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Synthesis and biological evaluation of a new class of glycoconjugated disulfides that exhibit potential anticancer properties

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ABSTRACT

A synthetic strategy, based on the in situ generation of sulfenic acids and their thermolysis in the presence of thiols, was developed for obtaining a collection of polyvalent disulfides in which a benzene scaffold accommodates two or three flexible arms connecting saccharide moieties. Targeting carbohydrate metabolism or carbohydrate-binding proteins may constitute important approaches in the discovery process of new therapeutic anticancer agents. Therefore, a preliminary screening to ascertain the cytostatic/cytotoxic potential of this new class of enantiopure glycoconjugated disulfides has been conducted. Among them, products with two disulfide arms, harbouring galactose rings, induced high levels of apoptosis on U937 histiocytic lymphoma cells, but lower levels of cell death on peripheral blood mononuclear cells from healthy donors. Further experiments indicated that apoptosis induced by these glycoconjugated bis(disulfides) in U937 cells corresponds to the Bcl-2-sensitive, intrinsic form of apoptotic cell death. The bioinvestigation was extended to a panel of human cancer cell lines with different levels of malignancy and resistance to chemotherapeutic agents. Compounds under study proved to induce detectable levels of cell death towards all the tested cancer cell lines.

1. Introduction

The biological role of carbohydrates is extremely important for a variety of cellular processes, including those involved in cell transformation and tumorigenesis. In fact, evidence accumulated in the last decades in a variety of cancer cell systems, as well as in specimens from cancer patients, indicates that modifications in carbohydrate-related structure, function or metabolism are common changes of cancer cells and especially of those showing resistance to traditional chemotherapy.[1] Thus, there is growing interest in the development of novel cancer therapeutics based on glycobiology.[2]

Functionalization, chirality, and structural diversity in carbohydrates allow the induction of several biological activities within a small number of structural variations so that they have been described as 'privileged structures'.[3] The principle of privileged structures matches well the idea of creating a collection of molecules in which the privileged skeleton allows a wide investigation in both medical and biological directions. Glycoconjugates in particular constitute fundamental tools in the discovery process of new therapeutic agents and in the research of information about complex biological processes, such as the recognition events.

In this paper we describe our synthetic strategy that involves transient sulfenic acids as intermediates in the obtainment of glycoconjugated disulfides, and a preliminary screening of the effect of the new compounds on cancer cell viability. In particular, we have synthesized a collection of new sugar molecules whose carbohydrate residues are joined by spacers of different length and flexibility through disulfide bonds, taking into account the pivotal role of apoptosis regulation in cancer progression and therapy,[4] and the potential of galactose-based small molecules as cancer therapeutics. In a previous work we have reported indeed a three-step synthesis of bis-β-D-glucopyranosides containing thioalkane and thioarene spacers, and two compounds of this family are endowed with a specific cytotoxic potential attributable to induction of cell death by apoptosis.[5]

Starting from these biological evidences, the control of molecular architecture and saccharide spacing playing a significant role on the enhancement of the apoptotic activity prompted us to introduce into the molecular skeleton of compounds—such as **1** and **2** (Fig. 1)—the disulfide bond, which is present in many biological systems and is hydrolysis-resistant. Moreover, glycosyldisulfides constitute a new class of carbohydrate derivatives with promising biological properties.[6] We have generated the disulfide bond by condensation of suitable sulfenic acids with thiols, a reaction that

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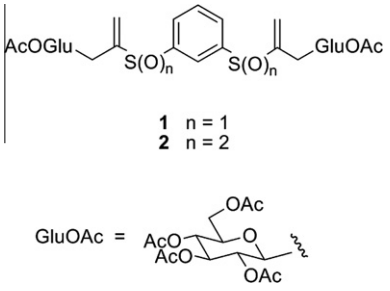


Figure 1. Glycoconjugated sulfoxide and sulfone.

is well known in the literature[7] but applied by us for the first time in the synthesis of bis(disulfides) bearing bioresidues.[8] While symmetric glycosyl disulfides are easily accessed following various straightforward procedures,[6] the synthesis of molecules in which each sulfur atom of the disulfide bond is linked to a different residue occurs through less efficient and direct approaches.[8] The collection of molecules that we have obtained following the synthetic procedure reported in this work may provide a basis for the development of a new series of pharmacologically active carbohydrate-based molecules.

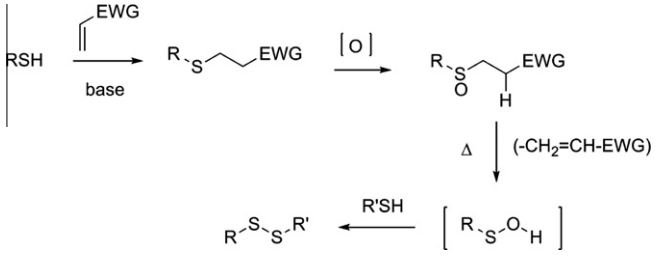
2. Results and discussion

2.1. Chemistry

In accordance with our research interests devoted to the use of sulfenic acids as intermediates in organic synthesis, we have developed the methodology for obtaining unsymmetrical disulfides by sulfenic acid/thiol condensation.[8] This synthetic strategy is based on the preparation of a suitable precursor of the desired sulfenic acid and its generation in situ by thermolysis in the presence of a thiol. The general procedure is depicted in Scheme 1 where thiols represent in turn the starting material for the sulfoxide precursor of the sulfenic acid and the condensation partner of the aforesaid sulfenic acid.

A sulfoxide possessing at least a hydrogen atom and an electron-withdrawing group β-situated with respect to the sulfur function acts as a good precursor of the corresponding sulfenic acid. The advantages of (i) easy modulability of this methodology and in particular (ii) possibility of choosing the suitable sulfenic acid precursor allow an efficient entry to sugar connecting disulfides in a concise manner. Following this procedure we have accomplished the synthesis of the collection of glycoconjugated bis and tris(disulfides), some of which protected at OH groups, shown in Figure 2. In each of them the benzene platform accommodates two or three flexible disulfide arms connecting the saccharide moieties, in analogy to the structural skeleton of compounds **1** and **2**.

The synthesis of disulfides **3**, **10**, **11**, **13**, and **15** has been already described.[8,9] Bis(disulfides) **4**–**6** and **8** have been obtained starting



Scheme 1. General synthetic procedure for unsymmetrical disulfides via sulfenic acids.

from the commercially available benzenedimethanethiols **16** and **17** that were reacted with methyl acrylate in the presence of a catalytic amount of benzyltrimethylamonium hydroxide (TRITON B), to give sulfoxides **20** and **21** after the controlled oxidation of the corresponding sulfides **18** and **19** (Scheme 2).

β-(Methoxycarbonyl)ethanesulfoxides, such as **20** and **21**, have been used in the synthesis of all compounds **3**–**9** (Fig. 2) since the 2-(methoxycarbonyl)ethyl residue, involved in the β-*syn*-elimination, allows the thermolysis to occur at the reflux temperature of 1,2-dichloroethane (DCE, 83 °C). Our β-(methoxycarbonyl)ethane sulfoxides,[9] described in the Supplementary data, can be kept on the bench without showing any trace of decomposition and can be easily purified by column chromatography.

In Scheme 3 the thermolysis of sulfoxides **20** and **21** is depicted. Sulfenic acids **22** and **23** were generated in situ, respectively, and reacted with 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-gluco- or -galactopyranose, leading to bis(disulfides) **4**, **6**, and **8**. Due to the high purity of precursors, the sulfenic acid/thiol condensation occurs in good yields in the presence of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose.

When the thermosensitive 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranose was used as sulfenic acid partner we observed a decrease in the yield of the corresponding bis and tris(disulfides) **5**, **8** and **15**[9] (Fig. 2 and Scheme 3) due to a minor availability of the easily decomposable aforesaid galactothiol to react with the transient sulfenic functions.

The choice of the starting thiol in the preparation of the sulfenic acid precursor is crucial in the efficiency of the total process. For instance, compound **3** can be prepared either by using 1,3-benzenedithiol (**24**) as condensation partner of the sulfenic acid **25**[8] or by involving thiol **27** in the condensation with sulfenic acid **26** generated in situ in three steps from dithiol **24** (Scheme 4) through 1,3-di-[(2-methoxycarbonylethyl)thio]benzene. This last way is described in the Supplementary data and leads to the acetylated glucoconjugate bis(disulfide) **3** in 85% yield against the 50% previously reported.[8]

The difference in yields between the two alternative routes of the same synthetic approach is mainly due to the difference in the sulfoxides used for generating sulfenic acids **25** and **26**.

The two sulfoxides, one of which is similar in structure to the sulfoxides shown in Scheme 3 and the other has been already described,[8] have two different alkane sulfinyl moieties involved in the β-elimination process. Sulfoxide precursor of sulfenic acid **25**, allows the generation of the transient species at mild temperature (40 °C), but undergoes readily decomposition and is difficult to handle. The more stable and well purified sulfoxide precursor of sulfenic acid **26** thermolyzed at higher temperature (83 °C) but, in this case, raised its reactivity as generator of the corresponding transient species, leading to a better total yield in the condensation reaction.

Compounds **7**, **9**, **12**, and **14** have been obtained in almost quantitative yield after deacetylation of the sugar residues in compounds **6**, **8**, **11,** and **13** respectively that was performed with aqueous ammonia (30%).[10] They were purified by flash chromatography ($CHCl_3$/MeOH 9:1).

Bis- and tris(disulfides) **3–15** have been all obtained in enantiopure form, fully characterized and subjected to biological studies described in the following section.

2.2. Biological data

In a first round of experiments we performed a preliminary screening to ascertain the cytostatic/cytotoxic potential of enantiopure bis and tris(disulfides) **3–15,** using the U937 histiocytic lymphoma cell line, that is, a tumor cell line showing moderate level of malignancy, as an experimental model. Disulfides **3–6**, **8**, **10**, **11**,

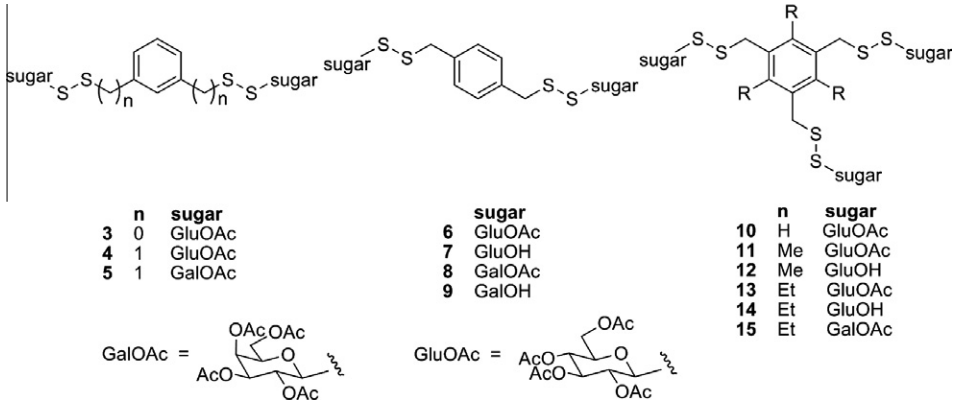
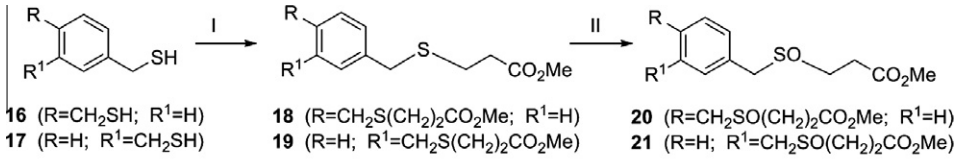
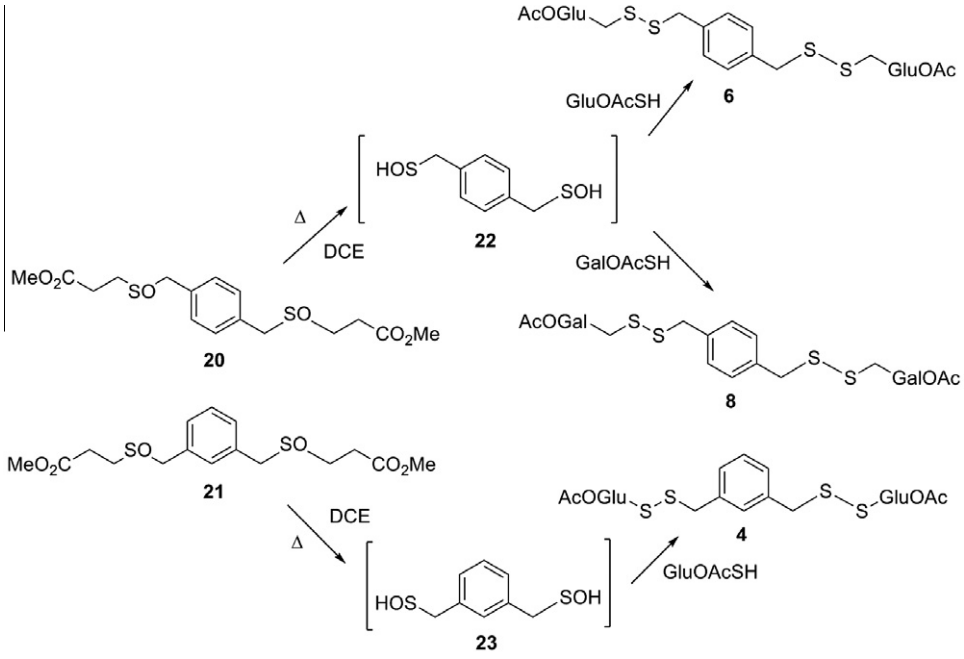


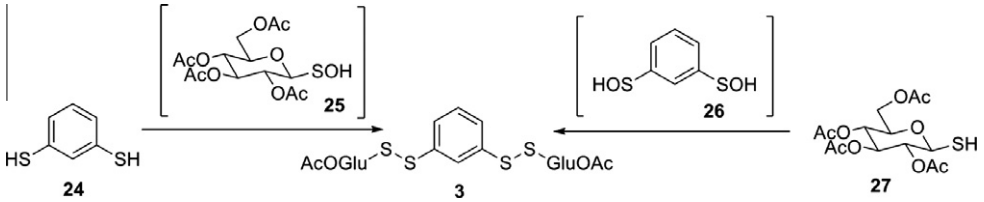
Figure 2. Glycoconjugated disulfides under biological evaluation.



Scheme 2. The synthesis of sulfoxides **20** and **21**.



Scheme 3. The generation in situ of sulfenic acids **22** and **23** and their reaction with suitable thiosugars.



Scheme 4. Two alternative applications of the same synthetic strategy towards disulfide **3.**

13, and **15** were dissolved in DMSO at the concentration of 20 mM, glycoconjugated disulfides **7, 9, 12**, and **14** were dissolved at the same concentration in RPMI-1640 medium. These mother solutions were further diluted in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin to reach a set of 1 in 10 serial dilutions ranging from 100 μM to 1 μM. The obtained samples were added to 5×10^4 U937 cells in 100 μL total volume and incubated at 37 °C in a CO_2 incubator. The same cells were exposed to vehicles alone, as a control experiment, in equal amounts to those employed for dissolving compounds **3–15**. After 24 h incubation, the absolute number of total cells and the percentage number of cells showing plasma membrane permeability were evaluated by microscopy analysis and trypan blue exclusion test, while percentage of apoptotic cells was evaluated by fluorescence microscopy analysis of cells stained with acridine orange.

Results shown in Figure 3 refer to the assayed tris(disulfides) **10–15**, while those shown in Figure 4 refer to glycoconjugated bis(disulfides) **3–9**. Data concerning 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranose (thiogal)[11] are also reported in Figure 2 for comparison. Mean values ± standard deviations are reported.

As shown in Figure 3A, tris(disulfides) **10** and **15** remarkably reduced the absolute number of cells, in a dose-dependent fashion, in comparison with vehicle treated cells. However, this reduction was not associated with a consistent induction of cell death by part of the same compounds, as shown in Figures 3B and C, suggesting a cytostatic rather than a cytotoxic activity. Tris(disulfides) **11**–**14** exerted the higher cytotoxic activity among this group of molecules, as shown in Figure 3B. Nevertheless, in neither case mean cell death values were beyond 25% nor remarkable levels of apoptosis were detected for any of the tris(disulfides) **10–15**.

Results shown in Figure 4 demonstrate that some of the bis(disulfides) **3–9** exerted an evident cytotoxic effect on U937 cells. In particular compounds **5** and **8**, that is, those harbouring galactose rings, induced very high levels of apoptosis at the higher concentration assayed (Fig. 4C; Supplementary data). After treatment with the same two compounds, dead cell levels, detected by trypan blue assay, were consistently lower than those of cells showing apoptotic morphology (Fig. 4B). This suggests that membrane permeability induced at 24 h by bis(disulfides) **5** and **8** was a late event of apoptosis that should be considered the only form of cell death induced by these compounds. Conversely, levels of trypan blue positive cells induced by bis(disulfides) **4** and **6** were higher than that of apoptotic cells detected for the same compounds. This suggests that forms of cell death other than apoptosis could contribute to the appreciable cytotoxicity exerted by compounds **4** and **6**.

In addition, bis(disulfides) **4** and **5** exerted the higher cytostatic effect, as assessed by reduction of total cell number (Fig. 4A). No remarkable difference was observed between apoptosis values detected in samples treated with thiogal[11] and vehicle treated samples, this suggesting that apoptosis induced by bis(disulfides) **5** and **8** is a consequence of their whole molecular structures rather than the effect of the single saccharide moieties.

The high, pro-apoptotic effect of compound **5** on U937 cells was then compared with that exerted on non-malignant cells, using peripheral blood mononuclear cells (PBMCs) from healthy donors and flow cytometry analysis following propidium iodide staining to detect hypodiploid, apoptotic nuclei. The results obtained by this last technique confirmed the capability of compound **5** to induce high levels of apoptosis on U937 cells, in a dose-dependent fashion, after 24 h incubation (Fig. 5). In contrast, levels of apoptosis detected on PBMCs after 24 h incubation with compound **5** were very modest (data not shown). Hypodiploid nuclei from these cells were appreciable only after 48 h incubation at the higher concentrations (Fig. 5) and even in this case they were significantly lower than those observed in U937 cells.

In a second round of experiments, we investigated the expression impact of the anti-apoptotic Bcl-2 protein on apoptosis induced by bis(disulfide) **8**, in order to further characterize the form of cell death induced by galactose-bearing glycoconjugates, such as **5** and **8**. We compared apoptosis levels following 24 h treatment with compound **8** in U937 stable transfectants over-expressing a functional active murine *bcl-2* gene (U937mBCL2) and in control cells transfected with an empty vector (U937pMEP).

Results shown in Figure 6 demonstrate that over-expression of Bcl-2 highly, even if not fully at the highest concentration assayed, protected U937mBCL2 cells from apoptosis induced by bis(disulfide) **8**. Conversely, U937pMEP cells were highly sensitive, as expected, to apoptosis induced by **8**. These experiments confirmed that galactose-bearing glycoconjugated bis(disulfides), such as **8**, have the ability to specifically induce cell death by apoptosis and indicated that apoptosis induced in U937 cells by this new class of compounds corresponds to the classical, Bcl-2-sensitive, mithocondrial-dependent, intrinsic form of apoptotic cell death.

We then focused our attention on compounds **5** and **8**, which showed the higher pro-apoptotic potential in U937 cells, and extended our investigation to a panel of human cancer cell lines with different characteristics. To this purpose, the following cell lines were utilized: THP-1 (established from the peripheral blood of a patient with acute monocytic leukaemia), MOLT-3 (established from the peripheral blood of a patient with acute lymphoblastic leukaemia), HeLa (established from cancer cells of a patient with cervical carcinoma), HT-29 (adenocarcinoma cells established from a patient with colorectal carcinoma), MCF7 (adenocarcinoma cells established from a patient with breast cancer) and HepG2 (hepatocyte carcinoma cells established from a patient with hepatocellular carcinoma). We gathered the cell lines into a first group of cells showing moderate malignancy/resistance to chemotherapy (THP-1, MOLT-3, and HeLa cells) and a second group with higher degree of malignancy/resistance (HT-29, HepG2, and MCF7 cells). For the first group of cancer cell lines, cytotoxicity was assessed as reported for U937 cells and results obtained with bis(disulfides) **5** and **8** were also compared with those obtained using glycoconjugated bis(disulfides) **7**, **9**, and thiogal[11] in some of the cell lines. For the second group of cell lines, all growing as adherent cells, cell death was detected by trypan blue exclusion test and a MTS assay performed directly in the culture wells after 48 h of treatment, to avoid possible bias in detection of death due to detachment procedures.

As shown in Table 1, cell lines of the first group exhibited variable sensitivity to the assayed glycoconjugates, and compounds **5** and **8** proved to specifically act as pro-apoptotic agents towards all tested cancer cell lines. In particular, MOLT-3 lymphoma cells were highly susceptible to apoptosis induced by these molecules. Furthermore cell number and viability were evidently reduced by bis(disulfides) **5** and **8** even in the second group of cancer cell lines, that is, in those generally considered as more malignant and resistant to chemotherapeutic agents (Table 2).

The cytotoxicity assays, adopted for HT-29, HepG2 and MCF7 cell lines, that is, a panel of cancer cells corresponding to malignancies of high worldwide incidence, do not allow us to assess the specific form of cell death induced by compounds **5** and **8** in these cells. However apoptosis remains a possible candidate on the basis of the results obtained with other cell lines.

3. Conclusions

We have synthesized, by an easy three-step procedure, a collection of molecules in which a benzene platform accommodates two or three flexible disulfide arms connecting saccharide moieties. This synthetic route allows: (i) the diversification of the carbohydrate residues within the organic scaffold; (ii) the introduction of

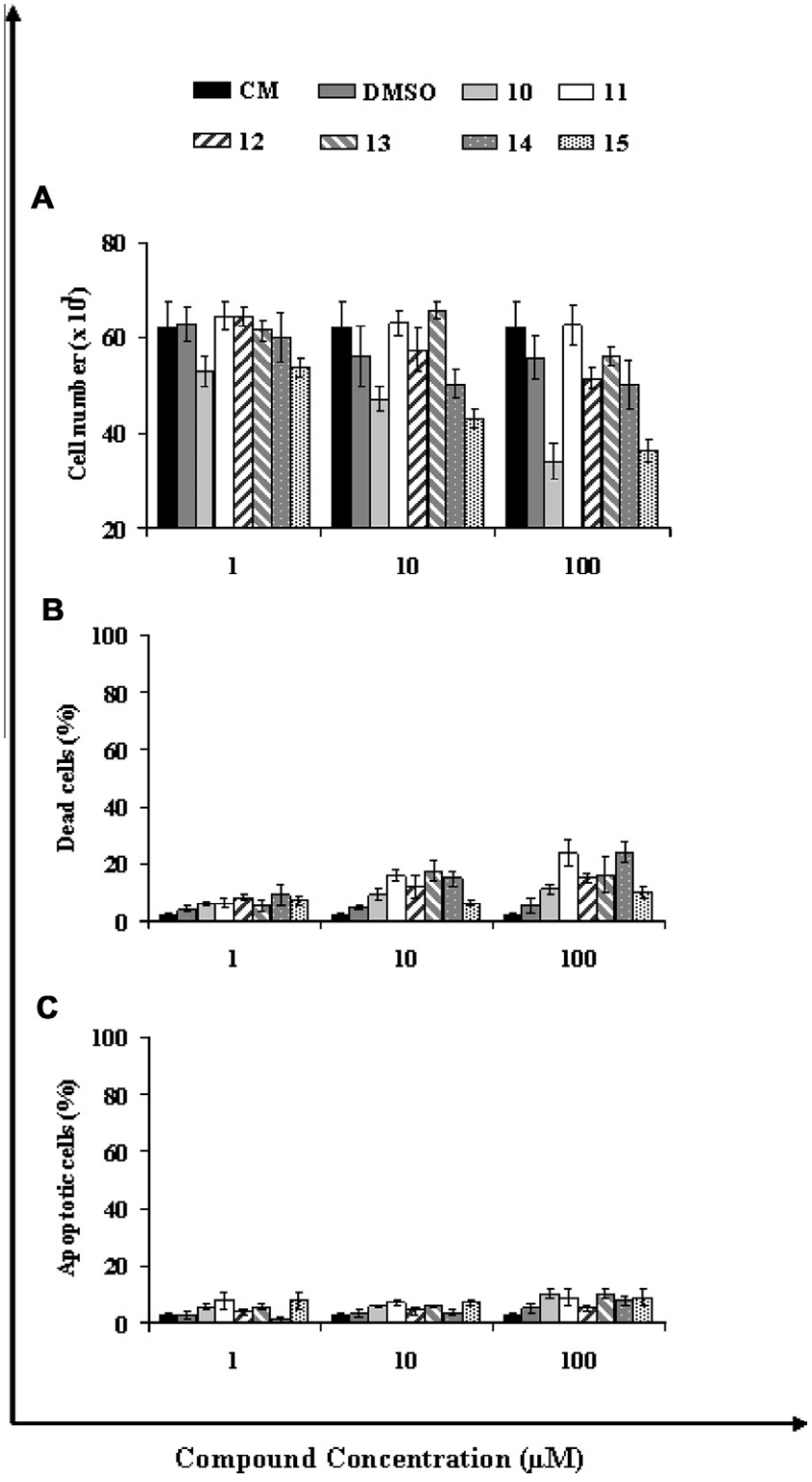


Figure 3. Effects of tris(disulfides) **10**–**15** on cytotoxicity in U937 cells after 24 h incubation. Each data point represents the mean and standard deviation from at least three different determinations. (A) Absolute number of total cells/well assessed by microscopy analysis using a haemocytometer. Comparisons of the means between treated samples and corresponding controls, by Hochberg's GT2 post-hoc multiple comparison One-Way ANOVA test: **10** l00 μM, p <0.001; **14** 10 μM and 100 μM, p = 0.050; **15** 10 μM, p = 0.007; **15** 100 μM, p <0.001; all other comparisons, not significant (NS). (B) Percentage of cells showing membrane permeability by trypan blue exclusion test. Comparisons of the means between treated samples and corresponding controls, by Hochberg's GT2 post-hoc multiple comparison One-Way ANOVA test: **11** l0 μM and l00 μM, p <0.001; **12** 10 μM, p = 0.006; **12** 100 μM, p <0.001; **13** l0 μM and l00 μM, p <0.001; **14** 10 μM and l00 μM, p <0.001; all other comparisons, NS. (C) Percentage nuclei showing apoptotic features at microscopy analysis following staining with acridine orange. Comparisons of the means between treated samples and corresponding controls, by Hochberg's GT2 post-hoc multiple comparison One-Way ANOVA test: **10** l00 μM, p = 0.012; **13** 100 μM, p = 0.012; **14** 100 μM, p = 0.028; other comparisons, NS.

the disulfide bond as biologically significant functional group; (iii) the modulability of the overall process through the use of suitable and easily accessible thiols (Scheme 3). Bis(disulfides), with a thread-like core and galactosyl groups at both ends of the molecule,

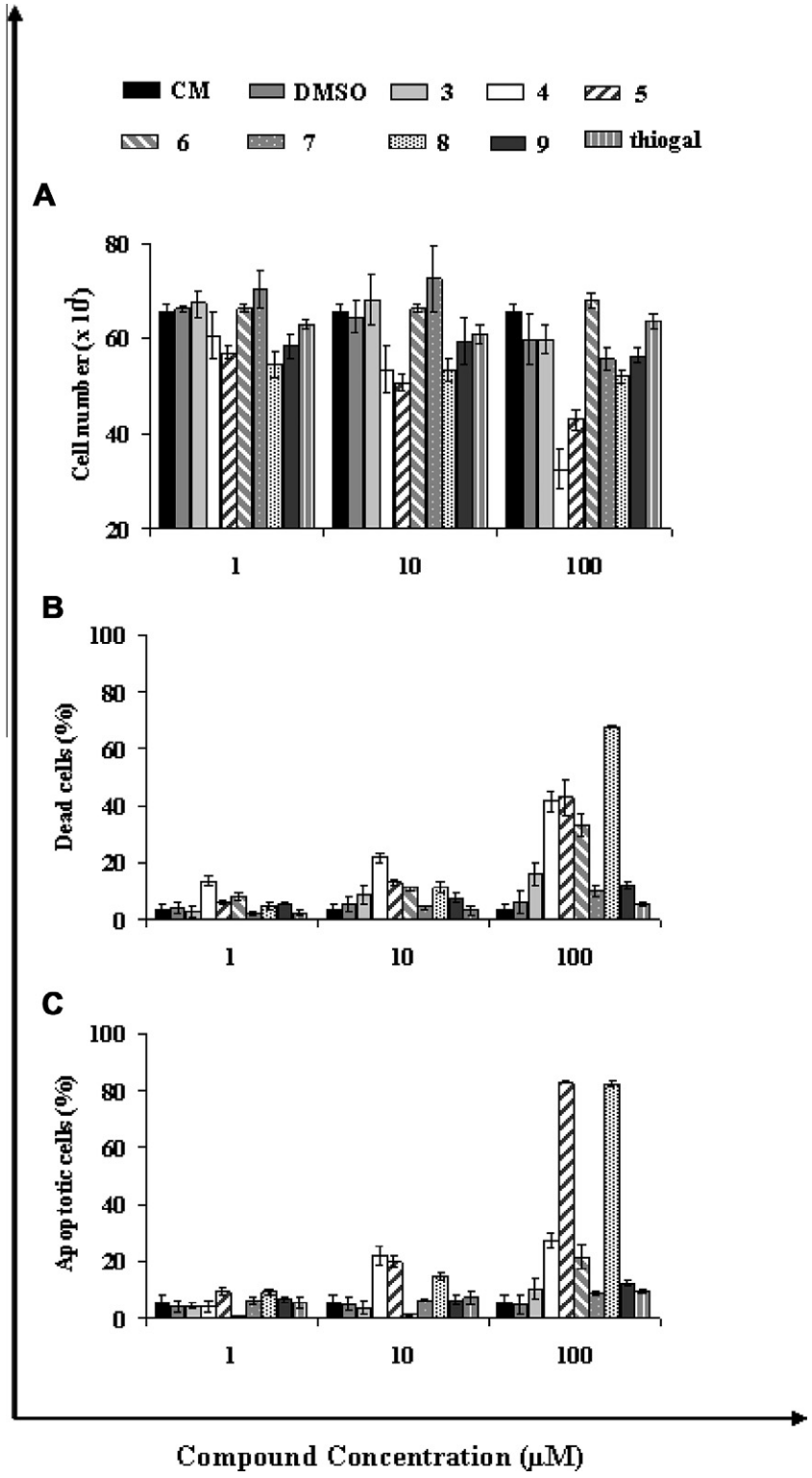


Figure 4. Effects of bis(disulfides) **3**–**9** and thiogal on cytotoxicity in U937 cells after 24 h incubation. Each data point represents the mean and standard deviation from at least three different determinations. (A) Absolute number of total cells/well assessed by microscopy analysis using a haemocytometer. Comparisons of the means between treated samples and corresponding controls, by Hochberg's GT2 post-hoc multiple comparison One-Way ANOVA test: **4** l0 μM, $p = 0.005$; **4** l00 μM, $p < 0.001$; **5** 10 μM and 100 μM, $p < 0.001$; **7** 100 μM, $p = 0.015$; **8** 1 μM, $p = 0.002$; **8** 10 μM, $p = 0.003$; all other comparisons, NS. (B) Percentage of cells showing membrane permeability by trypan blue exclusion test. Comparisons of the means between treated samples and corresponding controls, by Hochberg's GT2 post-hoc multiple comparison One-Way ANOVA test: **3** l00 μM, $p < 0.001$; **4** 1 μM, 10 μM and 100 μM, $p < 0.001$; **5** 10 μM, $p = 0.019$; **5** 100 μM, $p < 0.001$; **6** l00 μM, $p < 0.001$; **8** l00 μM, $p < 0.001$; all other comparisons, NS. (C) Percentage nuclei showing apoptotic features at microscopy analysis following staining with acridine orange. Comparisons of the means between treated samples and corresponding controls, by Hochberg's GT2 post-hoc multiple comparison One-Way ANOVA test: **4** l0 μM and l00 μM, $p < 0.001$; **5** 10 μM, $p = 0.01$; **5** 100 μM, $p < 0.001$; **6** 100 μM, $p < 0.001$; **8** l0 μM and 100 μM, $p < 0.001$; **9** 100 μM, $p = 0.043$; other comparisons, NS.

have proven to be cytotoxic, even if at relatively high concentrations, towards a panel of human cancer cells with different levels of malignancy and resistance to chemotherapeutic agents: importantly, also cell lines rather resistant to cytotoxic agent showed a

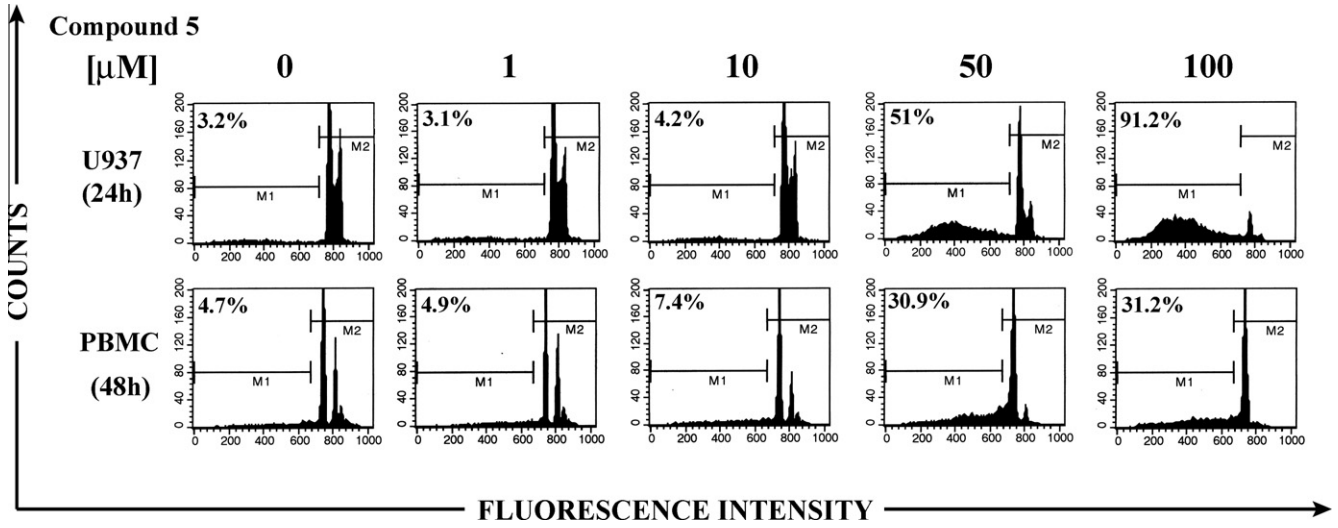


Figure 5. Effect of compound **5** on apoptosis in U937 cells and in peripheral blood mononuclear cells (PBMC). U937 cells and PBMC were treated with 0, 1, 10, 50 and 100 μM of compound **5** for 24 and 48 h, respectively, and apoptosis was evaluated by hypodiploid nuclei analysis after DNA staining with propidium iodide. Percentages of hypodiploid nuclei (M1) are reported in the cytograms. One experiment out of two performed with similar results, is shown.

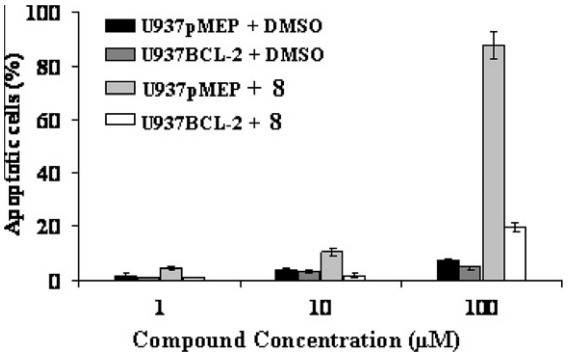


Figure 6. Effects of bis(disulfide) **8** on apoptosis in U937 transfectants overexpressing Bcl-2 (U937mBCL2) and in U937 control transfectants (U937pMEP), after 24 h incubation. Each data point represents the mean and standard deviation from three different determinations of percentage nuclei showing apoptotic features at microscopy analysis following staining with acridine orange. Comparisons of the means by Hochberg's GT2 post-hoc multiple comparison One-Way ANOVA test: l0 μM, U937pMEP+**8** versus U937pMEP+DMSO $p = 0.005$, U937mBCL2+DMSO versus U937pMEP+**8** $p < 0.001$, U937mBCL2+**8** versus U937pMEP+**8** $p < 0.001$; l00 μM, U937pMEP+**8** versus U937pMEP+DMSO $p < 0.001$, U937mBCL2+**8** versus U937pMEP+DMSO $p < 0.001$, U937pMEP+**8** versus U937mBCL2+DMSO $p < 0.001$, U937mBCL2+**8** versus U937mBCL2+DMSO $p < 0.001$, U937mBCL2+**8** versus U937pMEP+**8** $p < 0.001$; other comparisons, NS.

certain sensitivity to cell death induced by these compounds. Moreover, experiments using Bcl-2-overexpressing transfectants fully confirmed the apoptotic feature of death induced by the newly synthesized **5** and **8**. These experiments also showed that high expression of the Bcl-2 anti-apoptotic protein, which occurs in a wide variety of human cancers,[12] did not completely protect from apoptosis induced by the same bis(disulfides) **5** and **8**, giving an additional therapeutic value to our results. We cannot discern at the moment the mechanisms involved in induction of apoptosis by the galactose derivatives, and whether the action of these pro-apoptotic compounds is exerted at external membrane level or in the inner compartments of the cancer cells. However, we can notice that: (i) 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose did not induce apoptosis even at high concentrations, (ii) gluco- and galactoconjugates under study exert different effects, (iii) deprotected compound **9** did not exert the same pro-apoptotic effect exerted by the corresponding protected compound (Fig. 4C and Supplementary data). This allows us to hypothesize a highly specific effect related, but not simply attributed, to the galactosyl residues. One possible hypothesis is that our new galactoconjugates target cellular components that play fundamental roles in normal galactose metabolism, thus causing an irreversible cell damage. An attractive mechanism is that galactoconjugates could intracellularly bind to galectin-3, thus inhibiting the capacity of this protein to act as an anti-apoptotic, Bcl-2 mimetic.[13] Results obtained using the deprotected compound **9** seem to not support this hypothesis unless the binding to galectin-3 could prescind from classical hydrogen bonding interactions.

Further investigation is needed to clarify the relationships between apoptotic effects of the assayed compounds on cancer cells and related events. However, our data indicate that the new group of galactoconjugates bis(disulfides) specifically cause cell death ascribed to apoptosis on cancer cells of different origin. This aspect encourages us to search for improving the specific cytotoxic activity of this new family of compounds towards cancer cells. Results of these studies will elucidate the possibility of the therapeutic application of galactoconjugates bis(disulfides).

4. Experimental section

4.1. General methods for the synthetic procedures

Solvents were purified according to standard procedures. Light petroleum (petrol) used refers to the fraction boiling at 40–60 °C. All reactions were monitored by TLC on commercially available precoated plates (Aldrich Silica Gel 60 F254) eluted with acetone/$CHCl_3$, $CHCl_3$/MeOH, or EtOAc/MeOH, and the products were visualized with vanillin [1 g dissolved in MeOH (60 mL) and concd H_2SO_4 (0.6 mL)]. Silica gel used for column chromatography was Aldrich 60. ^1H and ^{13}C NMR spectra were recorded with a Varian Mercury 300 spectrometer at 300 and 75 MHz, respectively, in $CDCl_3$ or CD_3OD solutions; the assignments are supported by Attached Proton Test (APT) and homodecoupling experiments; proton and carbon nuclei identified by apex pertain to glucose or galactose residues in the bis(disulfides) **4**–**9** and tris(disulfides) **12** and **14**. The remaining glycoconjugated disulfides **3**, **10**, **11**, **13**, and **15** have been already described.[8,9] The purities of all tested compounds are higher than 95% by $^1H/^{13}C$ NMR spectroscopy and elemental analyses, which were reported to be within 0.4% of calculated values.

Table 1
Effects of bis(disulfides) **5**, **8**, **9**, and 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranose (thiogal) on cytotoxicity, as assessed by trypan blue exclusion test and detection of apoptotic cells, in MOLT-3, THP-1 and HeLa cancer cell lines

Cell lines	Compounds	IC_{50}[a] (μM)	CC_{30}[b] (μM)	AC_{30}[c] (μM)
MOLT-3	**5**	>100	54 (0.98)[d]	15 (0.73)
	8	>100	29 (0.98)	24 (0.97)
	9	>100	>100	>100
	Thiogal	>100	>100	>100
THP-1	**5**	>100	>100	92 (0.84)
	7	>100	>100	>100
	8	>100	93 (0.99)	88 (0.95)
	9	>100	>100	>100
	Thiogal	>100	>100	>100
HeLa	8	>100	>100	62 (0.98)

[a] Drug concentration able to cause inhibition of the total cell count by 50% (IC_{50}), after 24 h incubation.
[b] Drug concentration able to cause 30% cytotoxicity by trypan blue assay (CC_{30}), after 24 h incubation.
[c] Drug concentration able to cause 30% apoptotic cells by fluorescence microscopy analysis of acridine orange stained cells (AC_{30}), after 24 h incubation.
[d] Pearson's correlation coefficient shown in brackets.

Table 2
Effects of bis(disulfides) **5** and **8** on cytotoxicity, as assessed by trypan blue exclusion test and MTS assay, in HT-29, HepG2 and MCF7 cancer cell lines

Cell lines	Compounds	IC_{50}[a] (μM)	CC_{30}[b] (μM)	$MAIC_{30}$[c] (μM)
HT-29	**5**	60 (0.80) [d]	>100	12 (0.85)
	8	63 (0.80)	61 (0.80)	23 (0.90)
HepG2	**5**	54 (0.89)	77 (0.90)	41 (0.92)
	8	51 (0.90)	98 (0.90)	30 (0.92)
MCF7	5	42 (0.80)	>100	61 (0.99)
	8	38 (0.80)	69 (0.80)	63 (0.99)

[a] Drug concentration able to cause inhibition of the total cell count by 50% (IC_{50}), after 48 h incubation.
[b] Drug concentration able to cause 30% cytotoxicity by trypan blue assay (CC_{30}), after 48 h incubation.
[c] Drug concentration able to cause reduction of the formazan product formation (MTS assay) by 30% ($MAIC_{30}$), after 48 h incubation.
[d] Pearson's correlation coefficient shown in brackets.

4.2. Standard procedure for the preparation of bis(disulfides) 4–6, and 8

A solution of 1 mmol of bis(sulfoxide) **20** or **21** and 4 mmol of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-gluco- or -galactopyranose (2 mmol for each sulfinyl function) in 20 mL of 1,2-dichloroethane was maintained under stirring, at reflux temperature (83 °C). The reaction was monitored via TLC and ^{1}H NMR. All the obtained disulfides **4–6** and **8** were purified by flash chromatography. The excesses of glycosyl thiols were always recovered from the column, together with 5% maximum of octaacetyldiglycosyl disulfides.

4.2.1. 1,3-Di-{[(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)dithio] methyl}benzene 4

Yield 60%. TLC: R_f 0.72 (eluant EtOAc/MeOH 95:5). Mp 178–180 °C. ^{1}H NMR ($CDCl_3$): δ 7.2 (m, 4H, ArH), 5.29 (t, J_{vic} = 9.4 Hz, 2H) and 5.09 (t, J_{vic} = 9.6 Hz, 2H) (2 × H-2′,4′), 5.20 (t, J_{vic} = 9.0 Hz, 2H, 2 × H-3′), 4.49 (d, $J_{1',2'}$ = 10.0 Hz, 2H, 2 × H-1′), 4.23 and 4.15 (split AB system, $J_{5',6'A}$ = 4.4, $J_{5',6'B}$ = 2.4, $J_{6'A,6'B}$ = 12.4 Hz, 4H, 2 × H_2-6′), 3.96 (s, 4H, 2 × ArCH_2), 3.72 (ddd, $J_{4',5'}$ = 9.7 Hz, 2H, 2 × H-5′), 2.03, 1.99, 1.98, and 1.96 (four s, 24H, 8 × CH_3). ^{13}C NMR ($CDCl_3$): δ 170.3, 170.0, 169.2, and 168.9 8 × CO, 136.9 C-1,3, 130.1 (C-2), 128.6 and 128.4 (C-4-6), 87.4 (2 × C-1′), 75.8, 73.6, 68.8, and 67.8 (2 × C-2′-5′), 61.8 (2 × C-6′), 44.0 (2 × ArCH_2), 20.6, 20.5, 20.44, and 20.41 (8 × CH_3). Anal. Calcd for $C_{36}H_{46}O_{18}S_4$ (895.0): C, 48.31; H, 5.18. Found: C, 48.20; H, 5.22.

4.2.2. 1,3-Di-{[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) dithio]methyl}benzene 5

Yield 50%. TLC: R_f 0.51 (eluant acetone/$CHCl_3$ 1:9). Mp 78–80 °C. ^{1}H NMR ($CDCl_3$): δ 7.3 (m, 4H, ArH), 5.5–5.4 (m, 4H, 2 × H-2′,4′), 5.08 (dd, $J_{2',3'}$ = 10.2, $J_{3',4'}$ = 3.3 Hz, 2H, 2 × H-3′), 4.53 (d, $J_{1',2'}$ = 9.9 Hz, 2H, 2 × H-1′), 4.2–4.0 (m, 10H, 2 × H-5′, 2 × H_2-6′, 2 × ArCH_2), 2.19, 2.06, 2.05, and 2.00 (four s, 24H, 8 × CH_3). ^{13}C NMR ($CDCl_3$): δ 170.3, 170.1, 170.0, and 169.3 8 × CO, 137.2 C-1,3, 129.9 (C-2), 128.7 and 128.5 (C-4-6), 89.3 (2 × C-1′), 74.7, 71.7, 67.1, and 66.6 (2 × C-2′-5′), 61.5 (2 × C-6′), 44.4 (2 × ArCH_2), 20.73, 20.67, and 20.5 (8 × CH_3). Anal. Calcd for $C_{36}H_{46}O_{18}S_4$ (895.0): C, 48.31; H, 5.18. Found: C, 48.15; H, 5.27.

4.2.3. 1,4-Di-{[(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl) dithio]methyl}benzene 6

Yield 60%. TLC: R_f 0.74 (eluant EtOAc/MeOH 9.5:0.5). Mp 208–212 °C. ^{1}H NMR ($CDCl_3$): δ 7.26 (s, 4H, ArH), 5.34 (t, J_{vic} = 9.4 Hz, 2H) and 5.14 (t, J_{vic} = 9.7 Hz, 2H) (2 × H-2′,4′), 5.25 (t, J_{vic} = 9.1 Hz, 2H, 2 × H-3′), 4.52 (d, $J_{1',2'}$ = 9.9 Hz, 2H, 2 × H-1′), 4.28 and 4.19 (split AB system, $J_{5',6'A}$ = 4.7, $J_{5',6'B}$ = 2.4, $J_{6'A,6'B}$ = 12.5 Hz, 4H, 2 × H_2-6′), 4.01 (s, 4H, 2 × ArCH_2), 3.75 (ddd, $J_{4',5'}$ = 10.0 Hz, 2H, 2 × H-5′), 2.09, 2.05, 2.03, and 2.02 (four s, 24H, 8 × CH_3). ^{13}C NMR ($CDCl_3$): δ 170.5, 169.4, 169.1, and 170.2 8 × CO, 136.1 C-1,4, 129.6 (C-2,3,5,6), 87.7 (2 × C-1′), 76.1, 73.8, 69.1, and 68.0 (2 × C-2′-5′), 62.0 (2 × C-6′), 44.0 (2 × ArCH_2), 20.7, 20.64, 20.59, and 20.56 (8 × CH_3). Anal. Calcd for $C_{36}H_{46}O_{18}S_4$ (895.0): C, 48.31; H, 5.18. Found: C, 48.40; H, 5.08.

4.2.4. 1,4-Di-{[(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) dithio]methyl}benzene 8

Yield 55%. TLC: R_f 0.67 (eluant EtOAc/MeOH 9.5:0.5). Mp 76–84 °C. ^{1}H NMR ($CDCl_3$): δ 7.27 (m, 4H, ArH), 5.42–5.49 (m, 4H, 2 × H-2′,4′), 5.06 (dd, $J_{2',3'}$ = 10, $J_{3',4'}$ = 2.9 Hz, 2H, 2 × H-3′), 4.49 (d, $J_{1',2'}$ = 9.9 Hz, 2H, 2 × H-1′), 3.96–4.18 (m, 10H, 2 × H-5′, 2 × H_2-6′, 2 × ArCH_2), 2.19, 2.06, 2.05, and 1.99 (four s, 24H, 8 × CH_3). ^{13}C NMR ($CDCl_3$): δ 170.3, 170.1, 170.0, and 169.3 8 × CO, 136.2 C-1,3, 129.67 (C-2) 129,5 (C-4-6), 89.2 (2 × C-1′),

74.7, 71.7, 67.1, and 66.6 (2 × C-2′-5′), 61.5 (2 × C-6′), 44.2 (2 × ArCH$_2$), 20.74, 20.68, and 20.56 (8 × CH$_3$). Anal. Calcd for $C_{36}H_{46}O_{18}S_4$ (895.0): C, 48.31; H, 5.18. Found: C, 48.24; H, 5.01.

4.3. Standard procedure for the preparation of deprotected disulfides 7, 9, 12, and 14

To a solution of 1 mmol of acetylated disulfide in MeOH/THF (20 mL, 5:5) aqueous ammonia (5 mL) was added under stirring and stirring was maintained for 48 h, at room temperature. The reaction was monitored by TLC ($CHCl_3$/MeOH 9.5:0.5) and ^{1}H NMR. The solvent was evaporated and the deprotected glycoconjugated disulfides **7**, **9**, **12** and **14** were purified by column chromatography ($CHCl_3$/MeOH 8:2 up to 1:9).

4.3.1. 1,4-Di-{[(β-D-glucopyranosyl)dithio]methyl}benzene 7

Yield 53%. TLC: R_f 0.49 (eluant $CHCl_3$/MeOH 6:4). Mp 78–80 °C. ^{1}H NMR (CD_3OD): δ 7.30 (s, 4H, ArH), 4.32 (d, $J_{1',2'}$ = 9.4 Hz, 2H, 2 × H-1′), 4.08 (s, 4H, 2 × ArCH_2), 3.9–3.3 (m, 12H, 2 × H-2′-5′, 2 × H_2-6′). ^{13}C NMR (CD_3OD): δ 138.0 C-1,4, 130.6 (C-2,3,5,6), 92.0 (2 × C-1′), 82.5, 79.5, 72.8, and 71.4 (2 × C-2′-5′), 63.0 (2 × C-6′), 45.3 (2 × ArCH$_2$). Anal. Calcd for $C_{20}H_{30}O_{10}S_4$ (558.7): C, 42.99; H, 5.41. Found: C, 43.17; H, 5.18.

4.3.2. 1,4-Di-{[(β-D-galactopyranosyl)dithio]methyl}benzene 9

Yield 50%. TLC: R_f 0.47 (eluant $CHCl_3$/MeOH 6:4). Mp 103–110 °C. ^{1}H NMR (CD_3OD): δ 7.32 (s, 4H, ArH), 4.30 (d, $J_{1',2'}$ = 9.4 Hz, 2H, 2 × H-1′), 4.10 (s, 4H, 2 × ArCH_2), 3.9–3.5 (m, 12H, 2 × H-2′-5′, 2 × H_2-6′). ^{13}C NMR (CD_3OD): δ 138.0 C-1,4, 130.7 (C-2,3,5,6), 93.2 (2 × C-1′), 81.0, 76.2, 70.5, and 70.1 (2 × C-2′-5′), 62.8 (2 × C-6′), 45.3 (2 × ArCH$_2$). Anal. Calcd for $C_{20}H_{30}O_{10}S_4$ (558.7): C, 42.99; H, 5.41. Found: C, 42.77; H, 5.58.

4.3.3. 1,3,5-Tri-{[(β-D-glucopyranosyl)dithio]methyl}-2,4,6-trimethylbenzene 12

Yield 51%. TLC: R_f 0.16 (eluant $CHCl_3$/MeOH 7.5:2.5). Mp 129 °C. ^{1}H NMR (CD_3OD): δ 4.40 (d, $J_{1',2'}$ = 9.2 Hz, 3H, 3 × H-1′), 4.32 and 4.26 (AB system, J_{gem} = 11.4 Hz, 6H, 3 × ArCH_2), 3.9–3.3 (m, 18H, 3 × H-2′-5′, 3 × H_2-6′), 2.54 (s, 9H, 3 × ArCH_3). ^{13}C NMR (CD_3OD): δ 138.5 (C-1,3,5), 132.7 (C-2,4,6), 91.3 (3 × C-1′), 82.6, 79.4, 72.1, and 71.4 (3 × C-2′-5′), 62.9 (3 × C-6′), 42.4 (3 × ArCH$_2$), 17.1 (3 × ArCH$_3$). Anal. Calcd for $C_{30}H_{48}O_{15}S_6$ (841.1): C, 42.84; H, 5.75. Found: C, 43.03; H, 5.69.

4.3.4. 1,3,5-Tri-{[(β-D-glucopyranosyl)dithio]methyl}-2,4,6-triethylbenzene 14

Yield 52%. TLC: R_f 0.14 (eluant $CHCl_3$/MeOH 7:3). Mp 100 °C. ^{1}H NMR (CD_3OD): δ 4.42 (d, $J_{1',2'}$ = 9.4 Hz, 6H, 3 × H-1′), 4.31 and 4.20 (AB system, J_{gem} = 11.4 Hz, 6H, 3 × ArCH_2S), 4.0–3.3 (m, 18H, 3 × H-2′-5′, 3 × H_2-6′), 2.98 (q, J_{vic} = 7.5 Hz, 6H, 3 × CH_2CH$_3$), 1.28 (t, 9H, 3 × CH$_2$CH_3). ^{13}C NMR (CD_3OD): δ 145.4 (C-1,3,5), 131.9 (C-2,4,6), 91.2 (3 × C-1′), 82.8, 79.4, 72.3, and 71.7 (3 × C-2′-5′), 63.2 (3 × C-6′), 41.7 (3 × ArCH$_2$S), 24.3 (3 × ArCH$_2$CH$_3$), 17.1 (3 × ArCH$_2$CH$_3$). Anal. Calcd for $C_{33}H_{54}O_{15}S_6$ (883.2): C, 44.88; H, 6.16. Found: C, 44.53; H, 5.94.

4.4. Biological methods

4.4.1. Cells and treatments

The biological assays were performed on the following cell lines: U937 (established from the pleural effusion of a patient with histiocytic lymphoma), THP-1 (established from the peripheral blood of a patient with acute monocytic leukaemia), MOLT-3 (established from the peripheral blood of a patient with acute lymphoblastic leukaemia), HeLa (established from cancer cells of a patient with cervical carcinoma), HT-29 (adenocarcinoma cells established from a patient with colorectal carcinoma), MCF7 (adenocarcinoma cells established from a patient with breast cancer) and HepG2 (hepatocyte carcinoma cells established from a patient with hepatocellular carcinoma). In some experiments, peripheral blood mononuclear cells (PBMCs) from healthy donors, separated by density gradient centrifugation according to the standard technique, were also used. All cell lines were cultured in RPMI 1640 medium supplemented with 5–10% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin at 100 units/mL (GIBCO-Invitrogen, UK). For THP-1 cells, medium was supplemented with 10 mM HEPES, 1 mM sodium pyruvate and 4.5 g/L glucose (Sigma–Aldrich, USA). PBMCs were cultured at an initial density of 10^6 cells/ml, with the addition of human recombinant interleukin-2 (IL-2), 20 U/ml (Chiron corporation, Emeryville, CA). U937, THP-1 and MOLT-3 cell lines share common characteristics such as rapid growth in suspension, easily detectable apoptosis, relatively low level of malignancy and moderate sensitivity to cytotoxic agents. HeLa, HT-29, MCF7 and HepG2 cell lines share the common characteristics to grow as adherent cells and to present a relatively high level of malignancy and resistance to cytotoxic agents.

U937 cells expressing the vector containing the murine *bcl-2* gene (U937mBCL2) or the pMEP control vector (U937pMEP), have been previously described.[14] They were maintained in culture as were wild type U937 cells, except for the addition of hygromycin B (50 μg/mL).

For biological assays, glycoconjugated disulfides **3–15** in DMSO or in culture medium at a concentration of 20 mM. Cells were inoculated into 96-well microtiter plates at density of 10×10^4 or 50×10^4 cells/well, depending on the doubling time of individual cell lines, in 100 μl of culture medium containing increasing concentrations of the compounds to assay. Cells were then incubated at 37 °C and 5% CO_2 for 24 or 48 h. Control cultures received equivalent volumes of control medium and vehicle.

4.4.2. Evaluation of absolute cell number and of percentage cells showing membrane permeabilization and apoptosis

The absolute number of total cells and the percentage number of cells showing membrane permeabilization were evaluated by microscopy analysis in a haemocytometer chamber, using the trypan blue exclusion test as a standard viability assay. Apoptosis was evaluated by morphological analysis followed by staining with acridine orange and by flow cytometry analysis following staining with propodium iodide, as previously described.[15] For morphological analysis, over 600 cells, including those showing typical apoptotic characteristics, were analyzed using a fluorescence microscope. The identification of apoptotic cells was based on the presence of uniformly stained nuclei showing chromatin condensation and nuclear fragmentation. For flow cytometry analysis, isolated nuclei were analyzed by fluorescence and by forward- and side-angle-scatter multiparameter analysis on a Becton Dickinson FaCScalibur using the CELLQuest II software. A minimum of 5000 events were collected for each sample.

4.4.3. Evaluation of metabolic activity

The effects on the metabolic activity were examined by the MTS colorimetric method, using a commercial kit (MTS, Cell Titer 96 Aqueous One Solution, Promega). The assay was performed according to the manufacturer's protocol, by directly adding 20 μL of 'CellTiter 96 Aqueous One Solution Reagent' to the culture wells at the end of incubation period. After further 4 h incubation, absorbance was read at 490 nm.

4.4.4. Calculation of dose–response indexes

Inhibitory concentration 50 (IC_{50}, drug concentration able to cause inhibition of the total cell count by 50%), cytotoxic concentration 30 (CC_{30}, drug concentration able to cause 30% cytotoxicity

by trypan blue assay), apoptotic concentration 30 (AC_{30}, drug concentration able to cause 30% apoptotic cells by fluorescence microscopy analysis of acridine orange stained cells), and metabolic activity inhibitory concentration 30 ($MAIC_{30}$, drug concentration able to cause reduction of the formazan product formation, MTS assay, by 30%) were calculated according to the best-fit curve, y value versus log$x$, where y is the value of the examined function and x is the drug concentration. Results from at least three different determinations were used to calculate the dose–response curve. The 30% level for CC_{30}, AC_{30} and $MAIC_{30}$ ensures that values lie within the concentration range utilized.

4.4.5. Statistical analysis

Data analysis was performed using the SPSS statistical software system (version 17.0 for Windows, Chicago, IL). Comparison of means among sample groups was carried out using Hochberg's GT2 post-hoc multiple comparison One-Way ANOVA test. Differences were considered significant at p <0.05 and highly significant at p <0.001.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.03.070.

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