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Ergot Alkaloid Glycosides with Immunomodulatory Activities

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Abstract—New glycosides derived from ergot alkaloids elymoclavine and DH-lysergol were synthesized by chemoenzymatic methods. β-Glucosides were obtained either by chemical method or by transglycosylation (glycosidase from *Aspergillus oryzae*), lactosides were prepared by further extension of carbohydrate chain using β-1,4-galactosyltransferase (bovine milk) and α-5-*N*-acetylneuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→*O*)-elymoclavine was prepared using α-2,6-sialyltransferase (rat liver). Immunomodulatory activity of elymoclavine and 9,10-dihydrolysergol and their glycosylated derivatives on natural killer (NK) cell-mediated cytotoxicity of human resting and activated human peripheral blood mononuclear cells (PBMC) was investigated. Addition of ergot alkaloid glycosides to the mixtures of effector and target cells potentiated the PBMC cytotoxicity against both NK-sensitive and -resistant target cells. The glycoconjugates of elymoclavine enhanced cytotoxicity of PBMC against NK-resistant target cells. The glycoconjugates of DH-lysergol potentiated NK cytotoxicity of PBMC against NK-sensitive target cells. Copyright © 1996 Elsevier Science Ltd

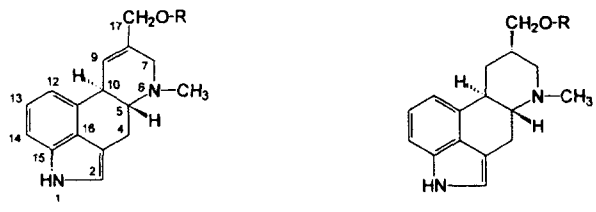
Introduction

The presence of 'hidden structures', resembling some important neurohumoral mediators (e.g., noradrenaline, serotonin and dopamine) in the molecules of ergot alkaloids (EA) could explain their interactions (agonistic or antagonistic) with the neurotransmitter receptors.[1] In addition to various medical applications of the EA (e.g., treatment of acromegaly, hypertension, hypergalactinemia, migraine, parkinsonism, senile cerebral disturbances, obstetric and psychiatric disorders), their immunomodulation effects have also been disclosed.[2,3b]

Immunomodulatory effects of EA[4,5] could result from their action on the membrane receptors of lymphoid cells.[6–8] Cytotoxic lymphocytes and especially natural killer (NK) cells form the effector arm of cell-mediated immune responses to infections and tumors and also the production of cytokines and other humoral immune factors.[8] From the point of view of EA modulation, they represent one of the most important fields of a study in immunology and neuroendocrinology.[9,10]

It has been documented that the NK cells bear the lectin receptors which through the binding to the carbohydrates on the surface of tumor cells mediate their killing.[11–13] β-Fructosides[3a] and β-galactosides[3b] of some EA were previously prepared and tested for stimulation of NK cytotoxic activity against NK-sensitive K562 target cells.[3b] Their stimulatory effect was dose dependent and considerably higher than that of their aglycons. Therefore, we decided to prepare some oligoglycosides of ergot alkaloids (e.g., β-lactosyl, β-*N*-acetyl-lactosaminidyl and α-sialyl; Scheme 1). A joint action of both bioactive moieties might be expected from these compounds.

DH-Lysergol (**2**) was chosen for this study as it is the simplest ergoline alkaloid. Most of the semisynthetic ergot alkaloids used in therapy contain the 9,10-dihydroergoline moiety in their molecules (e.g.,



1 R = H
1a R = β-Glc
1b R = β-Gal(1-4)β-Glc
1c R = β-Gal(1-4)β-GlcNAc
1d R = β-Glc(1-4)β-GlcNAc
1e R = Neu5Acα(2-6)Galβ(1-4)β-GlcNAc
1f R = β-Gal
1g R= β-GlcNAc
1h R= β-GalNAc

2 R = H
2a R = β-Glc
2b R = β-Gal(1-4)β-Glc
2f R = β-Gal

Scheme 1.

List of abbreviations: β-Glc, β-glucopyranosyl; β-Gal, β-galactopyranosyl; β-Fru, β-fructofuranosyl; Neu5Ac, 5-acetylneuraminyl; β-GlcNAc, 2-*N*-acetyl-2-deoxyglucopyranosyl; β-GalNAc, 2-*N*-acetyl-2-deoxygalactopyranosyl.

Nicergoline, Pergolide, Lisuride, dihydroergotamine, dihydro-α-ergocryptine, etc.). Elymoclavine (**1**) was chosen as the simplest representative of 9-ergolenes. These clavine alkaloids have been lightly investigated and interesting physiological activities[14] (also immunomodulatory) have been found in the elymoclavine and in its glycosides.[3b]

Here, we report our results in stepwise building of oligosaccharide chain by biotransformation using glycosidases and glycosyltransferases. The obtained compounds extended the set of ergot alkaloid glycosides tested for immunomodulatory effects on non-MHC restricted cytotoxic cells.

Results and Discussion

Chemoenzymatic sythesis of ergot alkaloid glycosides

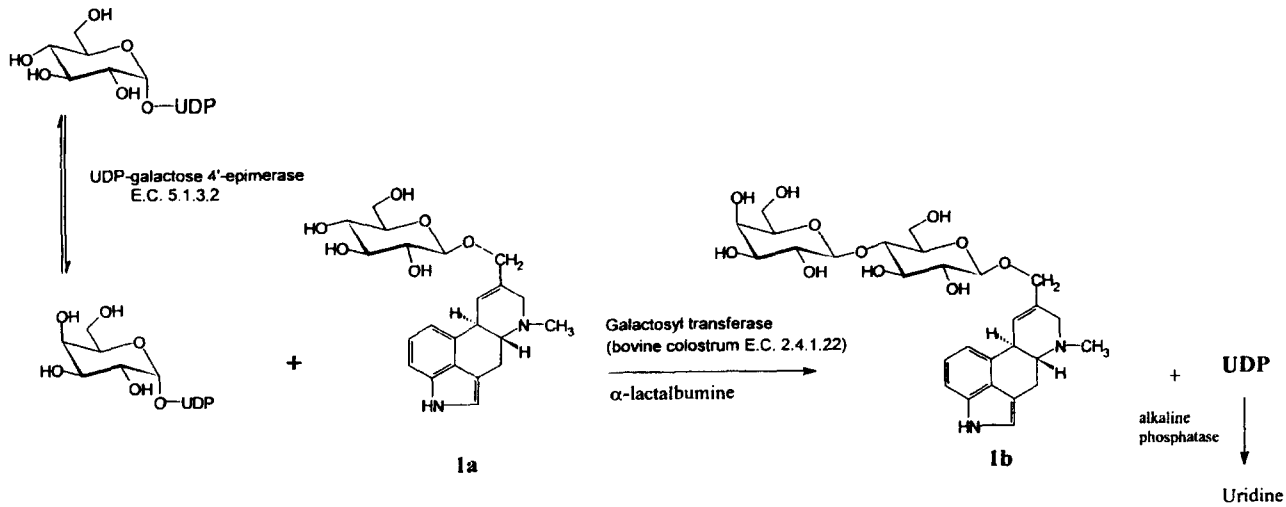
Elymoclavine *O*-β-glucoside (**1a**) was used as a starting material for the preparation of elymoclavine lactoside (Galβ(1→4)Glcβ *O*-elymoclavine), **1b** (Scheme 1).

Preparation of β-glucoside of **2**, using Koenigs–Knorr's reaction ($AgCO_3/SiO_2$, α-bromo-tetra-*O*-acetylglucose, 1,2-dichloroethane) has been described previously.[15] However, in our attempts to prepare **1a** and **2a**, mostly orthoesters were obtained and only traces of the desired glucoside were formed. Various modifications of this method tested [catalysts: $AgClO_4$, AgOTf, $Hg(CN)_2$, Fetizon reagent] also yielded orthoesters or acetylated aglycons. The use of peracetylated sugar and trimethylsilyl-triflate as a catalyst[16] gave β-glucoside in moderate yields (40–60%), in addition to a small amount (1–4%) of α-glycoside and acetate of aglycon (30%), presumably via the acidic cleavage of the orthoester formed. A small amount (5–10%) of aglycon was degraded.

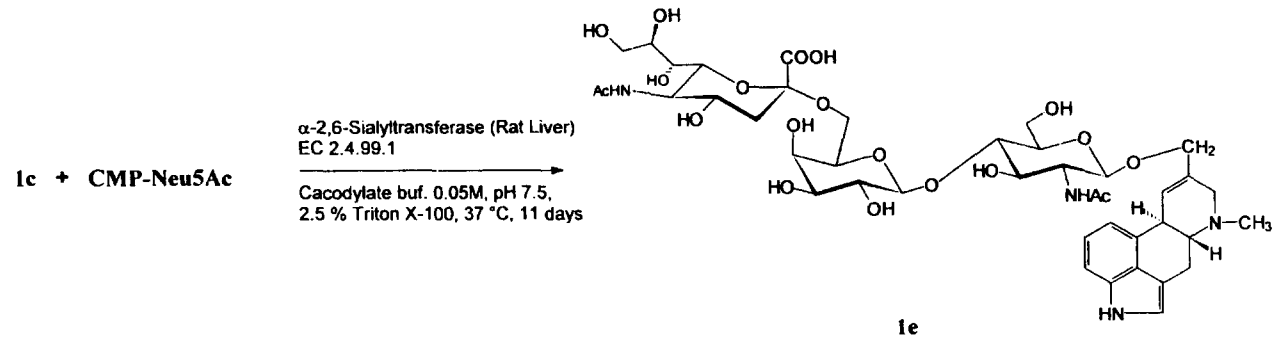
Enzymatic methods using glycosidases and activated glycosyl donors previously proved to be useful in preparation of EA β-galactosides[3b] (**1f** and **2f**), β-*N*-acetylglucosaminides, β-*N*-acetylgalactosaminides[17a] and α-mannosides.[17b] The use of *p*-nitrophenyl β-D-glucoside as a glucosyl donor and β-glucosidase from almonds (Sigma) was not successful. However, the use of β-galactosidase from *Aspergillus oryzae* (Sigma, grade XI) containing some β-glucosidase side activity[18] proved to be successful, although this reaction gave relatively low yields (3–12%) of β-glucosides without by-products and the unreacted aglycon could be quantitatively recovered.

Chemical glycosylation methods were completely excluded in the preparation of complex alkaloid oligoglycosides due to harsh conditions and complicated protecting/deprotecting procedures. Use of glycosidases would not ensure regioselectivity and the yields in these transfers seldom exceeded 30%. The use of β-1,4-galactosyl transferase (EC 2.4.1.22, bovine milk) proved to overcome both problems and the yield of the products **1b** and **2b** with the appropriate β(1–4) configuration was 70–95% (TLC; Scheme 2). α-2,6-Sialyltransferase (EC 2.4.99.1, rat liver) was used for preparation of **1e** (Scheme 3). This enzyme exhibits a narrow specificity towards the Galβ(1–4)GlcNAc moiety. We tested the possibility of the Neu5Ac transfer onto **1c** with the radioactive assay[19] routinely used with *N*-acetyllactosamine as the acceptor. The reaction rate (estimated from the radioactivity incorporated into the product within 30 min) was 20% higher than in the case of *N*-acetyllactosamine itself. This effect that could be explained by the favorable influence of the aglycon was also observed in enzymatic galactosylation of 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→*O*)-elymoclavine.[20]

Elementary composition of all reported compounds was checked by high-resolution measurement of the $[M+H]^+$ ions in their FAB mass spectra. The fragment ions observed in daughter spectra of these pseudomolecular ions are interpretable in terms of the proposed structures. New glycosides were further



Scheme 2.



Scheme 3.

characterized by ^1H and ^{13}C NMR spectra (Tables 1–3). According to $J_{1',2'}$ or $J_{1'',2''}$ couplings (Table 2), all anomeric centres have a β-configuration. Marked downfield glycosidation shifts of C-4′ resonances in **1b** and **2b** with respect to the parent compounds (Table 3) were evidence for a 1→4 linkage. Similarly, the downfield shift of C-6″ in **1e** with respect to that in **1c** documents[20] an attachment of sialic acid at this position. The assignment of anomeric configuration at C-2 (Neu5Ac) in **1e** relies upon the chemical shift of H-3*e* (Neu5Ac) that falls in the range typical for α-sialoglycosides.[21] A more reliable ^{13}C NMR-based method[22] was unfortunately not applicable because of limited sample amount.

Immunomodulatory activity of ergot alkaloid glycosides

NK cells can be activated via CD2 antigen[23] or by means of a low-affinity IgG receptor, Fc-γ-RIII (CD16).[8] However, neither CD2 nor CD16 appear to be essential for natural killing as NK cells lacking these antigens can still perform spontaneous cytotoxicity. Another surface molecule, the NKR-P1, identified in the rat, mouse and human NK cells has been shown to be relevant to natural killing.[24,25] Interaction of oligosaccharide ligands on the target cell surface with the NKR-P1 molecule is involved in the mechanism of NK cell triggering.[26] Binding and inhibition studies with neoglycoconjugates and monosaccharides indicate that NKR-P1 belongs to the C-type lectin family and binds onto saccharides with preference order GalNAc > GlcNAc ≫ Fuc ≫ Gal > Man.[27]

Compounds **1a–e**, **2a**, and **2b** were tested in cytotoxic reactions of resting and activated PBMC against tumor target cells differently sensitive to NK-cell mediated

Table 1(a). ^1H NMR chemical shifts (399.95 MHz, CD_3OD, 25 °C) aglycon part

Atom	1a	1b	1e	2a	2b
2	6.912	6.940	7.066	6.974	6.943
4*a*	2.718	2.759	n.d.[a]	2.796	2.756
4*e*	3.319	3.379	n.d.	3.537	3.508
5	2.491	2.549	n.d.	2.594	2.405
7*a*	2.922	3.008	n.d.	2.453	2.265
7*e*	3.603	3.614	4.109	3.500	3.393
8	—	—	—	3.250	2.380
9*a*	6.528	6.569	6.745	1.286	1.232
9*e*	—	—	—	2.731	2.727
10	3.774	3.804	n.d.	3.079	3.034
12	6.910	6.937	7.036	6.875	6.865
13	7.059	7.071	7.066	7.089	7.076
14	7.147	7.150	7.237	7.182	7.162
17*u*	4.272	4.293	4.322	3.707	3.958
17*d*	4.305	4.349	4.365	3.925	3.958
N-CH_3	2.479	2.256	3.071	2.740	2.633

[a]n.d. not determined.

Table 1(b). ^1H NMR chemical shifts (399.95 MHz, CD_3OD, 25 °C) sugar part

	1	2	3	4	5	6d	6u	sugar
1a	4.309	3.252	3.376	3.203	3.256	3.886	3.633	Glc
1b	4.356	3.315	3.540	3.482	3.400	3.985	3.771	Glc
	4.238	3.552	3.489	3.825	3.596	3.792	3.708	Gal
1e[a]	4.662	3.616	3.810	3.449	3.717	4.045	3.761	GlcNAc
	4.312	3.530	3.476	n.d.[b]	n.d.	n.d.	n.d.	Gal
2a	4.314	3.242	3.398	3.690	3.230	3.994	3.531	Glc
2b	4.341	3.320	[c]	[c]	3.479	3.959	3.789	Glc
	4.389	[c]	[c]	3.913	3.611	3.809	3.723	Gal

[a]Additional signals: 1.685 (dd, H-3‴a), 2.102 (3H, s, GlcNAc), 2.035 (3H, s, Neu5Ac); 2.035 (3H, Neu5Ac), 2.793 (H-3‴e), 3.648 (1H, m, H-4‴), 3.847 (1H, m, H-5‴).
[b]Not determined.
[c]3.562–3.577 ppm.

Compounds **1a–e**, **2a**, and **2b** were tested in cytotoxic reactions of resting and activated PBMC against tumor target cells differently sensitive to NK-cell mediated

Table 2. Selected coupling constants (sugar residues only)

	1,2	2,3	3,4	4,5	5,6*d*	5,6*u*	6*d*,6*u*	sugar
1a	7.8	9.3	8.3	9.9	1.9	6.8	−12.0	Glc
1b	7.9	9.0	8.5	9.3	2.2	5.8	−12.1	Glc
	7.4	9.8	3.2	1.1	7.5	4.6	−11.4	Gal
1e[a]	8.5	n.d.[b]	n.d.	n.d.	2.9	7.8	−10.1	GlcNAc
	7.0	9.6	n.d.	n.d.	n.d.	n.d.	n.d.	Gal
2a	7.8	9.0	9.0	8.2	4.6	6.9	−10.2	Glc
2b	7.8	9.6	n.d.	n.d.	2.5	4.3	−12.1	Glc
	7.5	n.d.	2.2	0.9	7.5	4.6	−11.4	Gal

[a]$J_{3''u,3''e} = -12.2$, $J_{3''u,4''} = 11.7$, $J_{3''e,4''} = 4.8$.
[b]Not determined.

lysis. All compounds were tested in the concentration range from 10^{-6} to 10^{-15} M. The maximum immunomodulatory effect was obtained at the concentration 10^{-10} M. Higher and lower concentrations (results not shown) were without significant changes. Therefore, 10^{-10} M concentration was selected in all the following assays.

In the first experiments GalNAc (*N*-acetylgalactosamine) was selected for its high degree of lectin binding activity to determine cytotoxic activity of human peripheral blood mononuclear cells (PBMC) in co-stimulatory experiments in combination with elymoclavine (**1**) and dihydrolysergol (**2**).

To ascertain whether the receptor sites responsible for the recognition of carbohydrate groups on target cells or neuroendocrine receptors on effector cells would be influenced, compounds **1** or **2** or their combinations **1** + GalNAc, **2** + GalNAc or GalNAc alone (concentrations see above) were added into the 4 h cytotoxic assay against NK-sensitive K562 target cells. Little inhibition of lysis was observed in all cases. The decrease of cytotoxic activity to 63% of the unstimulated control was observed only after administration of **1** + GalNAc.

In the next experiments, the influence of **1** and **2** on NK cell activity before the administration of GalNAc was ascertained. After 30 min preincubation of the effector cells with **1** or **2**, a significant decrease of cytotoxic activity (ca. 50% of control values) was observed. However, if the GalNAc was present during cytotoxic reaction with EA-pretreated PBMC the decrease of cytotoxic activity was more pronounced (to 40% of control with **1** and to 10% of control with **2**). In cases where **1** and **2** decrease the cytotoxic functions of effector cells, the presence of GalNAc may support this effect by limiting signalling pathways among carbohydrate groups on tumor cells and the lectin receptor.

In the following experiments we compared the effects of **1**, **1a**, **1b**, **1f** and **2**, **2a**, **2b**, **2f**, glucose, galactose or lactose alone on either freshly isolated human PBMC or activated NK-enriched cell population as effector cells against MOLT4 T lymphoma cells (resistant to lysis by fresh PBMC and sensitive to activated lymphocytes). After addition of single saccharides to effector–target cell mixture, the cytotoxic activity of resting fresh lymphocytes was enhanced in all cases. The most potent stimulation was observed with glucose (see Fig. 1). The glycosylation of **1** does not influence cytotoxic activity of PBMC as we have not detected any differences between **1** alone and its glycoconjugates. On the contrary, the glycosylation of **2** substantially potentiates the NK cell-mediated lysis of tumor cells. Concerning the activated NK cell population an inhibition of cytotoxicity by both aglycons (**1** and **2**) and free saccharides was detected as well. Glycosylation of **1** and **2** restored the inhibitory activity of free saccharides and aglycons as well. Figure 1 shows that the glycosylated derivatives of EA did not substantially influence the lytic capability of activated NK cells.

Consequently we have screened the effects of **1** and **2** in comparison with their glycoconjugates previously used and further **1c**, **1d**, **1e**, **1g**, and **1h** on the cytotox-

Table 3(a). ^{13}C NMR chemical shifts (100.58 MHz, CD_3OD, 25 °C) aglycon part

Atom	1a	1b	1e	2a	2b
2	119.87	119.84	120.87	120.08	119.70
3	111.99	112.06	109.30	110.01	110.98
4	27.66	27.79	26.28	27.05	27.54
5	66.21	66.27	70.45	69.19	69.23
7	58.26	58.37	65.10	61.21	61.69
8	135.52	135.59	135.63	37.04	37.34
9	125.15	125.19	125.28	31.29	31.88
10	42.52	45.55	41.50	40.93	41.28
11	132.82	132.35	129.37	132.10	132.65
12	113.25	113.23	113.89	114.04	113.83
13	123.66	123.66	124.05	123.97	123.81
14	110.24	110.21	111.20	110.52	110.17
15	134.85	134.82	132.64	135.37	133.10
16	127.79	127.84	127.21	127.29	127.47
17	73.79	73.68	72.48	73.71	74.28
N-Me	41.52	41.58	48.05	42.72	43.32

Table 3(b). ^{13}C NMR chemical shifts (100.58 MHz, CD_3OD, 25 °C) sugar residues

							Ac		
	1	2	3	4	5	6	CH_3	C=O	sugar
1a	103.57	75.32	78.52	72.30	78.65	63.67			Glc
1b	103.44	75.01	76.88	81.41	72.55	62.78			Glc
	105.48	72.80	75.11	70.58	77.39	62.85			Gal
1e[a]	105.03	57.74	74.56	82.36	76.07	64.92	23.03	174.52	GlcNAc
	101.97	72.68	75.10	70.90	77.09	66.27			Gal
	175.25	101.94	31.04	70.27	54.26	73.65	23.71	174.78	Neu5Ac
2a	105.33	75.45	78.37	71.97	78.37	63.07			Glc
2b	105.13	75.06	76.70	80.96	76.80	62.21			Glc
	105.37	72.83	75.10	70.57	77.36	62.77			Gal

[a]Additional signals: 70.27 d (C-7), 73.44 d (C-8), 63.28 t (C-9).

icity of resting PBMC against the NK-sensitive K562 and NK-resistant RAJI target cells.

NK cell-mediated lysis against K562 target cells was substantially increased by addition of **2**. Compounds **2a** and **2b** exhibited a lower stimulatory effect than **2** (Fig. 2).

On the contrary, **2** did not influence cytotoxicity of PBMC against RAJI target cells. A slight stimulation was observed after addition of **2f** and compounds **2a** and **2b** inhibited the cytotoxicity (Fig. 2).

In the series of experiments concerning **1** and its glycosylated derivatives **1a–h**, only **1a**, **1f** and **1g** showed an enhancement of cytotoxic activity in comparison to **1** against K562 cells and RAJI cells, whereas **1f** potentiated the lytic activity of effector cells predominantly against K562 target cells (about 60%) and **1a** and **1e** predominantly against RAJI cells (ca. 70%; see Fig. 2).

From these results it could be generally deduced that the functional activities of glycosylated molecules of **1** and **2** are dependent on the aglycon molecule and the terminal saccharide, further on the status of effector cells (resting or activated), and type of target cells. From experimental design used, it could not be deduced which of the EA glycosylated derivatives may interfere with the processes accompanying the lysis of cells.

Conclusions

Glycosylation of ergot alkaloids **1** and **2** profoundly changed their immunomodulatory properties. In human PBMC, elymoclavine (**1**) displays weaker stimulatory effects, whereas the DH-lysergol (**2**) stimulates their cytotoxic activity only against the NK-sensitive (K562) cells. The cytotoxicity of human PBMC against NK-resistant (RAJI) target cells is predominantly stimulated by the glycoconjugates of **1** whereas the cytotoxicity against NK sensitive (K562) cells is stimulated by glycoconjugates of **2**.

One of the most important effects of EA is known to be in the regulation of pituitary hormone secretion (e.g., prolactin).[28] It is known that the mutually opposite effects of **1** and **2** have a qualitatively different mechanism of action on production and deliberation of prolactin hormone by endocrine cells. A slight inhibitory effect of **1** is more prolonged in comparison with more rapid and intensive onset but short duration of action of **2**.[29] It may be supposed that the regulation of NK cell activity by EA acts through

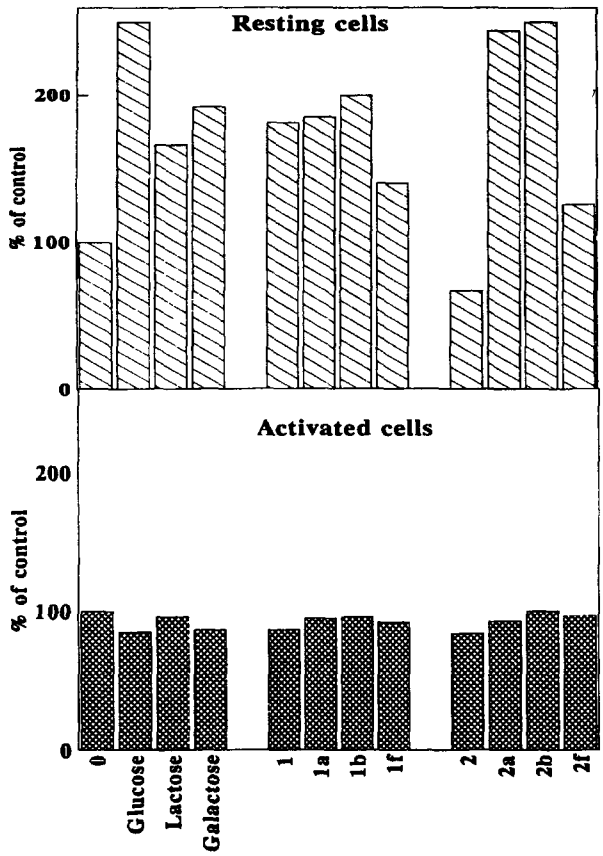


Figure 1. Comparison of immunomodulatory effects of single saccharides, EA aglycons and their glycosylated derivatives on human NK cell-mediated cytotoxicity against MOLT4 target cells. Values are presented in % of controls. For resting cells control = 2.7%, for activated cells control = 41.9% of specific cytotoxicity.

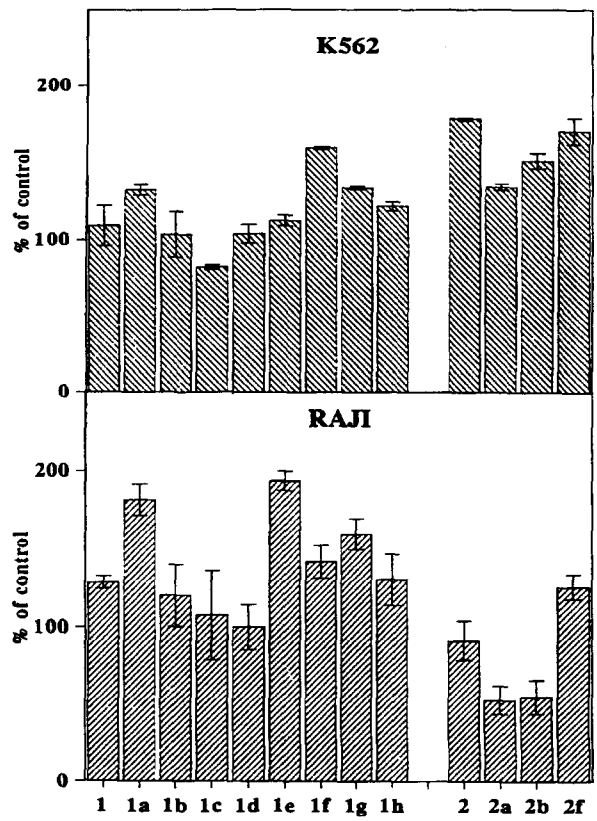


Figure 2. Influence of EA and EA glycosides on human NK cell-mediated cytotoxicity against K562 and RAJI target cells. Values are presented in % of controls. In K562 control = 17.3%, in RAJI control = 1.6% of specific cytotoxicity. Average values from six experiments are shown.

prolactin[30,31] binding sites (K_d=300 pM, B_{max}=660 sites/cell) that are present on large granular lymphocytes.[32]

The possibility of an interaction of terminal saccharide groups of glycoprotein and glycolipid antigens on the surface of various kinds of tumor cells with the EA glycosides must be also considered.[33,34]

Experimental

Elymoclavine (**1**) and 9,10-dihydrolysergol (**2**) were kindly donated by Galena Pharmaceuticals Ltd (Opava, Czech Republic).

β-D-Glucopyranosyl-(1→*O*)-elymoclavine (1a). Compound **1** (254 mg, 1 mmol) and glucose-β-pentaacetate (390 mg, 1 mmol) were dried in vacuo over P_2O_5 and dissolved in 15 mL of a mixture of nitromethane:CH_2Cl_2 (2:1, dry), and molecular sieve (4 Å) were added. TMS-triflate (450 μL, 1.6 mmol) was slowly added to the stirred mixture (0 °C, under nitrogen) and the stirring was continued for 3 h at room temperature. Triethylamine (400 μL) was added to quench TMS-triflate, the mixture was diluted with CH_2Cl_2 and washed twice with saturated $NaHCO_3$ and with H_2O. After drying and evaporation, flash chromatography (silica gel 7–35 μm, SDS, France; hexane:ethyl acetate:CH_2Cl_2:MeOH 25:25:40:10) afforded tetra-*O*-acetyl-β-D-glucopyranosyl-(1→*O*)-elymoclavine (274 mg, 46%). The compound was deacetylated at room temperature by the mixture Et_3N:MeOH:H_2O (30 h) and purified by chromatography (SiO_2; CH_2Cl_2:MeOH:NH_3 8:2:0.01) which yielded **1a** (164 mg, 85%).

β-D-Glucopyranosyl-(1→*O*)-9,10-dihydrolysergol (2a). Compound **2** (200 mg, 0.78 mmol), citric acid monohydrate (90 mg, 0.44 mmol), *p*-nitrophenyl-β-D-glucopyranoside (170 mg, 0.56 mmol) and McIlvain buffer (13 mL, 0.07 M, pH 5.1) were heated at 80 °C for dissolution. After cooling, β-galactosidase from *A. oryzae* (580 mg, Sigma, grade XI) was added and the mixture was incubated for 330 min at 29 °C. The reaction was stopped by heating (100 °C, 3 min), and **2a** was isolated as previously described[20] to afford 20 mg of **2a** (7%). The rest of unreacted aglycon was nearly quantitatively recuperated.

β-D-Galactopyranosyl(1→4)-β-D-glucopyranosyl-(1→*O*)-elymoclavine (lactosyl-elymoclavine) (1b). A solution of **1a** (21 mg, 0.05 mmol) and uridine 5′-diphosphoglucose (Sigma, 15 mg, 0.025 mmol) in Na-cacodylate buffer (950 μL, 0.05 M, pH 7.5, containing 5 mM $MnCl_2$) was adjusted to pH 7.5 by 1 N NaOH. A solution of α-lactalbumin (100 μL, 10 mg/mL), galactosyltransferase (Sigma, 0.4 U), UDP-Glc 4′-epimerase (Sigma, 2 U) and calf intestinal alkaline phosphatase (Boehringer, 10 U) were added to avoid a feedback inhibition by UDP[35] and the mixture was incubated at 37 °C. After 48 h UDP-Glc (6 mg, 0.01 mmol) was added (pH adjusted) and more enzymes, i.e., galactosyltransferase (0.15 U) and UDP-Glc 4′-epimerase (0.5 U) (Sigma), were supplemented. These additions were repeated every 48 h until the reaction was stopped (after 290 h) by quick freezing and lyophilization. The reaction was monitored by TLC and worked up as previously described.[20] The analytical yield of **1b** was 87%, isolated yield 30%.

β-D-Galactopyranosyl(1→4)-β-D-glucopyranosyl-(1→*O*)-9,10-dihydrolysergol(lactosyl-DH-lysergol) (2b). A solution of **2a** (15 mg, 0.036 mmol) and uridine 5′-diphosphoglucose (20 mg, 0.033 mmol) in Na-cacodylate buffer (66 μL, 0.05 M, pH 7.5, containing 5 mM $MnCl_2$) was adjusted to pH 7.5 by 1 N NaOH and a solution of α-lactalbumin (66 μL, 10 mg/mL). Galactosyltransferase (0.27 U), UDP-Glc 4′-epimerase (1.4 U) and calf intestinal alkaline phosphatase (15 U) were added and the mixture was incubated at 37 °C. After 48 h, UDP-Glc (3 mg, 5 μmol) was added (pH adjusted) and more enzymes, i.e., galactosyltransferase (0.1 U) and UDP-Glc 4′-epimerase (0.5 U), were supplemented. These additions were repeated every 48 h until the reaction was completed (100 h). The reaction was monitored by TLC and worked up as described previously.[20] The analytical yield of **2b** was almost quantitative, isolated yield 67%.

α-5-*N*-Acetylneuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→*O*)-elymoclavine (1e). Compound **1c**[20] (5.2 mg, 8.4 μmol) and CMP-Neu5Ac[19] (6 mg, 10 μmol) were dissolved in water (30 μL). Bovine serum albumin solution (30 μL, 5 mg/mL), cacodylate buffer (30 μL, 0.25 M, pH 7.5 containing 2.5% of Triton X-100) and α-2,6-sialyltransferase (6 mU) from rat liver (Boehringer) were added and the mixture was incubated at 37 °C. CMP-Neu5Ac (3 mg, 5 μmol) and sialyltransferase (6 mU) were added again after 48 h. These additions were repeated three times, each after 48 h. The reaction was completed after 260 h by freezing and lyophilization. The product formation was followed by TLC (SiO_2; *n*-propanol:nitromethane:H_2O 10:9:2). The product was separated by flash chromatography on silica gel with the mixture CH_2Cl_2:MeOH (8:2) with a slow gradient of methanol yielding **1e** (3.5 mg, 47%).

The preparation of compounds **1f** and **2f** has been described elsewhere.[3b] Derivatives **1g** and **1h** were prepared according to ref 17a.

Spectral characterization of the compounds

^1H and ^{13}C NMR spectra were measured on a Varian VXR-400 spectrometer (399.952 and 100.577 MHz, respectively) in CD_3OD at 25 °C. Residual solvent signal (δH 3.33 and δC 49.3) served as an internal reference. Chemical shifts are given in the δ-scale; digital resolution was 0.0002 and 0.006 ppm, respectively. *J* values are given in Hz. Carbon signal multiplicity was determined by an APT (Attached Proton Test) experiment. Manufacturer's software was used

for 2-D NMR (COSY, ROESY, HOM2DJ, HETCOR).

Positive-ion FABMS were recorded on a double-focusing Finnigan MAT 90 instrument of BE geometry (magnetic sector preceding the electrostatic one) under previously described conditions.[36] For **1b** and **2b** *m*-nitrobenzyl alcohol (Aldrich) was used as a matrix, **1e** was measured in glycerol (Sigma).

Alkaloid numbering is given in Scheme 1. The letters *a* and *e* denote axial or equatorial protons, respectively. Letters *d* and *u* indicate the downfield or upfield resonating protons in diastereotopic methylene groups, respectively. Primed numbers were used for the first sugar residue, double primed numbers for the second one and triple primed for the Neu5Ac residue in **1e**.

According to observed $J_{1',2'}$ or $J_{1'',2''}$ values (see Table 2), all newly formed glycosidic linkages have a β-configuration. Characteristic downfield glycosidation shifts of C-4′ resonances in **1b** and **2b** with respect to the parent compounds are evidence for a 1→4 linkage (see Table 3). Similarly, the downfield shift of C-6″ in **1e** with respect to **1c** documents a 2→6 attachment of sialic acid. Judging from the downfield shifts of N-Me, H-5 and H-4 signals compared with those in **1** and **2**, these compounds were obtained as N-6 protonated salts.

All prepared compounds exhibited the expected protonated molecular ions in their mass spectra. The assigned structures are supported by fragment ions present in the daughter spectra of these ions. FABMS: **1b** $[M+H]^+$ ($C_{28}H_{39}N_2O_{11}$) calcd 579.2554, measured 579.2503, daughter ions (rel. int.) 561 (23), 445 (18), 417 (32), 399 (14), 283 (8), 255 (53), 237 (100), 167 (2), 154 (1); **2b** $[M+H]^+$ ($C_{28}H_{41}N_2O_{11}$) calcd 581.2710, measured 581.2766, daughter ions (rel. int.) 563 (42), 447 (94), 419 (100), 401 (33), 285 (76), 257 (76), 239 (19), 167 (12), 154 (10); **1e** $[M+H]^+$ ($C_{41}H_{59}N_4O_{19}$) calcd 911.3774, measured 911.3902, daughter ions (rel. int.) 893 (7), 657 (4), 620 (100), 237 (5), 819 (8). MS data of **1a** and **2a** have been reported elsewhere.[20,36]

Cell preparations

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood (Heparine, Spofa, Czech Repuplic, 5 IU/mL) of individual healthy donors after separation on Ficoll–Hypaque (Lymphoprep, Sigma, U.S.A.) density gradient. The cells were resuspended in a complete culture medium. Activated NK-enriched lymphoblasts were generated after 10 days in PBMC culture with a 50Gy gamma-irradiated RPMI8866 B lymphoblastoid cell line.[37]

Long-term cultures and other experiments were performed in RPMI-1640 medium enriched with L-glutamin (2 mM), antibiotics (penicillin 100 IU/mL and streptomycin sulphate 100 μg/mL, Spofa, Czech Republic) and supplemented with 10% fetal calf serum (Biocom, Brno, Czech Republic). Incubation was carried out at 37 °C in a humified atmosphere containing 5% CO_2 (IR 1500-Flow Laboratories).

Tumor cell lines used as targets for estimation of human NK cell activity: K562 [ECACC, #85011407], human myeloid leukemic lymphoblast, RAJI [ECACC, #85011429], human Burkitt lymphoma lymphoblast, and MOLT4 [ECACC, #85011413], human T-cell leukemia lymphoblast were obtained from ECACC (Salisbury, U.K.).

Effector cells at concentrations 2×10^5 were incubated with 1×10^4 ^{51}Cr-labeled (60 min) target cells for 4 h at 37 °C in round-bottomed 96-well plastic microtiter plates for tissue cultures. After incubation the radioactivity in 0.1 mL of cell free supernatants was measured in gamma scintillation counter. All tests were performed in triplicate. Spontaneous ^{51}Cr release was determined when cultivation medium was used instead of effector cells. This baseline was 2–5% of the total ^{51}Cr incorporated into the target cells. The percentage of specific lysis was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{exp. cpm} - \text{spont. cpm}}{\text{max. cpm} - \text{spont. cpm}} \times 100,$$

where exp. (experimental) cpm is the mean cpm released in the presence of the effector cells, spont. (spontaneous) cpm is the mean cpm released by target cells incubated alone and max. (maximal) cpm is 40% of the total amount of ^{51}Cr incorporated into target cell.

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