-dideoxy-*N*-alkyl iminosugars and demonstrated IFN-γ and IL-4 inhibition activity in some of them. None of the above studies tested non-derivatized DNJ as a control (benchmark) (**1**), which we consider a drawback.

Here we are reporting new results on the potential immunoactivation effect of DNJ (**1**) which could be of significant importance for the treatment of viral infections, during which the activation of NK cells represents one of the critical elements of the immune response.[21,22] This pilot study indicates that DNJ and mainly its derivatives currently used in therapy should be further tested for their immunostimulating activities in more complex cellular systems, and in vivo.

Acknowledgments

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13. All new compounds were fully characterized with NMR and gave correct elemental analysis. **8**: syrup, $[\alpha]_D$ −64.6 (*c* 1.06, $CHCl_3$), R_f 0.41 (8:2 $CHCl_3$–MeOH); **9**: syrup, $[\alpha]_D$ −65.4 (*c* 1.12, $CHCl_3$), R_f 0.25 (95:5 $CHCl_3$–MeOH); **10**: solid foam, $[\alpha]_D$ −29.4 (*c* 1.14, $CHCl_3$), R_f 0.40 (EtOAc); **11**: solid foam, $[\alpha]_D$ −46.4 (*c* 1.1, $CHCl_3$), R_f 0.24 (4:6 hexane–EtOAc); **4**: solid foam, $[\alpha]_D$ −17.2 (*c* 1.0, MeOH); **5**: solid foam, $[\alpha]_D$ −21.8 (*c* 1.04, MeOH).
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16. Compound **4**: ^{1}H NMR (CD_3OD–D_2O, 600 MHz): δ 4.72 (br s, 1H, H-1′), 4.40 (br d, 1H, $J_{2',3'}$ 3.9 Hz, H-2′), 3.96 (dd, 1H, $J_{5,6a}$ 4.4 Hz, $J_{6a,6b}$ 12.2 Hz, H-6a), 3.85–3.80 (m, 3H, H-2, H-6b, H-6′b), 3.79–3.70 (m, 4H, H-4, H-6′a, H-3′, H-4′), 3.57 (m, 1H, H-5′), 3.54 (t, 1H, $J_{2,3} = J_{3,4}$ 8.5 Hz, H-3), 3.32 (dd, 1H, $J_{1eq,2}$ 4.9 Hz, $J_{1ax,1eq}$ 11.7 Hz, H-1*eq*), 3.21 (m, 1H, H-5), 2.89 (t, 1H, $J_{1ax,2}$ 11.7 Hz, H-1*ax*), 1.98 (s, 3H, *Me*CON); ^{13}C NMR (CD_3OD–D_2O, 62.9 MHz): δ 174.4 (C=O), 102.1 (C-1′), 79.0 (C-4), 78.0 (C-5′), 76.1 (C-3), 69.5 (C-2), 68.9, 68.4 (C-3′, C-4′), 62.6 (C-6′), 60.3 (C-5), 58.3 (C-6), 53.9 (C-2′), 46.8 (C-1), 23.4 (*Me*CON). Compound **5**: ^{1}H NMR (CD_3OD–D_2O, 600 MHz): δ 4.82 (br s, 1H, H-1′), 4.55 (br d, 1H, $J_{2',3'}$ 4.1 Hz, H-2′), 3.89 (dd, 1H, $J_{5,6b}$ 2.1 Hz, $J_{6a,6b}$ 11.8 Hz, H-6b), 3.87 (dd, 1H, $J_{5,6a}$ 4.1 Hz, H-6a), 3.80 (m, H, H-6′a, H-6′b), 3.77 (dd, 1H, $J_{3,4}$ 8.9 Hz, $J_{4,5}$ 9.1 Hz, H-4), 3.73 (ddd, 1H, $J_{1eq,2}$ 4.9 Hz, $J_{1ax,2}$ 11.7 Hz, $J_{2,3}$ 8.9 Hz, H-2), 3.65 (dd, 1H, $J_{2',3'}$ 4.1 Hz, $J_{3',4'}$ 9.6 Hz, H-3′), 3.52 (t, 1H, $J_{3,4}$ 8.9 Hz, H-3), 3.47 (t, 1H, $J_{4',5'}$ 9.6 Hz, H-4′), 3.31 (m, 1H, H-5′), 3.29 (dd, 1H, $J_{1ax,1eq}$ 11.7 Hz, H-1$_{eq}$), 3.21 (m, 1H, H-5), 2.88 (t, 1H, H-1$_{ax}$), 2.01 (s, 3H, *Me*CON); ^{13}C NMR (CD_3OD–D_2O, 62.9 MHz): δ 176.0 (C=O), 101.1 (C-1′), 78.7 (C-5′), 78.4 (C-4), 76.2 (C-3), 73.9 (C-3′), 68.4 (C-2), 68.2 C-4′), 61.9 (C-6′), 60.3 (C-5), 58.3 (C-6), 54.5 (C-2′), 47.0 (C-1), 22.8 (*Me*CON).
17. For the preparation of soluble rNKR-P1A, an optimized construct was employed involving amino acids A_{90} to K_{215} in the extracellular ligand binding domain. After expression in bacteria, the protein precipitated into inclusion bodies, which were dissolved in 6 M guanidine chloride in pH 9.0, 50 mM Tris–HCl and 10 mM DTT, and denatured by rapid dilution into a 100-fold excess of refolding buffer composed of pH 9.0, 50 mM Tris–HCl containing 1 M L-arginine, 100 mM $CaCl_2$, 9 mM cysteamine, 3 mM cystamine, and 1 mM NaN_3. The refolding mixture was dialyzed against a low salt buffer composed of 15 mM Tris–HCl pH 9.0 with 9 mM NaCl and 1 mM NaN_3. The diluted protein was recovered by anion exchange chromatography on Q-Sepharose FF, and eluted using a gradient elution increasing the concentration of NaCl from 20 mM to 1 M. The protein, eluted as a symmetrical peak at 0.2 M NaCl, was concentrated and further purified by gel filtration in a Superdex 200 HR column followed by a second anion exchange chromatography stage in a SOURCE 15Q 4.6/100 PE column. The final preparation was homogenous on SDS PAGE, displaying a significantly higher mobility under nonreducing conditions, and ESI-FT-ICR mass spectrometry producing homogenous protein species at $[M+H]^+ = 14233.99$. The thermal and long-term biochemical stability of the protein was investigated using spectral techniques and mass spectrometry, and the protein proved stable for at least 7 days at 30 C or 37 °C.
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19. For plate binding and inhibition assays, soluble rNKR-P1 and hCD69 proteins were labeled by the covalent attachment of fluorescent labels through reaction with *N*-hydroxysuccinimide fluorescein and rhodamine (Pierce, Rockford, IL, USA), respectively. Four moles of fluorescein and five moles of rhodamine were attached per mole protein as confirmed by quantitative spectrophotometry, MALDI-MS, and ion cyclotron FT-MS. Flexible 96-well round-bottomed polyvinyl chloride plates (BD Biosciences) were coated with

$GlcNAc_{17}$BSA ligand, blocked with 2% BSA, and then incubated in the presence of fluorescein-rNKR-P1A or rhodamine-hCD69 (0.1 μg/ml, corresponding to half-saturation under the given experimental conditions) and serial dilutions of the inhibitor. After incubation at 4 °C for 2 h, plates were washed four times with PBS, and drained. The ligand-bound receptor was dissociated by overnight incubation in 100 μl of 0.1 M sodium acetate buffer supplemented with 0.1% octyl-β-glucoside and 0.1% Triton X-100, the solution transferred to 96-well flat-bottomed UV transparent plates (UV Star, BioOne, Greiner), and the amount of bound protein determined by fluorescence using standard fluorescein and rhodamine settings for a Safire[2] spectrofluorometer (Tecan, AT). Complete inhibition curves were constructed using SigmaPlot software, and the IC_{50} values were calculated from at least three independent experiments.

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