Glycoconj J (2010) 27:277–285
DOI 10.1007/s10719-009-9276-3

Cytotoxicity and enzymatic activity inhibition in cell lines treated with novel iminosugar derivatives

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Received: 26 March 2009 / Revised: 11 August 2009 / Accepted: 9 December 2009 / Published online: 30 December 2009

Abstract Iminosugars are monosaccharide analogues that have been demonstrated to be specific inhibitors for glycosidases and are currently used therapeutically in several human disorders. *N*-alkylated derivatives of D-fagomine and (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol with aliphatic chains were tested in eight human cancer cell lines to analyze their cytotoxicity and the inhibitory effect in the activities of specific glycosidases. Results indicate that these compounds were more cytotoxic as the length of the alkyl chain increases. *N*-dodecyl-D-fagomine inhibited specifically the α-D-glucosidase activity in cell lysates, whereas no effect was detected in other glycosidases. The *N*-dodecyl derivative of (2*R*,3*S*,4*R*,5*S*)-2-(Hydroxymethyl)-5-methylpyrrolidine-3,4-diol induced specific inhibition against α-L-fucosidase in cell lysates. Our results indicated that the length of the alkyl chain linked to the iminosugars determine their cytotoxicity as well as the inhibitory effect on the enzymatic activities of specific glycosidases, in human cancer cell lines.

Keywords Iminosugar · *N*-alkylated iminosugars · Cytotoxicity · Glycosidase

Introduction

Glycosylation is a post-translational modification that has been detected to be altered in several pathologies as cancer, cystic fibrosis, inflammatory diseases among others [1]. It is a highly specific sequential process determined by the glycosylation and de-glycosylation enzymes, glycosyltransferases and glycosidases. Iminosugars are monosaccharide analogues with a nitrogen atom in place of the ring oxygen. Several iminosugars isolated from natural sources and the corresponding synthetic derivatives have been demonstrated to be inhibitors of specific glycosidases and are used therapeutically in several diseases [2]. Lysosomal glycosphingolipidoses disorders characterized by deficiencies in the catabolic activity of glycosidases in the lysosome can affect the maturation and transport of glycoproteins and glycolipids and, as a consequence, leads to lysosomal storage of glycosphingolipids. These diseases can be treated with *N*-alkylated iminosugars that act as inhibitors of the *N*-glycans biosynthesis with a reduction in the lysosomal concentration [1, 3]. Specifically, the inhibitory effect of the iminosugar *N*-butyl-1-deoxynojirimycin has been clinically tested to treat the type I of Gaucher disease, caused by deficiency of the lysosomal enzyme, glucocerebrosidase, that leads to the accumulation of glucocerebroside [1, 4], whereas α-1-C-octyl-1-deoxynojirimycin has been analyzed in Gaucher cell lines [5]. Also, 1-deoxynojiromycin, a potent α-D-glucosidase inhibitor, has anti-HIV activities by the inhibition of the HIV entry [6], although the complete mechanism has not been fully characterized. The effects in the inhibition of α-glucosidase activity of several compounds are currently being used as therapeutic agents in type 2 diabetes [7]; and sialic acid analogues are effective in the decrease of neuraminidase activity of influenza A and B viruses inhibiting their replication [8]. In tumor cells, the invasion and metastasis processes involve the glycan structures located at the cell membrane that mediate cell-cell and cell-extracellular matrix interactions. To alter the biosynthesis of these oligosaccharides, several approaches have been assessed. From them, iminosugars, that act as competitive inhibitors of specific glycosidases, have been suggested as therapeutic tools to

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inhibit tumor metastasis (reviewed in [9]). As an example, *gem*-diamine 1-*N*-iminosugars have been reported to suppress invasion of B16 melanoma and 3LL lung carcinoma cells [10]. Also, pyrrolidine-3,4-diol derivatives bearing aromatic and aliphatic amino side chains that are selective inhibitors of α-mannosidase, have been described to inhibit the growth of human glioblastoma and melanoma cells [11, 12].

D-fagomine, a polyhydroxylated piperidine analogue ((2*R*,3*R*,4*R*)-2-hydroxymethylpiperidine-3,4-diol) is a naturally occurring iminosugar that has been reported to have inhibitory effects against α-, β-glucosidase and α-, β-galactosidase from mammals [13]. *N*-alkylated derivatives of D-fagomine have been synthesized using fructose-6-phosphate aldolase and tested for their inhibitory effects *in vitro* against α-glucosidase from rice and β-D-galactosidase from bovine liver, showing that their effects increase with the aliphatic chain length [14]. Also, the (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol is an iminosugar analogue of L-fucose that has been previously synthesized [15], and it is a specific inhibitor of α-L-fucosidase from bovine kidney (manuscript in preparation).

Here, *N*-alkylated derivatives of D-fagomine and (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol have been tested in several human epithelial cancer cell lines to analyze their cytotoxicity and inhibitory effect of specific glycosidase activities in cell lysates.

Materials and methods

Synthesis of sugar analogues

A) D-Fagomine and *N*-alkylated-D-fagomine derivatives

D-fagomine (**1**) and the corresponding *N*-alkylated derivatives (**1a**–**1e**), were synthesized using D-fructose-6-phosphate aldolase with the previously described procedure [14]. Five different molecules were obtained by modification on the nitrogen atom with an aliphatic chain of several lengths from four to 12 carbons. The structures of the D-fagomine (**1**) and the *N*-alkylated-D-fagomine derivatives (**1a**–**1e**) are shown in Fig. 1a.

B) (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol (2) and *N*-(2*R*,3*S*,4*R*,5*S*)-1-dodecyl-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol (2a)

(2*R*,3*S*,4*R*,5*S*)-2-(Hydroxymethyl)-5-methylpyrrolidine-3,4-diol (**2**) was synthesized in our laboratory following a previously reported procedure [13]. The *N*-dodecyl derivative (**2a**), was prepared as described for the synthesis of *N*-alkylated-D-fagomine derivatives [14], (Fig. 1b).

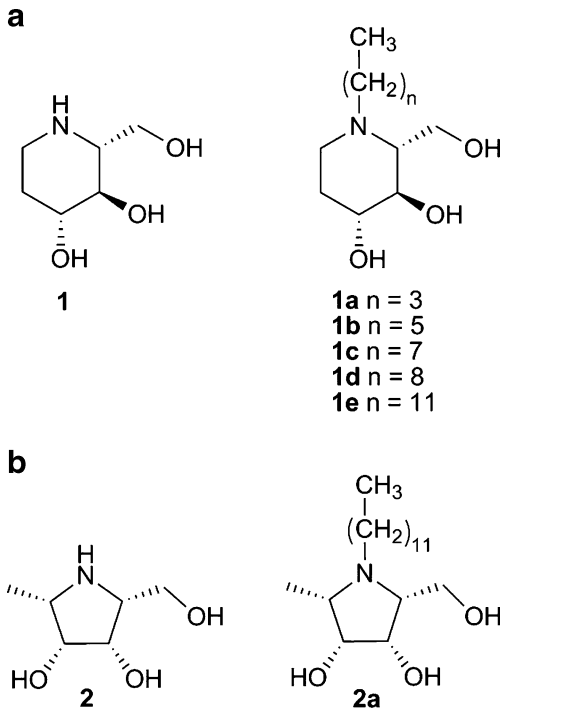


Fig. 1 Structure of the synthesized compounds used in this study. **a** D-fagomine (**1**) and the corresponding *N*-alkylated derivatives (**1a**–**1e**). **b** (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol (**2**) and *N*-(2*R*,3*S*,4*R*,5*S*)-1-dodecyl-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol (**2a**)

Cell lines and cell-treatments

Stomach (GP220 and MKN45), colon (HT-29/M3 and LS174T), pancreas (MDA-Panc28 and Capan-1) and breast (MCF-7 and SK-BR-3) human cancer cell lines were used to test compounds **1, 1a**–**1e** and **2**–**2a**. Cells were seeded at 2–4×10^4 cells/cm^2 and cultured using standard protocols in D-MEM (Invitrogen, Carlsbad, CA) −10% FBS. Cells were routinely checked for *Mycoplasma* contamination (Venor-GeM, Minerva Biolabs, Germany). Cell treatments with the synthesized molecules were performed at aproximately 70% of cell confluence.

Cytotoxic effects of each product were evaluated by incubating the cells with the different compounds at several concentrations (from 2 μM to 5 mM) and times (24 h, 48 h and 72 h). Growth rates were determined by cell viability using the trypan blue exclusion test. Phenotypical alterations of the treated cells were visualized and pictures taken in a LEICA DMIRB microscope using the Leica IM50 image manager software.

All the assays were done by duplicate and at least two different experiments were performed.

Glycosidase enzymatic inhibition assays in cell lysates

Fresh cell lysates (50 μg) were incubated in 96-well plates with or without the sugar analogue in 150 μL of acetic

buffer pH 5 for 1 h at RT with shaking. The substrate (50 μL) was added and incubated at 37°C. The reaction was stopped by increasing the pH by addition of 50 μL of 1 M Tris solution (pH 10). The amount of *p*-nitrophenol formed was determined at 405 nm.

The specific conditions for each enzyme were: glucosidase activity was determined after 3 h incubation using *p*-nitrophenyl α-D-glucopyranoside at 10 mM, 5 mM, 2 mM, 1 mM and 0.5 mM. Mannosidase activity was determined after 3 h incubation with *p*-nitrophenyl α-D-mannopyranoside at 5 mM, 2 mM, 1 mM and 0.5 mM. Fucosidase activity was determined after 3 h incubation using *p*-nitrophenyl α-L-fucopyranoside at 2 mM, 1 mM and 0.5 mM. Galactosidase activity was determined after 1 h incubation with *p*-nitrophenyl β-D-galactopyranoside at 5 mM, 2 mM, 1 mM and 0.5 mM. All the substrates were purchased from Sigma, except *p*-nitrophenyl β-D-galactopyranoside (AnaSpec). Results are shown as the effects induced on the concentration of the specific substrates by the incubation of the cell lysates with or without the iminosugar. All the assays were undertaken at least in duplicate and two independent experiments were done.

Results

D-fagomine derivatives

Cytotoxic effects and phenotypical alterations

To test the effects in cultured cell lines, D-fagomine (**1**), and the corresponding derivatives (**1a–1e**) were added to the

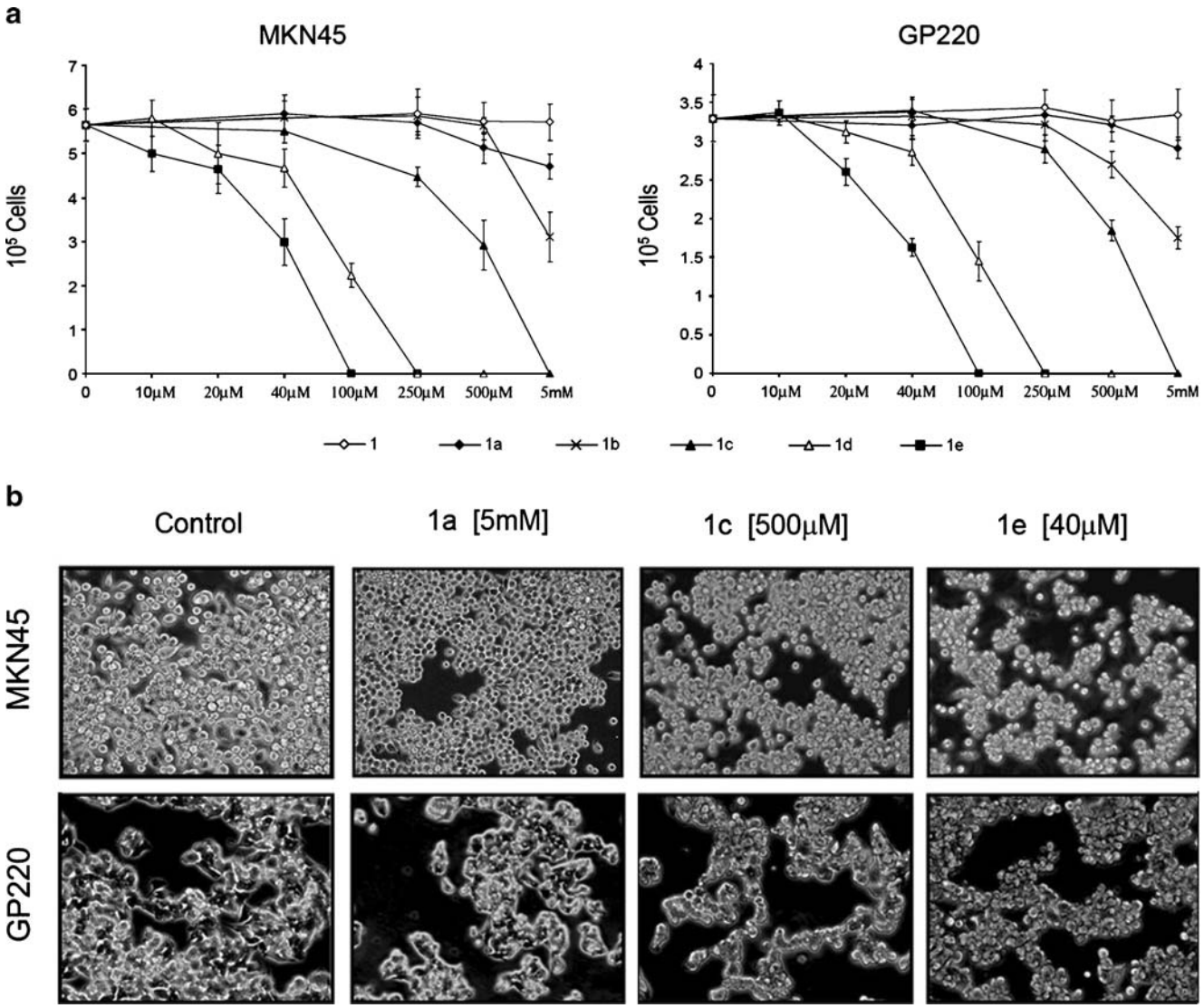


Fig. 2 Effects and phenotypic alterations on MKN45 and GP220 cell lines treated for 24 h with D-fagomine (**1**) and different *N*-alkyl-D-fagomine derivatives (**1a**–**1e**). **a** Cell viability curves. **b** Phenotypical appearance of non-treated and treated cells

media at different concentrations, from 2 μM to 5 mM for 24 h, 48 h, and 72 h to determine the optimal dose and time for each product in two gastric cancer cell lines, GP220 and MKN45. Further experiments were done at concentrations from 10 μM to 5 mM for 24 h. Results indicate that in these cell lines, as the alkyl chain enlarges, the cytotoxic effects were most relevant. *N*-dodecyl-D-fagomine (**1e**) induced the highest cytotoxicity (Fig. 2a), whereas no effect was observed with D-fagomine (**1**) and *N*-butyl-D-fagomine (**1a**) treatments. After treatment with D-fagomine derivatives, phenotypical changes were observed and these alterations were more apparent for compounds with the largest aliphatic chains, being *N*-dodecyl-D-fagomine (**1e**) the compound that induced the most important changes. *N*-dodecyl-D-fagomine-

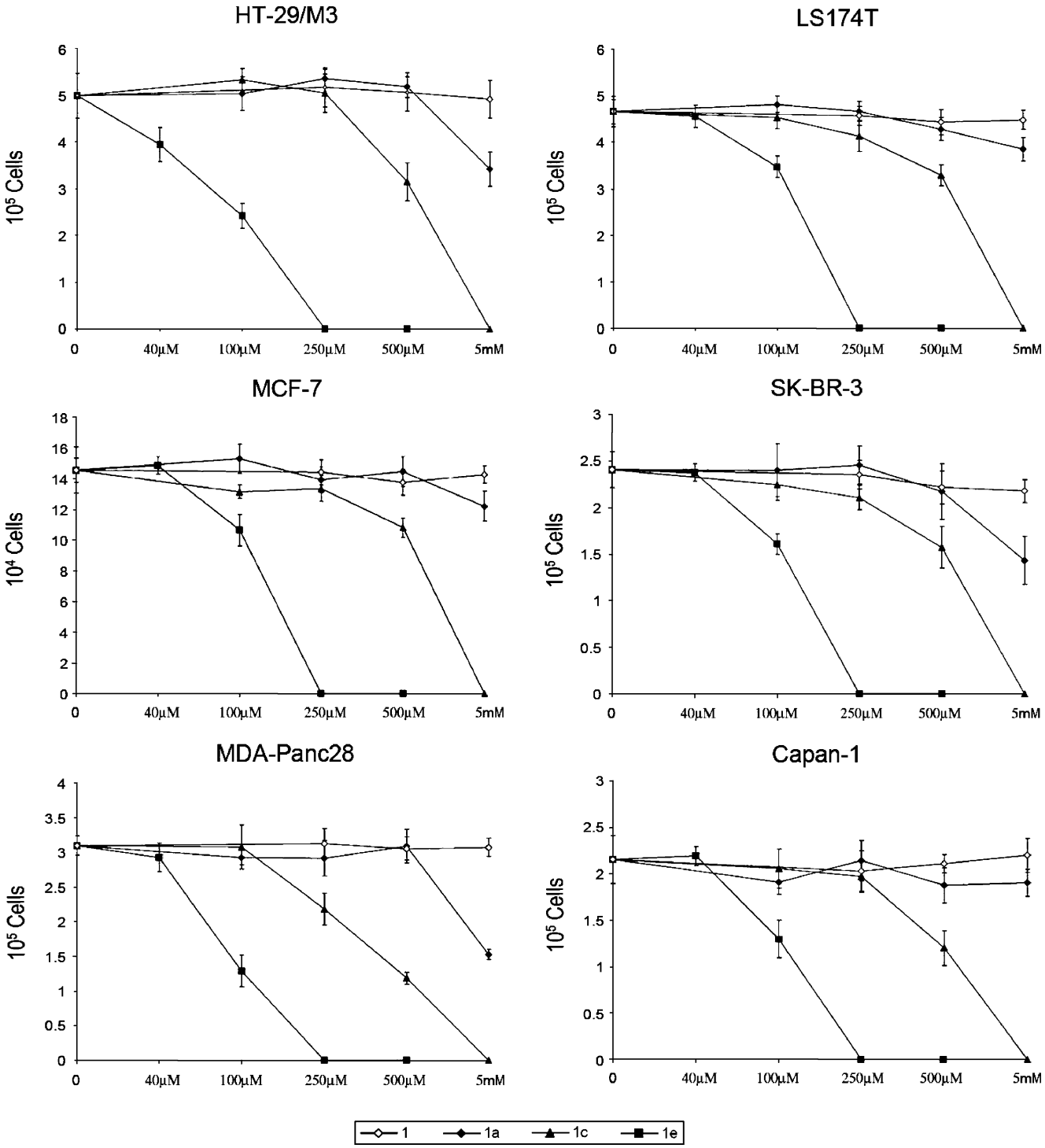


Fig. 3 Cell viability curves of six human cancer cell lines treated with D-fagomine (**1**) and *N*-alkyl-D-fagomine derivatives (**1a, 1c** and **1e**)

treated cells detached from the plastic plates, and under microscopic observation they appeared with rounded shape, and large cytoplasmic vesicles were detected (Fig. 2b). These phenotypic alterations reverted when the compounds were removed and, after 24 h, cells look again as the untreated ones.

Similar results were obtained in other epithelial cancer cell lines of colon (HT-29/M3, LS174T), breast (MCF-7, SK-BR-3) and pancreas (MDA-Panc28, Capan-1) treated with different amounts (from 10 μM to 5 mM) of D-fagomine (**1**), *N*-butyl-D-fagomine (**1a**), *N*-octyl-D-fagomine (**1c**), and *N*-dodecyl-D-fagomine (**1e**). The corresponding viability curves are shown in Fig. 3.

Enzymatic activities after treatment with D-fagomine derivatives

Previous studies from our group [14] showed that D-fagomine (**1**) and the corresponding *N*-alkylated derivatives (**1a**–**1e**) were inhibitors of α-D-glucosidase from rice and β-D-galactosidase from bovine liver, whereas no inhibition was observed against α-D-glucosidase from baker's yeast, β-D-glucosidase from almond, α-D-mannosidase from jack beans, α-L-rhamnosidase from *Penicillium decumbens* and α-L-fucosidase from bovine kidney. Among the *N*-alkylated derivatives, *N*-nonyl- (**1d**) and *N*-dodecyl-D-fagomine (**1e**) were the best inhibitors against α-D-glucosidase, whereas **1e** was a good inhibitor for the β-galactosidase [14].

The capacity of the *N*-alkylated-D-fagomine derivatives (**1a**–**1e**) to inhibit the activity of specific human glycosidases in MKN45 and GP220 cell lysates was analyzed. Cell lysates were first treated with several amounts of *N*-dodecyl-D-fagomine (**1e**) and the activity of different glycosidases (α-D-glucosidase, β-D-galactosidase, α-D-mannosidase, and α-L-fucosidase) was evaluated measuring the levels of the specific substrates. No effects were detected in the concentrations of *p*-nitrophenyl α-L-fucopyranoside, *p*-nitrophenyl α-D-mannopyranoside, and *p*-nitrophenyl β-D-galactopyranoside, as it is shown for MKN45 cell lysates in Fig. 4a. Interestingly, **1e** had an inhibitory effect on the glucosidase activity, and this inhibition is most important at higher doses of *N*-dodecyl-D-fagomine (**1e**), showing 18.39% and 30.63% of inhibition for MKN45 cells and, 11.87% and 43.88% for GP220 cells

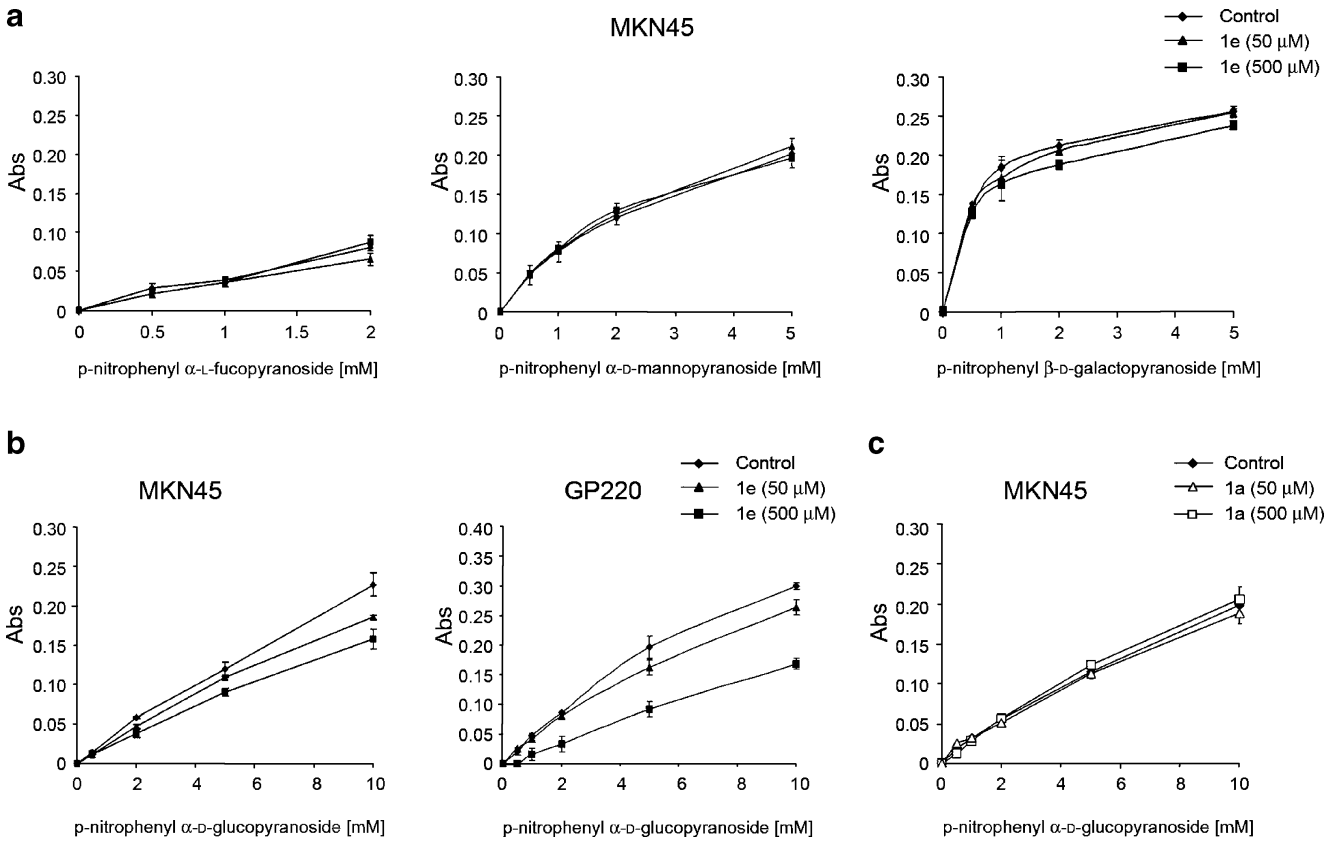
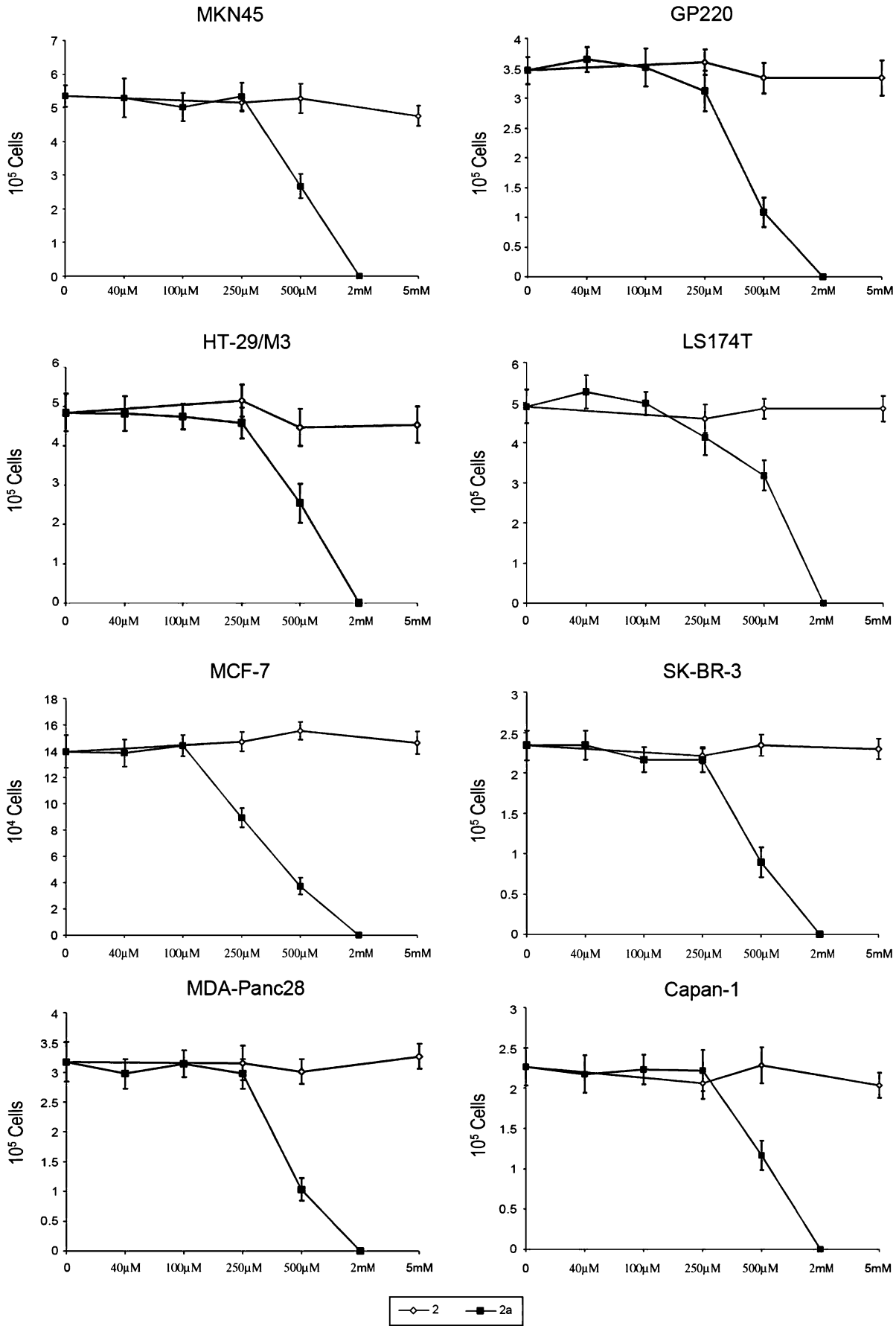


Fig. 4 Glycosidase enzymatic activities in MKN45 and GP220 cell lysates non-treated and treated with D-fagomine derivatives. **a** Effects on *p*-nitrophenyl α-L-fucopyranoside, *p*-nitrophenyl α-D-mannopyranoside, and *p*-nitrophenyl β-D-galactopyranoside concentration on MKN45 cell lysates treated with *N*-dodecyl-D-fagomine (**1e**). **b** *p*-nitrophenyl α-D-glucopyranoside concentration in MKN45 and GP220 cell lysates treated with **1e** compound. **c** *N*-butyl-D-fagomine (**1a**) effects in α-D-glucosidase activity in MKN45 cell lysates


MKN45
GP220
HT-29/M3
LS174T
MCF-7
SK-BR-3
MDA-Panc28
Capan-1
10^5 Cells
10^4 Cells
0
40μM
100μM
250μM
500μM
2mM
5mM
2
2a

◀ **Fig. 5** Cytotoxic effects on eight human cancer cell lines treated for 24 h with several amounts of **2** and the corresponding *N*-dodecyl derivative (**2a**)

at 50 μM and 500 μM, respectively. Representative results in the *p*-nitrophenyl α-D-glucopyranoside concentration in MKN45 and GP220 cell lysates are shown in Fig. 4b.

No inhibitory activity was detected when MKN45 cell lysates were treated with *N*-butyl-D-fagomine (**1a**) (Fig. 4c), thus indicating that the alkyl chain length is of paramount importance for the activity against α-D-glucosidase of these compounds on the cell lines studied.

(2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol (2) and *N*-dodecyl derivative (2a)

Cytotoxic effects and phenotypical alterations

To evaluate if the length of the alkyl chain can be also associated to the effects of other sugar analogues, the fucose analogue iminosugar (**2**) and its corresponding derivative **2a**, bearing a dodecyl aliphatic chain were synthesized. Cell lines were treated with 10 μM, 40 μM, 250 μM, 500 μM, 2 mM and 5 mM of **2**, and **2a** for 24 h. The effects on cell viability were dose dependent for **2a**, showing that at 2 mM induces 100% of cell death (Fig. 5), while compound **2** was not cytotoxic. Moreover, the phenotypical changes of the cells treated with **2a** were similar to those described above for *N*-dodecyl-D-fagomine (**1e**), although higher concentrations were needed (40 μM vs 500 μM, respectively, data not shown).

These results indicated again the importance of the length of the alkyl chain on the cytotoxic effects of the iminosugar derivatives.

Enzymatic activities after treatment with N-(2R,3S,4R,5S)-1-dodecyl-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol (2a)

The previously synthesized product **2** was found to be an inhibitor against α-L-fucosidase from bovine kidney (IC_{50}= 0.009 μM), whereas no effect was detected against α-

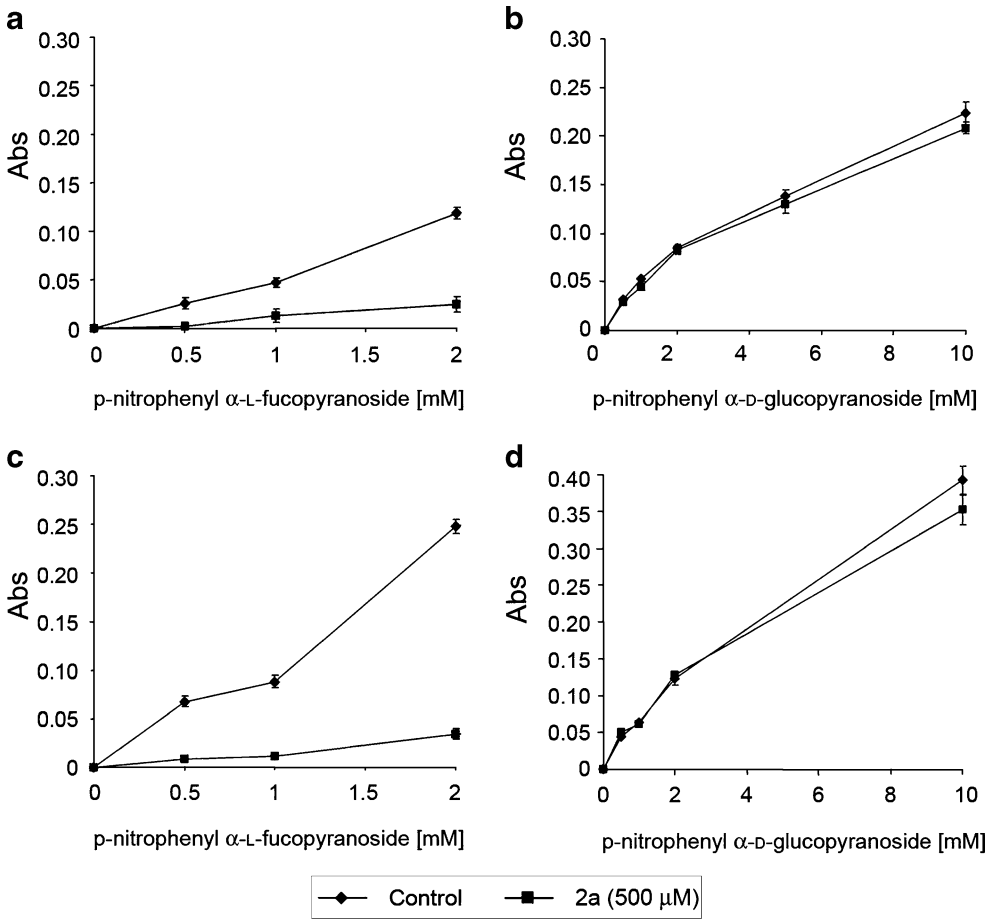


Fig. 6 Effect on *p*-nitrophenyl α-L-fucopyranoside (**a**,**c**) and *p*-nitrophenyl α-D-glucopyranoside (**b**,**d**) concentration in cell lysates treated and non-treated with *N*-(2*R*,3*S*,4*R*,5*S*)-1-dodecyl-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol (**2a**). **a**,**b** MKN45 cells; **c**,**d** GP220 cells

glucosidase from baker yeast, β-glucosidase from almond, and β-galactosidase from *Aspergillus oryzae* [15] (data not shown).

Similar to the experiments with D-fagomine derivatives, compound **2a** was also assayed as inhibitor of specific glycosidases. MKN45 and GP220 cell lysates were treated with 500 μM of **2a** and the activity of α-D-glucosidase and α-L-fucosidase was evaluated. Results indicate that **2a** was a good inhibitor against α-L-fucosidase: 79.2% and 86.0% of inhibition for the MKN45 and GP220 cells, respectively; whereas no inhibitory effect on α-D-glucosidase was found (Fig. 6).

Discussion

Structural modifications, as the addition of different chemical groups at the imino function, have been devised to increase their efficiency in the inhibition of specific glycosidases. Among them, the introduction of an alkyl chain with different number of C atoms to the nitrogen atom of naturally occurring iminosugars and their inhibitory activity effects against glycosidases have been reported [16].

The alkylated iminosugars include the sugar analogue moiety that would recognize the specific glycosidase in the endoplasmic reticulum, and the associated alkyl chain that would increase the interaction with the ER membrane [17]. The more extended interaction with the cell membrane is a result of the increase of hydrophobicity of the compound given by the *N*-alkyl chain. As a consequence, higher cellular association and cytotoxicity have been reported in the *N*-alkylated-derivatives of the natural glucose analogue deoxynojirimycin [18]. Also, the biological effects associated to the increase of the *N*-alkyl chain length in deoxynojirimycin derivatives, augment their cellular retention and therefore higher longevity and organ retention were obtained probably due to the slower release of these products [19]. In addition, the inhibition of the *N*-acetyl β-hexosaminidase, important in osteoarthritis, has been reported to be higher in the compounds with longer *N*-alkyl chain [20].

In our work, we have tested the cytotoxic effects of a panel of *N*-alkylated D-fagomine derivatives and the *N*-dodecyl derivative of (2*R*,3*S*,4*R*,5*S*)-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol in eight human cancer cell lines. Results indicated that the longer alkyl chain induced the higher cytotoxicity and the associated phenotypic alterations, being the derivative with 12 C-atoms chain the most active one. No important differences were found regarding to the monosaccharide analogue to which they are associated, indicating that these effects are only related to the alkyl chain. Moreover, our studies did not detect differences regarding to the different organ origin of the cell lines tested.

We have verified that the length of the *N*-alkyl chain is important in the inhibition of the glycosidase activities when cell lysates were treated with the derivatives tested in this study. α-Glucosidase inhibition was detected when the cell lysates were treated with *N*-dodecyl-D-fagomine, whereas no inhibition was found with the *N*-butyl derivative. In this system, these compounds do not alter the activity of other glycosidases in contrast with our previous data indicating that *N*-dodecyl-D-fagomine was also active against β-galactosidase purified from bovine liver [14].

The *N*-(2*R*,3*S*,4*R*,5*S*)-1-dodecyl-2-(hydroxymethyl)-5-methylpyrrolidine-3,4-diol, a derivative of the fucose analogue, is able to inhibit the α-L-fucosidase activity in cell lysates, whereas no α-glucosidase inhibition was detected. These results correlate with the inhibitory data obtained against α-L-fucosidase from bovine kidney, whereas no effect was observed in the activity of β-D-galactosidase from *Aspergillus oryzae* α-D-glucosidase from bakers yeast and β-D-glucosidase from sweet almond (manuscript in preparation).

In summary, we have analyzed the biological effects of novel iminosugar derivatives and we have corroborated that the length of the *N*-alkylated chain is responsible for the cytotoxicity and the associated phenotypic alterations. In addition, the longer *N*-alkylated chain attached to the iminosugar induced the higher inhibition on glycosidase activities in cell lysates, although the glycosidase specificity for each compound remains unaffected.

Acknowledgments Authors thank Marta Garrido and Pedro Fuentes for excellent technical assistance, and the financial support of Marató de TV3 (050930), Instituto de Investigación Carlos III (PI061421), Spanish MCINN (CTQ2006-01345/BQU and CTQ2006-01080), and Generalitat de Catalunya DURSI (2005-SGR-00698) and ESF project COST (CM0701).

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