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Glycopeptide dendrimer colchicine conjugates targeting cancer cells

Emma M. V. Johansson [a], Joëlle Dubois [b], Tamis Darbre [a], Jean-Louis Reymond [a,*]

[a] Department of Chemistry and Biochemistry, University of Berne, Freiestrasse 3, CH-3012 Berne, Switzerland
[b] ICSN-CNRS, avenue de la Terrasse, 91198 Gif sur Yvette Cedex, France

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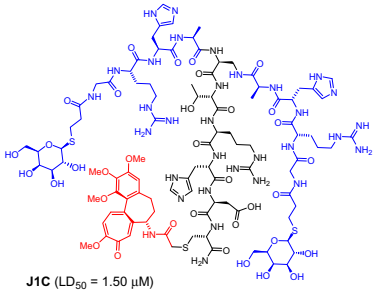
ABSTRACT

Screening of a 65,536-member one-bead-one-compound (OBOC) combinatorial library of glycopeptide dendrimers of structure $((\beta Gal)_{n+1}X^8X^7X^6X^5)_2DapX^4X^3X^2X^1(\beta\text{-}Gal)_m$ (βGal = β-galactosyl-thiopropionic acid, X^{8-1} = variable amino acids, *Dap* = L-2,3-diaminopropionic acid, *n*, *m* = 0, or 1 if X^8 = Lys resp. X^1 = Lys) for binding of Jurkat cells to the library beads in cell culture, resynthesis and testing lead to the identification of dendrimer **J1** $(\beta Gal\text{-}Gly\text{-}Arg\text{-}His\text{-}Ala)_2$*Dap*-Thr-Arg-His-Asp-CysNH_2 and related analogues as delivery vehicles. Cell targeting is evidenced by FACS with fluorescein conjugates such as **J1F**. The colchicine conjugate **J1C** is cytotoxic with LD_{50} = 1.5 μM. The β-galactoside groups are necessary for activity, as evidenced by the absence of cell-binding and cytotoxicity in the non-galactosylated, acetylated analogue **AcJ1F** and **AcJ1C**, respectively. The pentagalactosylated dendrimer **J4** βGal_4(*Lys*-Arg-His-Leu)$_2$*Dap*-Thr-Tyr-His-Lys(βGal)-Cys) selectively labels Jurkat cell as the fluorescein derivative **J4F**, but its colchicine conjugate **J4C** lacks cytotoxicity. Tubulin binding assays show that the colchicine dendrimer conjugates do not bind to tubulin, implying intracellular degradation of the dendrimers releasing the active drug.



1. Introduction

Dendrimers are tree-like synthetic macromolecules with various applications in technology and medicine.[1] One of the frequently envisioned applications for dendrimers is targeted drug delivery of cytotoxic drugs in cancer therapy, with the aim of increasing the therapeutic ratio by avoiding unwanted toxicity in non-target tissues.[2] Targeting to cancer cells can be obtained by using macromolecular carriers with MW >40 kDa due to the enhanced permeation and retention (EPR) effect.[3] In such applications a PEGylated dendrimer or polymer carrier is typically loaded with multiple copies of the drug,[4] and may carry a targeting groups such as folic acid,[5] biotin,[6] or integrin ligands.[7] Ligand-based targeting is also used in non-dendritic delivery devices, for example based on antibodies[8] or vitamin B_{12}.[9]

We have recently developed the chemistry of peptide dendrimers[10] and glycopeptide dendrimers[11] as artificial protein models.[12] Peptide dendrimers resemble the molten globule state of proteins,[13] and can display various functions such as enzyme-like catalysis,[14] metal and cofactor binding,[15] and inhibition of bacterial biofilms mediated by multivalent lectin binding.[16,17] We have also started to probe the potential of glycopeptide dendrimers as drug delivery agents by focusing on colchicine.[18,19] Colchicine is a relatively inexpensive natural product too toxic for use in cancer

* Corresponding author. Fax: +41 31 631 80 57.
E-mail address: jean-louis.reymond@ioc.unibe.ch (J.-L. Reymond).

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therapy and which might benefit from combination with a targeting device, or a prodrug strategy as shown for close analogues.[20] Initial experiments with glycopeptide dendrimer conjugates of colchicine[18] showed that they can be cytotoxic to cancer cells by disrupting cell division by the tubulin binding mechanism of colchicine,[21] however with only weak activities (LD_{50} >5 μM). In our efforts to optimize the system, we now report a combinatorial study of glycopeptide dendrimers carrying two to five copies of a β-galactoside as targeting device and a colchicine molecule at their core. On-bead screening for binding to Jurkat cells lead to identification of dendrimer **J1** as delivery device for colchicine as a cysteine-thioether conjugate **J1C** (Fig. 1). The corresponding fluorescein conjugate **J1F** shows strong binding to these cells. The cytotoxicity of the colchicine conjugate **J1C** is presumably mediated by binding to galactose specific receptors such as galectins,[22,23] internalization, and degradation to release the active drug, as for other glycosylated delivery devices based on polymeric *N*-acetyl-galactosamine[24] and galactosylated[25] ligands.

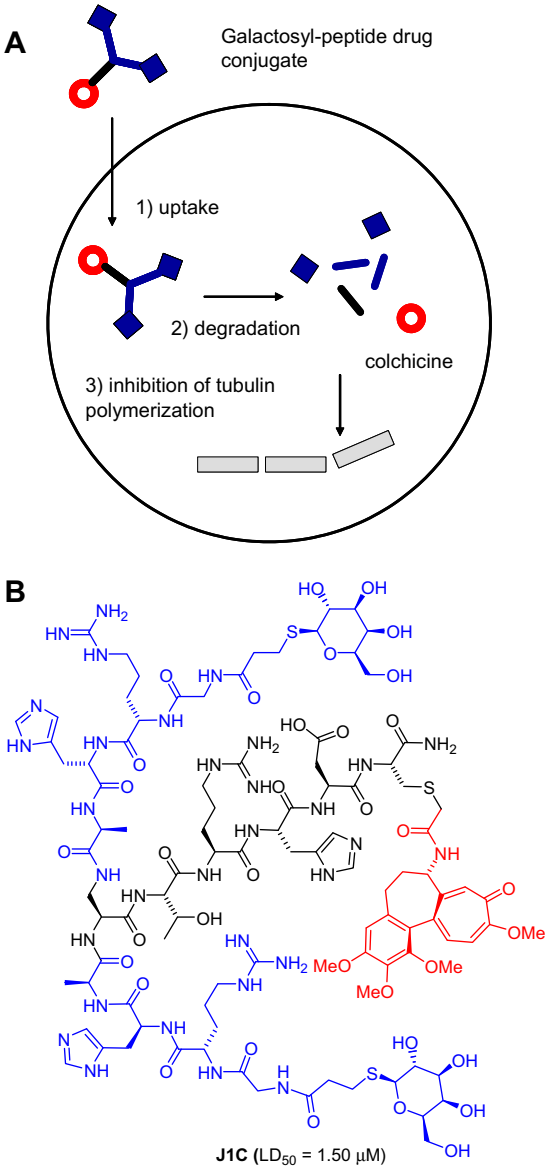
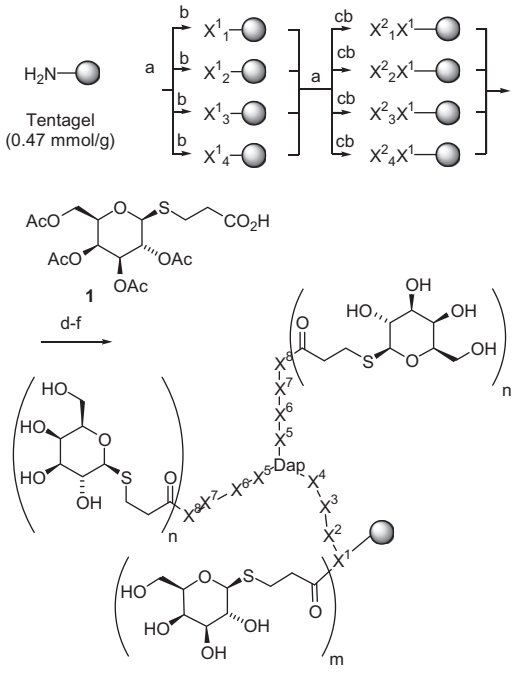


Figure 1. (A) Principle of degradable drug delivery device. (B) Structural formula of glycopeptide dendrimer colchicine conjugate **J1C**.

2. Results and discussion

2.1. Combinatorial design and selection

A 65,536-membered combinatorial library was prepared following our previously reported combinatorial design allowing decoding by amino acid analysis,[26] featuring a first generation dendrimer with three tetrapeptide arms separated by a 2,3-diaminopropionic acid branching unit. We used four variable amino acids at each of the eight variable positions X^1–X^8 and attached a galactose residue by standard amide bond coupling of the known[27] β-thiogalactosyl propionic acid **1** at the N-terminus (Scheme 1). A side-chain alloc protected lysine and a bis-Fmoc protected lysine were placed as one of the four variable amino acids at position X^1 and X^8, respectively, resulting in dendrimers displaying galactose residues in copy number two (no lysine at either X^1 or X^8, 56.25% of the library), three (lysine at X^1 but not at X^8, 18.75% of the library), four (lysine at X^8 but not at X^1, 18.75% of the library), or five (lysine at both X^1 and X^8, 6.25% of the library). We have successfully used this first generation dendrimer design with variable multivalency previously to identify multivalent dendrimers binding to a fucose-specific lectin.[28] The relatively long tetrapeptide arms are



Library **βGalL** (n = 1 or 2, m = 0 or 1)

X^8	X^7	X^6	X^5	X^4	X^3	X^2	X^1
Lys*	Asp	Ser	Thr	Ser	Arg	Phe	Lys**
Phe	Asn	His	Ala	Asn	Gly	Glu	Asp
Gly	Arg	Ile	Leu	Thr	Tyr	His	Val
Tyr	Val	Glu	Pro	Pro	Ile	Leu	Ala

Scheme 1. Synthesis and structure of the library **βGalL**. The solid support is tentagel and the branching unit is L-2,3-diaminopropanoic acid (Dap). Lys* = alloc protected L-lysine, Lys** = bis-Fmoc protected L-lysine. Reagents and conditions: (a) suspend the whole resin batch in NMP/DCM (2:1, v/v), mix via nitrogen bubbling for 15 min, and split the batch in four equal portions 1–4; (b) in each portion X^a_b (a = 1–8; b = 1–4): 3.0 equiv Fmoc–X^a_b–OH, 3.0 equiv PyBOP, 6.0 equiv DIEA, DCM/NMP(1:1, v/v), 1.5 h; (c) DMF/piperidine (4: 1, v/v), 2 × 10 min. Steps a–c are repeated nine times, the fifth coupling being with only Dap; (d) Alloc/Fmoc removal: 25 equiv $PhSiH_3$, 0.2 equiv $Pd(PPh_3)_4$, DCM 2 × 20 min under nitrogen followed by washing with 10 mL DCM, dioxane/H_2O (9:1) DMF and DCM, then DMF/piperidine (4:1, v/v), 2 × 10 min; (e) 5 equiv **1**, 5 equiv DIC, 5 equiv HOBt, DCM/NMP(1:1, v/v), 24 h, or for the control acetylated library: Ac_2O/CH_2Cl_2 1:1, 30 min; (f) TFA/TIS/H_2O (95:2.5:2.5), 4 h, then MeOH/NH_4/H_2O (v/v 8:1:1), 24 h.

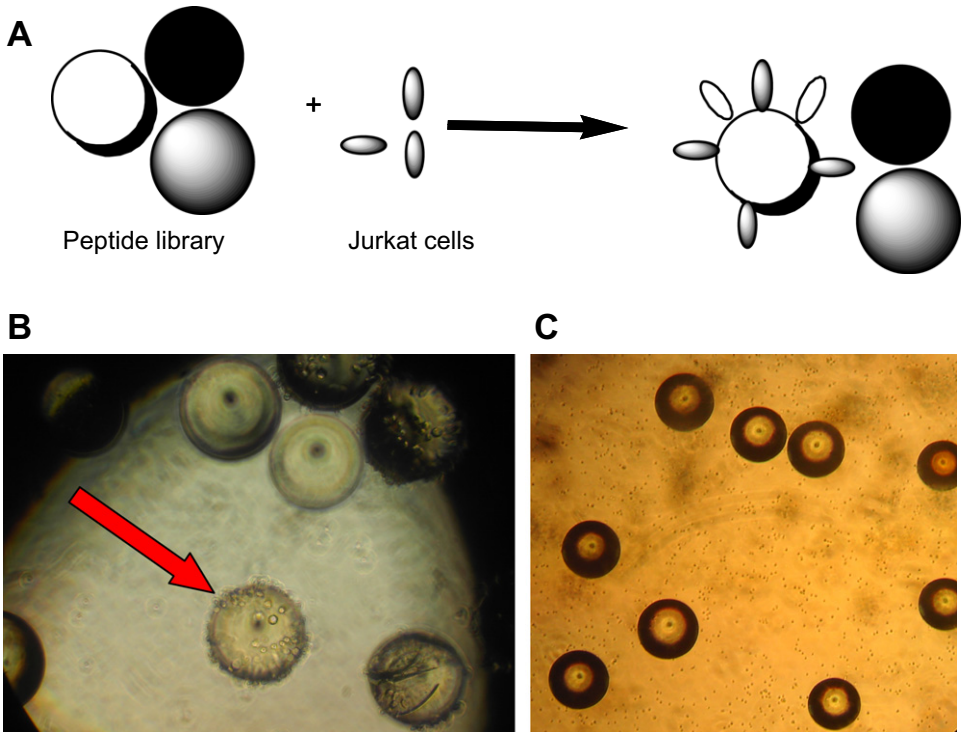


Figure 2. On-bead assay for Jurkat cells binding to peptide dendrimers. (A) Schematic representation of the solid-phase test using Jurkat cells. (B) Images captured using a microscope of library **βGalL**. Cells are seen as granulation on the surface of some of the beads (red arrow). (C) Picture of the acetylated library **AcL**. No cells are seen at the bead surface.

thought to provide a conformationally flexible spacer displaying various functional groups as the amino acid side-chains that should be favorable for both carbohydrate multivalency and secondary binding interactions.[17]

The dendrimer library was assembled on a one gram batch of non-cleavage tentagel (TG) resin (0.47 mmol/g, 90 μm Φ beads). After coupling the last four amino acids at position X^8, the library was pooled and treated with $Pd(PPh_3)_4/PhSiH_3$ to remove the alloc protecting group of lysine residues at position X^1 and with piperidine to effect removal of the Fmoc protecting groups at the N-termini. The resin was then split into two portions and the free N-termini were either coupled with the acetylated β-thiogalactosyl propionic acid building block **1** or acetylated. Side-chain deprotection under acidic conditions and methanolysis of the galactosyl acetate ester finally gave the libraries **βGalL** (Scheme 1) and the control acetylated library **AcL** for testing.

The cell-binding potential of the dendrimers was assessed by an on-bead assay consisting in simply adding the polymer beads to a culture medium containing Jurkat cells. Jurkat cells were selected as standard cancer cells because they grow as suspension and are known to express galectins. Under optimized conditions, binding of Jurkat cells to a few percent of the polymer beads of library **βGalL** was visible under a light microscope after 24 h incubation in the cell culture (Fig. 2). This procedure is similar to other reported cell-binding assays with OBOC combinatorial libraries.[29] Interestingly, the Jurkat cells did not bind at all to the beads of the control library **AcL**, the empty beads, or a related focusylated combinatorial dendrimer library[16] under the same conditions, showing that the presence of a galactose residue was essential for cell-binding. The cell-decorated beads detected with the assay of library **βGalL** were manually picked with a pipette, washed with aqueous phosphate buffer and methanol to remove cell debris, transferred to individual analysis tubes and subjected to amino acid analysis for sequence determination.[30] Analysis of the sequences showed a strong preference for Asp/Asn at position X^7 (Table 1). On the other hand, the hits contained cationic, anionic, and neutral sequences, as well as sequences displaying two, three, four and five galactose residues.

2.2. Synthesis of selected dendrimers and their colchicine and fluorescein conjugates

Sequences **J1–J10** displaying all valency possibilities and a diversity of amino acid sequences were selected for resynthesis. Each dendrimer was prepared by solid-phase peptide synthesis on rink-amide resin incorporating a cysteine residue as an additional residue at the first position. The dendrimers were then converted to the colchicine and fluorescein conjugates by coupling the cysteine side-chain with chloroacetyl-colchicine (**4**)[31] and iodoacetyl-fluorescein (**5**), respectively, as described earlier.[18] An acetylated version of **J1** was also prepared as a control. All products were obtained in sufficient yields and purities for biological studies after purification by RP-HPLC (Table 2). The chloroacetyl-colchicine (**4**) was synthesised by a modification of a reported procedure as shown in Scheme 2.[31] Direct deacetylation of colchicine results in isomerisation of the carbonyl and methoxy group in the adjacent unsaturated cycloheptane. Therefore, the acetyl group is activated by addition of a Boc-protecting group followed by stepwise removal of the acetyl group (to **2**) and Boc-protecting group to give the free amine derivative (**3**). In the final step, the free amine derivative is reacted with chloroacetic anhydride in dichloromethane to give chloroacetyl-colchicine **4** in an overall yield of 34% from colchicine. In the published Boc protection step[31] the yield was 65% and around 25% of the starting material was recovered. We found that changing the solvent from dichloromethane to THF and having a constant temperature of 45 °C improved the yield to 90%.

Table 1
Amino acid sequences of peptide dendrimers binding to Jurkat cells selected for resynthesis

No.[a]	*n*Gal[b]	X^8	X^7	X^6	X^5	X^4	X^3	X^2	X^1
J1	2	Gly	Arg	His	Ala	Thr	Arg	His	Asp
J2	2	Phe	Asp	Ser	Ala	Thr	Tyr	Leu	Asp
J3	2	Phe	Asn	Ser	Ala	Thr	Tyr	Leu	Asp
J4	5	Lys	Arg	His	Leu	Thr	Tyr	His	Lys
J5	4	Lys	Asp	Glu	Pro	Thr	Gly	Phe	Asp
J6	4	Lys	Asn	Glu	Pro	Thr	Gly	Phe	Asp
J7	3	Gly	Asp	Glu	Thr	Asn	Arg	Phe	Lys
J8	3	Gly	Asn	Glu	Thr	Asn	Arg	Phe	Lys
J9	4	Lys	Asp	Glu	Pro	Pro	Ile	Glu	Val
J10	4	Lys	Asn	Glu	Pro	Pro	Ile	Glu	Val

[a] Dendrimers **J2**/**J3**, **J5**/**J6**, **J7**/**J8** and **J9**/**J10** represent the Asp and Asn version at position X^7, which were both possible sequences from the amino acid analysis results.
[b] *n*Gal = number of β-thiogalactosyl-propionyl groups on the dendrimers.

2.3. Binding of peptide dendrimers to Jurkat cells

The binding to Jurkat cells was investigated by fluorescence activated cell sorting (FACS) using fluorescein and its dendrimer conjugates **J1F**, **AcJ1F**, **J2F**, **J3F**, and **J4F**, displaying between zero and four β-thiogalactoside residues. The cells were plated at a concentration of 50,000 cells per mL and grown overnight in order to produce a healthy growing population, treated with the various ligands to a final concentration of 5 μM, and incubated for further two days before analysis. The results from these experiments are shown in Figure 3 as single parameter histograms. Co-staining with propidium iodide (PI) showed no increase in dead cells upon addition of ligands, implying that the fluorescein–dendrimer conjugates were non-cytotoxic. The fluorescence intensity was significantly higher with galactosylated ligands compared to non-galactosylated ligands, with cells incubated with the pentavalent dendrimer **J4F** showing the strongest fluorescence intensity. Selective staining by the galactosylated dendrimers over fluorescein was also evident under the fluorescent microscope (Fig. 4).

2.4. Cytotoxicity studies

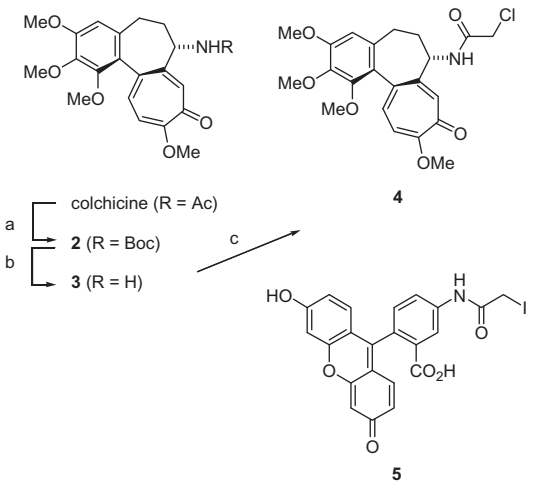
Having ascertained that the glycopeptide dendrimers selected in the on-bead cell-binding assay bound to Jurkat cells as the fluorescein conjugates, we next investigated the cytotoxicity of the corresponding colchicine–dendrimer conjugates. The colchicine dendrimer conjugates were added at 5 μM final concentration to an overnight culture of cells that had a starting concentration of 50,000 cells per mL. After incubation for 48 h, the cells were stained with propidium iodide (PI) and healthy and dead cells were counted by FACS. LD_{50} values were subsequently determined for all dendrimers showing significant cytotoxicity (Fig. 5 and Table 3).

Strikingly, only the colchicine dendrimer conjugates displaying two galactose residues, namely **J1C**, **J2C**, and **J3C**, showed measurable cytotoxicity. The acetylated control dendrimer **AcJ1C** was not cytotoxic, in agreement with the weak cell-binding of the fluorescein conjugate **AcJ1F** (Fig. 3) and suggesting that cell internalization of the galactosylated dendrimers was dependent on galactose recognition and not caused by passive diffusion as is the case for colchicine itself. More surprisingly, the dendrimers displaying three, four or five galactose residues did not show measurable cytotoxicity. This is surprising considered that the corresponding fluorescein-labeled dendrimers showed strong binding to Jurkat cells (Fig. 3). It should be noted that **J2C** bearing an aspartate residue at X^7 was threefold more active than its asparagine analogue **J3C**, in line with the stronger labeling of Jurkat cells

Table 2
Synthesis of peptide dendrimers and conjugates

No.	Sequence[a]	Yield[b] mg, %	MS calcd	MS[c] obsd
J1	$(\beta GalGRHA)_2$*Dap*TRHDCNH$_2$	15 mg, 12%	2058.86	2059.0
J1C	$(\beta GalGRHA)_2$*Dap*TRHDC(Col)NH$_2$	1.8 mg, 46%	2457.01	2457.0
J1F	$(\beta GalGRHA)_2$*Dap*TRHDC(Fluo)NH$_2$	0.9 mg, 38%	2446.93	2446.0
AcJ1	$(AcGRHA)_2$*Dap*TRHDCNH$_2$	35 mg, 36%	1642.78	1642.9
AcJ1C	$(AcGRHA)_2$*Dap*TRHDC(Col)NH$_2$	2.3 mg, 53%	2040.93	2040.0
AcJ1F	$(AcGRHA)_2$*Dap*TRHDC(Fluo)NH$_2$	2.2 mg, 42%	2030.85	2030.0
J2	$(\beta GalFDSA)_2$*Dap*TYLDCNH$_2$	5 mg, 4%	2040.7	2039.2
J2C	$(\beta GalFDSA)_2$*Dap*TYLDC(Col)NH$_2$	0.5 mg, 42%	2962.2	n.d.
J2F	$(\beta GalFDSA)_2$*Dap*TYLDC(Fluo)NH$_2$	1.1 mg, 46%	2426.8	2427.0
J3	$(\beta GalFNSA)_2$*Dap*TYLDCNH$_2$	6 mg, 5%	2038.77	2037.5
J3C	$(\beta GalFNSA)_2$*Dap*TYLDC(Col)NH$_2$	0.2 mg, 17%	2964.2	n.d.
J3F	$(\beta GalFNSA)_2$*Dap*TYLDC(Fluo)NH$_2$	0.8 mg, 34%	2424.5	2425.4
J4	βGal_4(*K*RHL)$_2$*Dap*TYHK(βGal)CNH$_2$	11 mg, 6%	3056.3	3056.0
J4C	βGal_4(*K*RHL)$_2$*Dap*TYHK(βGal)C(Colch)NH$_2$	1.6 mg, 31%	3453.43	3453.3
J4F	βGal_4(*K*RHL)$_2$*Dap*TYHK(βGal)C(Fluo)NH$_2$	0.7 mg, 13%	3443.36	3443.4
J5	βGal_4(*K*DEP)$_2$*Dap*TGFDCNH$_2$	13.1 mg, 9%	2565.7	2566.0
J5C	βGal_4(*K*DEP)$_2$*Dap*TGFDC(Col)NH$_2$	2.0 mg, 56%	2963.2	2965.0
J6	βGal_4(*K*NEP)$_2$*Dap*TGFDCNH$_2$	25 mg, 16%	2587.7	2586.5
J6C	βGal_4(*K*NEP)$_2$*Dap*TGFDC(Col)NH$_2$	2.8 mg, 84%	2961.2	2961.0
J7	$(\beta GalGDET)_2$*Dap*NRFK(βGal)CNH$_2$	11 mg, 8%	2306.4	2308.0
J7C	$(\beta GalGDET)_2$*Dap*NRFK(βGal)C(Col)NH$_2$	1.1 mg, 34%	2703.9	2708.0
J8	$(\beta GalGNET)_2$*Dap*NRFK(βGal)CNH$_2$	20 mg, 14%	2305.5	2305.0
J8C	$(\beta GalGNET)_2$*Dap*NRFK(βGal)C(Col)NH$_2$	1.4 mg, 40%	2701.9	2703.0
J9	βGal_4(*K*DGP)$_2$*Dap*PIEVCNH$_2$	18.6 mg, 13%	2583.8	2584.0
J9C	βGal_4(*K*DGP)$_2$*Dap*PIEVC(Col)NH$_2$	1.9 mg, 55%	2981.3	2982.0
J10	βGal_4(*K*NGP)$_2$*Dap*PIEVCNH$_2$	9.0 mg, 6%	2582.9	2582.8
J10C	βGal_4(*K*NGP)$_2$*Dap*PIEVC(Col)NH$_2$	0.52 mg, 23%	2978.3	2980.0

[a] Peptide sequences with one-letter code amino acids, see Scheme 1 for detailed structural formulae. βGal = β-thiogalactosyl-propionyl. *K* indicates L-lysine acylated at both amino groups at the N-terminus, *Dap* is the L-2,3-diaminopropanoic acid branching unit. K(βGal) indicates lysine with a β-thiogalactosyl-propionyl group acylated on the ε-amino group. C(Col) indicates cysteine alkylated at the thiol group with chloroacetyl-colchicine. C(Fluo) indicates cysteine alkylated at the thiol group with iodoacetyl-fluorescein.
[b] Isolated yields from calculated resin loading after cleavage and purification by preparative RP-HPLC.
[c] ESI MS, see Section 4 for details.



Scheme 2. Synthesis and structure of ligands for conjugation. Reagents and conditions: (a) (i) Boc_2O, Et_3N, DMAP, THF, 45 °C, 24 h (95%); (ii) NaOMe, MeOH, 0 °C, 1.5 h (quant); (b) TFA: CH_2Cl_2 (1:10), 4 h (80%); (c) 3 equiv $(ClCH_2CO)_2O$, Et_3, DMAP, CH_2Cl_2, 45 °C, 2 h (45%).

with **J2F** than with **J3F**. The higher activity of **J2C** suggests that the bead selected in the cell-binding assay carried the sequence of **J2** with an aspartate at X^7, and highlights the critical role of the amino acid sequence in activity.

Additional cytotoxicity studies were performed with mouse embryonic fibroblasts (MEF) cells as a model of healthy cells. FACS analysis was not reliable with these cells due to aggregation, and cytotoxicity was estimated instead by visual cell counting under the microscope. The data indicated that both free colchicine and the dendrimer conjugates exhibited a similarly slightly higher cytotoxicity against Jurkat cells compared to MEF (Table 4), showing that the galactose-dependent toxicity of the dendrimer conjugates did not result in a more selective toxicity.

2.5. Tubulin binding assay and mechanistic hypothesis

Considering that only the bis-galactosylated, colchicine conjugated dendrimers show measurable cytotoxicity, one must assume that this activity involves a selective uptake and intracellular release of the dendrimers followed by a colchicine-type inhibition of mitosis as previously observed with related glycopeptide dendrimer colchicine conjugates.[18] A tubulin polymerization inhibition assay was performed to test the ability of the glycopeptide dendrimer conjugates to directly interact with tubulin. While colchicine itself exhibits micromolar activity in this assay (IC_{50} = 1.6 μM), there was no detectable inhibition with dendrimer conjugates such as **J1C** or **J4C**. However partial polymerization inhibition was observed with the tripeptide colchicine conjugate **His-Asp-Cys(S-Colch)NH_2** corresponding to the core sequence of **J1C** (33% at 1 mg/ml), as well as with the chloroacetyl-colchicine **4** used to prepare the conjugates (IC_{50} = 17 μM). These data suggest that the glycopeptide dendrimers are degraded after internalization to release short peptide conjugates of colchicine capable of tubulin binding and cytotoxicity, albeit more weakly than colchicine itself. The degradation process should not be problematic if proteases are present, as we have recently shown in a systematic reactivity study.[32] Weaker tubulin binding by dendrimer fragments compared to colchicine might explain the difference in cytotoxicity between colchicine (LD_{50} = 0.02 μM) and the dendrimers (LD_{50} = 1.5–5.3 μM) (Table 3). A degradation mechanism for activation suggests that the lack of activity observed in tri-, tetra- and pentavalent dendrimers might reflect either the absence of degradation or the formation of inactive fragments due to the particular core residues present in these dendrimers. On the other hand, multivalent tight binding to galactose receptors inhibiting internalization and degradation might also play a role.

3. Conclusion

Although the peptide dendrimers may be considered as relatively complex structures compared to other dendrimer types, it should be stressed that they are relatively easy to prepare, not only as combinatorial library, which is not possible with other dendrimer types, but also as single purified products, as illustrated by the synthesis of 27 different dendrimers for the present study (Table 1). Thus, glycopeptide dendrimers selected from the combinatorial library by an on-bead cell-binding assay showed good affinity to Jurkat cells mediated by β-thiogalactoside residues at the branches ends, as evidenced by FACS using fluorescein-labeled dendrimers. The presence of the β-galactoside groups was necessary for efficient cell-binding. When considering the cytotoxicity of the corresponding colchicine conjugates however, only the three peptide dendrimers **J1C**, **J2C**, and **J3C**, each carrying two β-galactoside residues in their branches, showed activity. Dendrimers with different sequences including higher multivalency of galactose or non-galactosylated dendrimers were not cytotoxic. Tubulin polymerization inhibition assays showed no tubulin binding activity for the complete dendrimers but weak binding for fragments. The inactive glycopeptide dendrimers are thus probably degraded intracellularly to release small fragments whose affinity might be significantly weaker than colchicine itself, explaining the higher LD_{50} values of the glycopeptide dendrimers conjugates compared to free colchicine. Although the galactose-dependent cell-binding and cytotoxicity of the dendrimer–cochicine conjugates excludes significant proteolytic degradation prior to cell uptake, future improvements of the system will need to address the question of degradation by serum proteases for an in vivo situation. Tubulin binding itself could be optimized by searching for cell-internalizing glycopeptide dendrimer colchicine conjugates retaining strong tubulin binding without the need for degradation, or by designing conjugates that can release colchicine itself.

4. Experimental section

4.1. Synthesis of the split-and-mix combinatorial library βGalL

The peptide dendrimer library **βGalL** was prepared from a 1 g resin batch of TentagGel HL NH_2 (loading: 0.47 mmol/g) divided equally in four reactors. In four reactors the resin was acylated with one of the four amino acids (3 equiv) in the presence of PyBOP (3 equiv) and *N,N*-diisopropylethylamine (DIEA) (5 equiv). Amino acids were acylated for 1.5 h, and Dap was coupled in all reactors for 2 h. After each coupling, the four resin batches were mixed together and again split into four parts, equally introduced in the four reactors. These split-and-mix steps were repeated after each amino acid coupling. After each coupling the resin was successively washed with *N*-methylpyrrolidone (NMP), MeOH, CH_2Cl_2 (3 times with each solvent) and subsequently checked for free amino groups with the trinitrobenzene sulfonate (TNBS test). Proline coupling was checked with the chloranile test. If the test indicated the presence of free amino groups, the coupling was repeated. The Fmoc protecting groups were removed with a solution of 20% piperidine in DMF (two times 10 min) and the solvent was removed by filtration. At the end of the synthesis the Fmoc protected resin was dried and stored at -20 °C. A random selection of 25 of beads were picked, analysed and decoded to determine the dendrimer sequence to confirm the distribution of amino acids in each

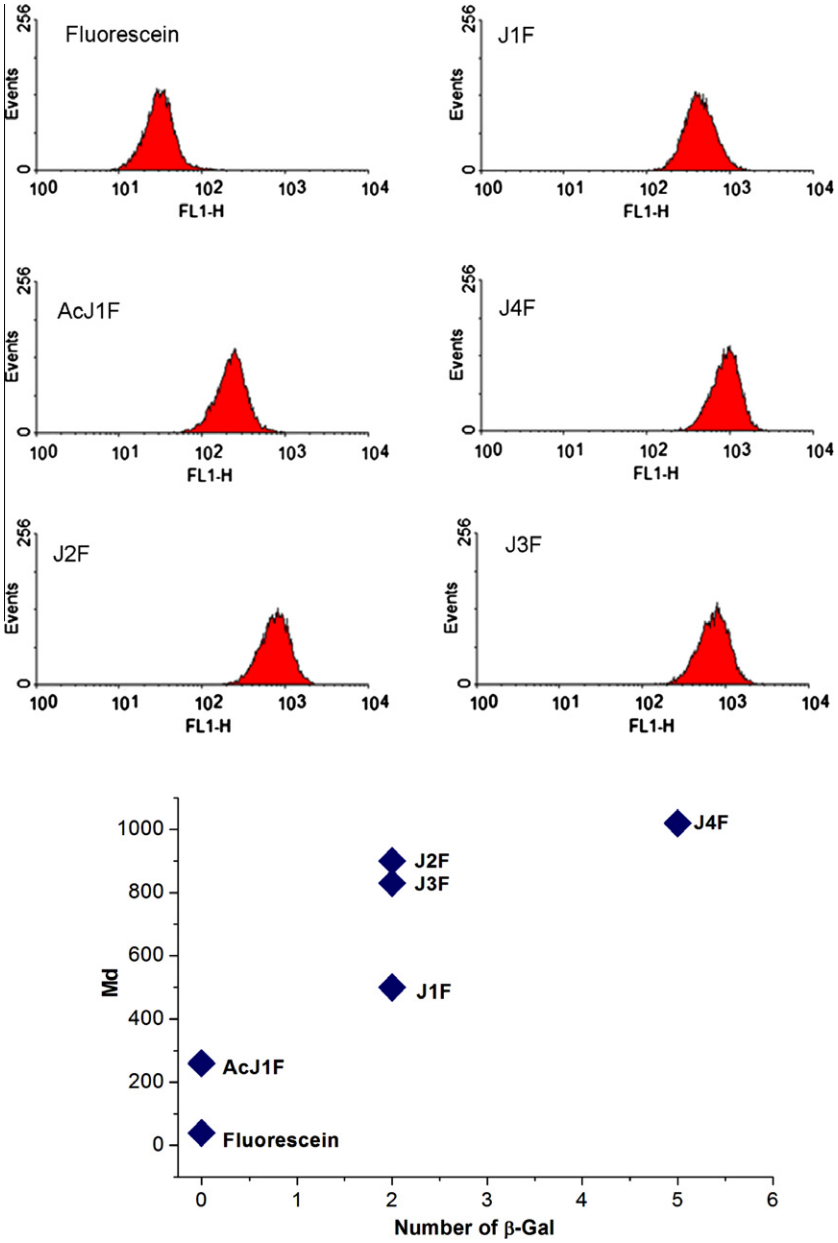


Figure 3. FACS of Jurkat cells binding to fluorescein and the fluorescein–dendrimer conjugates. One parameter distribution plots of fluorescence intensity versus cell count. The *x*-axis represents emission intensity (excited at 488 nm, emission observed at 510–560 nm) and the *y*-axis is the number of cells. Md = median fluorescence intensity. Fluorescence levels were negligible without added ligand (Md = 2.8) or with the non-labeled dendrimer **J1** (Md = 3.0).

position as expected. Immediately before screening, the Fmoc protecting groups were removed and the library was capped with a β-D-thiogalactoside residue **1** (prepared following the procedure of Magnusson et al.[27] 5 equiv) of DIC (5 equiv) and HOBt (5 equiv) in CH_2Cl_2/NMP (2:1, v/v) overnight. The side-chain protecting groups were removed with TFA/TIS/H_2O (95/2.5/2.5) for 4 h. Then the carbohydrate was deacetylated with a solution of MeOH/NH_4/H_2O (v/v 8:1:1) for 24 h, resulting in the glycopeptide dendrimer library on beads **βGalL**.

4.2. Synthesis of N-acetylated control library AcL

Fmoc and Alloc protecting groups were removed and the library was capped with Ac_2O/CH_2Cl_2 1:1 for 30 min. The side-chain protecting groups were removed with TFA/TIS/H_2O (95:2.5:2.5) for 4 h to give a library with acetylated *N*-termini and amine side-chain groups.

4.3. Cell culture

Jurkat cells were grown in RPMI medium with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin, and 1% (v/v) L-glutamine. Mouse embryonic fibroblasts (MEFs) were grown in DMEM (Dulbeccos modified Eagle medium) with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin, 1% (v/v) HEPES buffer, and 1% (v/v) L-glutamine. All cells were grown in a humidified incubator at 37 °C and 5% CO_2. Passage of the cells were performed every 2–3 days to preserve the exponential growth of the cells. The MEFs cells were split by removing the media and then wash with sterile PBS buffer. Antiadherent Trypsin/EDTA solution was added and the cells were incubated at 37 °C for 20 min.

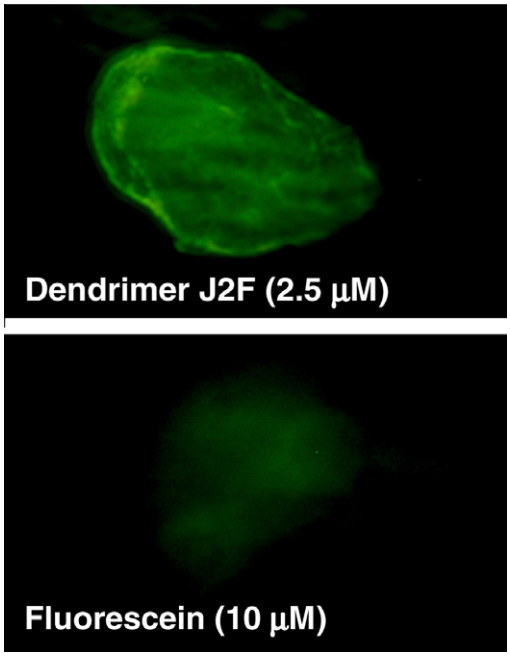


Figure 4. Fluorescence image of Jurkat cells stained with dendrimer **J2F** at 2.5 μM and fluorescein at 10 μM for 4 h indicating the increased affinity of the dendrimers to the cell.

Table 3
LD_{50} values for cytotoxic colchicine dendrimer conjugates against Jurkat cells

No.	Sequence	*n*Gal[a]	LD_{50} (μM)
—	Colchicine		0.020 ± 0.004
J1C	$(\beta GalGRHA)_2$*Dap*TRHDC(Col)NH_2	2	1.50 ± 0.04
AcJ1C	$(AcGRHA)_2$*Dap*TRHDC(Col)NH_2	0	—[b]
J2C	$(\beta GalFDSA)_2$*Dap*TYLDC(Col)NH_2	2	1.53 ± 0.06
J3C	$(\beta GalFNSA)_2$*Dap*TYLDC(Col)NH_2	2	5.52 ± 0.15
J4C	βGal_4(*K*RHL)$_2$*Dap*TYHK(βGal)C(Colch)NH_2	5	—[b]
J5C	βGal_4(*K*DEP)$_2$*Dap*TGFDC(Col)NH_2	5	—[b]
J6C	βGal_4(*K*NEP)$_2$*Dap*TGFDC(Col)NH_2	4	—[b]
J7C	$(\beta GalGDET)_2$*Dap*NRFK(βGal)C(Col)NH_2	3	—[b]
J8C	$(\beta GalGNET)_2$*Dap*NRFK(βGal)C(Col)NH_2	3	—[b]
J9C	βGal_4(*K*DGP)$_2$*Dap*PIEVC(Col)NH_2	4	—[b]
J10C	βGal_4(*K*NGP)$_2$*Dap*PIEVC(Col)NH_2	4	—[b]

[a] Number of β-thiogalactosyl-propionyl groups in the dendrimer.
[b] These dendrimer conjugates showed negligible cytotoxicity (2–14% dead cells) at 5 μM.

4.4. Solid phase screening with Jurkat cells

The glycodendrimer library **βGalL** (50 mg), was washed three times with 3 mL of PBS buffer. The cells were pelleted and resuspended in new RPMI medium with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 1% (v/v) L-glutamine. The cells were added to the library in a sterile Petri dish and incubated at 37 °C, 5% CO_2 for 24 hours, first gently shaken (60 rpm) for 4 h then static incubation for the remaining 24 h. Resin beads with cells attached were man-

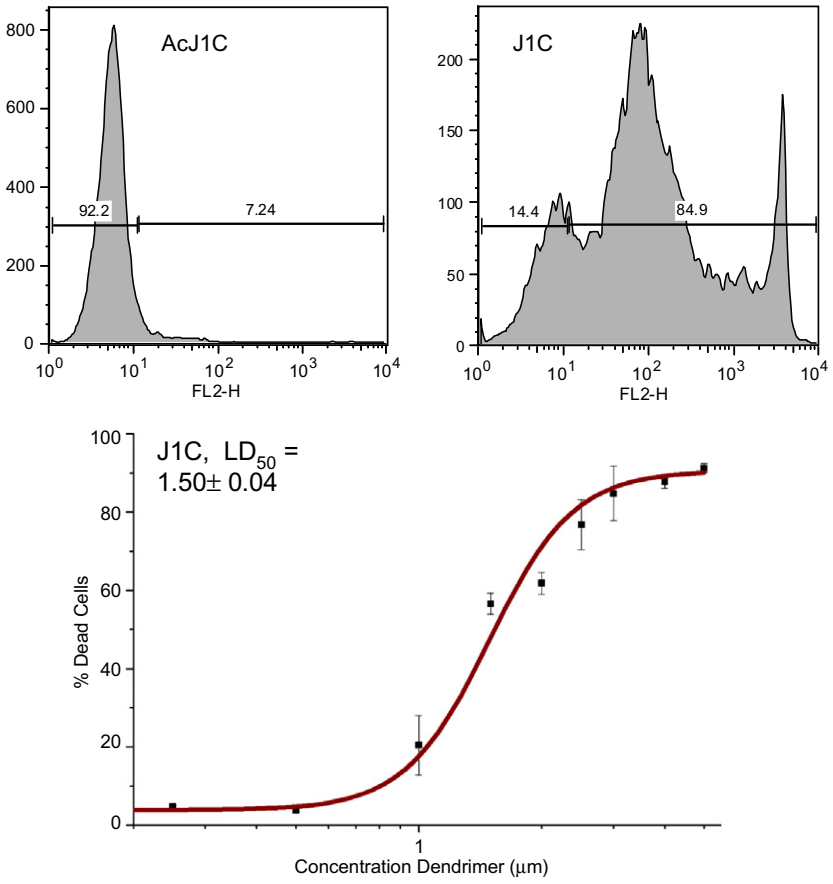


Figure 5. Cytotoxicity assays. FACS analysis of Jurkat cells treated with colchicine dendrimer conjugates at 5 μM for 48 h followed by staining with propidium iodide. The *x*-axis is the intensity of emitted light by the propidium iodide–DNA complex, measured at 580–650 nm, from excitation at 488 nm. Propidium iodide penetrates only into dead cells and complexes the DNA, leading to a strong fluorescence only in dead cells. An LD_{50} curve obtained by this assay for **J1C** is shown as a representative example.

Table 4
Cytotoxicity of colchicine-linked dendrimers against Jurkat cells and MEF cells

	% Dead cells at 5 μM[a]	
	Jurkat cells (%)	MEF cells (%)
Colchicine	85	57
J1C	91	51
J2C	91	68
J3C	19	6

[a] The cells were incubated for 2 days at 37 °C in the presence of 5 μM colchicine or dendrimer. Live and dead cells were quantified by counting.

ually picked and washed with 70% ethanol (10 1 mL), water (10 mL), MeOH (two times), CH_2Cl_2 (five times), MeOH (two times), and water (five times) in order to remove bound cells. Picked beads were analysed by amino acid analysis to determine their sequence.

4.5. *N*-((*tert*-Butoxy)carbonyl)-colchicine

Colchicine (0.60 g, 1.5 mmol) was dissolved in anhydrous THF. Di(*tert*-butyl)pyrocarbonate (1.5 g, 7 mmol), triethylamine (0.2 mL, 1.43 mmol) and DMAP (0.15 g, 1.2 mmol) was added. The mixture was stirred at 45 °C for 24 h. The solvent was removed under reduced pressure to give a yellow oil. Purification using column chromatography (silica gel, ethyl acetate) and concentration of the appropriate fractions gave *N*-((tert-butoxy)carbonyl)-colchicine (0.56 g, 95%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.52 (s, 1H,), 7.26 (d, J = 10.6 Hz, 1H), 6.72 (d, J = 10.7 Hz, 1H), 6.49 (s, 1H) 5.10 (q, J = 6.0 Hz, 1H), 3.91 (s, 1H), 3.88 (s, 1H), 3.84 (s, 1H), 3.61 (s, 1H), 2.70–2.45 (m, 3H), 2.22 (s, 3H), 1.98–1.80 (m, 1H), 1.51 (s, 9H); MS (ES+): calcd for $C_{27}H_{34}NO_8$ $[M+H]^+$: 499.6, found: 500.2.

4.6. *N*-((*tert*-Butoxy)carbonyl)-deacetylcolchicine (2)

An excess of sodium (500 mg, 22 mmol) carefully dissolved in methanol (5 mL) was added to compound *N*-((tert-butoxy)carbonyl)-colchicine (0.042 g, 0.084 mmol) and the solution was stirred for 2 h at 0 °C. The solvent was removed under reduced pressure and the residue was re-dissolved in ethyl acetate (10 mL). The organic phase was washed with H_2O (10 mL) and brine (10 mL), dried over $MgSO_4$ and the solvent was removed under reduced pressure to give-((*tert*-butoxy)carbonyl)-deacetylcolchicine (**2**) as a yellow oil, which was used without further purification.

4.7. Deacetylcolchicine (3)

The crude *N*-((*tert*-butoxy)carbonyl)-deacetylcolchicine (0.10 g, 0.22 mmol) was dissolved in anhydrous dichloromethane (10 mL) and concentrated HCl (1 mL) was added. The reaction mixture was stirred at 45 °C for 3 h. Toluene was added as a co-solvent and removed under reduced pressure to give crude deacetylcolchicine as a dark orange oil. Preparative RP-HPLC was used to obtain deacetylcolchicine (**3**) as a yellow powder (0.063 g, 80%). Analytical-HPLC: t_r = 7.4 (λ = 214 nm, A/B = 70:30 to 0:100 in 15 min); ^{1}H NMR (300 MHz, CD_3OD): δ = 7.44 (d, J = 10.7 Hz, 1H,), 7.25 (d, J = 10.7 Hz, 1H), 7.20 (s, 1H), 6.78 (s, 1H), 4.04 (s, 3H), 3.91 (s, 3H), 3.87 (s, 3H), 3.69 (s, 3H), 2.80–2.60 (m, 1H), 2.50–2.35 (m, 2H), 2.08–1.95 (m, 1H); MS (ES+): calcd for $C_{20}H_{23}NO_5$ $[M+H]^+$: 358.16, found: 358.4.

4.8. Chloroacetyl-colchicine (4)

Deacetylcolchicine (**3**) (129 mg, 0.36 mmol) was dissolved in anhydrous dichloromethane (5 mL) together with triethylamine (65 μL, 4.6 mmol) at 0 °C. Chloroacetic anhydride (500 mg, 0.189 mmol) was added drop wise and the resulting mixture was stirred for 2 h. The reaction was quenched with ice water and organic phase was washed with brine. The organic phase was dried over $MgSO_4$ and then removed to give **4** as yellow oil. Preparative RP-HPLC was used to obtain **4** as a pale yellow powder 74 mg, 47%). Analytical-HPLC: t_r = 8.4 (λ = 350 nm, A/B = 70:30 to 0:100 in 15 min); ^{1}H NMR (300 MHz, CD_3OD): δ = 8.98 (d, J = 6.0 Hz, 1H, NH), 7.43 (d, J = 12.0 Hz, 1H), 7.38 (s, 1H), 7.22 (d, J = 12.0 Hz, 1H), 6.75 (s, 3H), 4.51 (m, 1H), 4.09 (s, 2H), 4.01 (s, 3H), 3.90 (s, 3H), 3.88 (s, 3H), 3.60 (s, 3H), 2.70–2.60 (m, 1H), 2.50–2.25 (m, 2H), 2.08–1.95 (m, 1H); MS (ES+): calcd for $C_{22}H_5ClNO_6$ $[M+H]^+$: 434.1, found: 434.2.

4.9. Dendrimer synthesis

Peptide syntheses were performed manually in a glass reactor or plastic syringes (5 or 10 ml). The resin NovaSyn TGR (loading: 0.18–0.29 mmol/g) was acylated with each amino acid or diamino acid (3 equiv) in the presence of BOP or PyBOP (3 equiv) and DIEA (5 equiv) for 1.5 h (3 h after the first generation). After each coupling, the resin was successively washed with NMP, MeOH, and CH_2Cl_2 (three times with each solvent), then checked for free amino groups with the TNBS test. If the TNBS test indicated the presence of free amino groups, the coupling was repeated. After each coupling, the potential remaining free amino groups were capped with acetic anhydride/CH_2Cl_2 for 10 min. The Fmoc protecting groups were removed with a solution of 20% piperidine in DMF (2310 min) and the solvent was removed by filtration. In the end of the sequence the resin was capped with β-thiogalactosyl propionic acid (**1**) (5 equiv) in the presence of DIC (5 equiv) and HOBt (5 equiv) or DIEA (5 equiv) and HCTU (3 equiv) in NMP overnight. The carbohydrate was deprotected with a solution of MeOH/NH_3/H_2O (v/v 8:1:1) for 24 h. The resin was dried and the cleavage was carried out with TFA/TIS/H_2O (95:2.5:2.5) for 4 h. The peptide was precipitated with methyl *tert*-butyl ether then dissolved in a water/acetonitrile mixture. All dendrimers were purified by preparative HPLC with detection at λ = 214 nm. Eluent A contained water and TFA (0.1%); eluent B contained acetonitrile, water, and TFA (3/2/0.1%). The yields (mg, %) and MS data are given in Table 2.

4.10. Synthesis of dendrimer conjugates

Conjugation of chloroacetyl-colchicine **4** and iodoacetyl-fluorescein **5** derivative to the dendrimers was performed in PB-buffer (50 mM, pH 8.5) acetonitrile (70:30), stirring at 40 °C for 24–48 h under nitrogen. The reaction took place in the minimum amount of solvent required to dissolve starting materials and always with an excess of either **4** (2–4 mg) or **5** (4 mg) compared to the dendrimer (1–5 mg). The final dendrimer conjugates were obtained after semi-preparative HPLC. The yields (mg, %) and MS data are given in Table 2.

5. Cytotoxicity and fluorescein staining FACS experiments

One day prior to the addition of test compounds (colchicine or fluorescein derivatives), cells (5×10^4 cells mL^{-1}) were seeded on to six-well plates (2 mL/well). A volume of a stock solution in sterile MilliQ water of the test compound (1–50 μL) was added to the wells with a micropipette. After another two days of incubation the medium with cells were transferred to a FACS tube. In the case of the cytotoxicity experiment, 50 μL of propidium iodide (0.4 $mg.mL^{-1}$) was added 2 min before the sample was analysed. Acquisition and analysis was performed using a FAC-Scan Flow cytometer and the Cell Quest software. List mode data are acquired using the Flow Cytometry Standard 2.0 (FCS). The excitation wave-

length was 488 nm and the emission was collected at 550 nm. The raw data was processed further with WinMDI 2.9 software (Joseph Trotter, The Scripps Research Institute, USA). The MEF cells medium was removed to the FACS tube. The cells were washed with sterile PBS and antiadherent Trypsin/EDTA solution (0.5 mL) was added and incubated at 37 °C for 20 min after which the solution was added to the FACS tube.

5.1. Tubulin Polymerization Assay

Sheep brains were obtained from the INRA (Institut National de Recherche Agronomique) from freshly slaughtered animals and tubulin isolation was started in the next hour. Thus, sheep brain microtubule proteins were purified by two cycles of assembly/disassembly at 37 °C/0 °C in MES buffer: 100 mM MES (2-[*N*-morpholino]-ethanesulfonic acid, pH 6.6), 1 mM EGTA (ethyleneglycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid), 0.5 mM $MgCl_2$. All samples were dissolved in DMSO. The evaluated compound (1 μL) was added to microtubular solution (150 μL) that was incubated at 37 °C for 10 min and at 0 °C for 5 min before evaluation of the tubulin assembly rate. The tubulin assembly assay was realized according to a slightly modified Guénard's protocol[33] using colchicine as reference compound.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.026.

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