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# New oleyl glycoside as anti-cancer agent that targets on neutral sphingomyelinase

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ABSTRACT

We designed and synthesized two anomeric oleyl glucosaminides as anti-cancer agents where the presence of a trifluoroacetyl group close to the anomeric center makes them resistant to hydrolysis by hexosaminidases. The oleyl glycosides share key structural features with synthetic and natural oleyl derivatives that have been reported to exhibit anti-cancer properties. While both glycosides showed antiproliferative activity on cancer cell lines, only the α-anomer caused endoplasmic reticulum (ER) stress and cell death on C6 glioma cells. Analysis of sphingolipids and glycosphingolipds in cells treated with the glycosides showed that the α-anomer caused a drastic accumulation of ceramide and glucosylceramide and reduction of lactosylceramide and GM3 ganglioside at concentrations above a threshold of 20 μM. In order to understand how ceramide levels increase in response to α-glycoside treatment, further investigations were done using specific inhibitors of sphingolipid metabolic pathways. The pretreatment with 3-*O*-methylsphingomyelin (a neutral sphingomyelinase inhibitor) restored sphingomyelin levels together with the lactosylceramide and GM3 ganglioside levels and prevented the ER stress and cell death caused by the α-glycoside. The results indicated that the activation of neutral sphingomyelinase is the main cause of the alterations in sphingolipids that eventually lead to cell death. The new oleyl glycoside targets a key enzyme in sphingolipid metabolism with potential applications in cancer therapy.



## 1. Introduction

We have described the synthesis of glycolipids with antitumoral activity [1]. Among the variety of glycosides obtained with different hydrocarbon chains, the highest activities were shown by compounds containing an oleyl group, the glycoside **1** being the most potent inhibitor (Fig. 1A). These results were in line with other reports describing molecules composed by an oleyl group linked to a hydroxylated moiety that show anticancer properties. Thus, the iminosugar **7** (Fig. 1B), with an oleyl substituent, was the most active anticancer compound among a family of 1,4-iminoribitols [2]. The oleylamide **8** was described as a ceramide analog able to induce apoptosis in human cancer cells [3]. Natural oleyl derivatives with hydroxylated moieties, such as compounds **9** [4] and **10** [5] have also been reported to exhibit potent antitumor activity. Even a simple structure like 2-hydroxyoleic acid (**11**) was shown to inhibit the growth of cancer cells and exhibit antitumor activity in animal models of cancer [6,7]. The results of these investigations indicate the existence of a structure-activity relationship between anticancer activity and the conjugation of a polar hydroxylated head to an oleyl group. However, the precise target for the activity of these compounds still remains unknown. In order to understand the mechanism behind the activity of **1** we did further studies [8,9], using cell lines A549 and C6. Glycoside **1**

*Abbreviations:* UPLC-MS, ultra performance liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance; MTT, methylthiazol tetrazolium; IC50, 50% inhibitory dose; UPRE, unfolding protein response element; ERSE, endoplasmic reticulum stress element; Cer, ceramide; SMs, phingomyelin; GlcCer, glucosylceramide; GCS, glucosylceramide synthase; SMS, sphingomyelin synthase; XBP-1, X-box binding protein 1.

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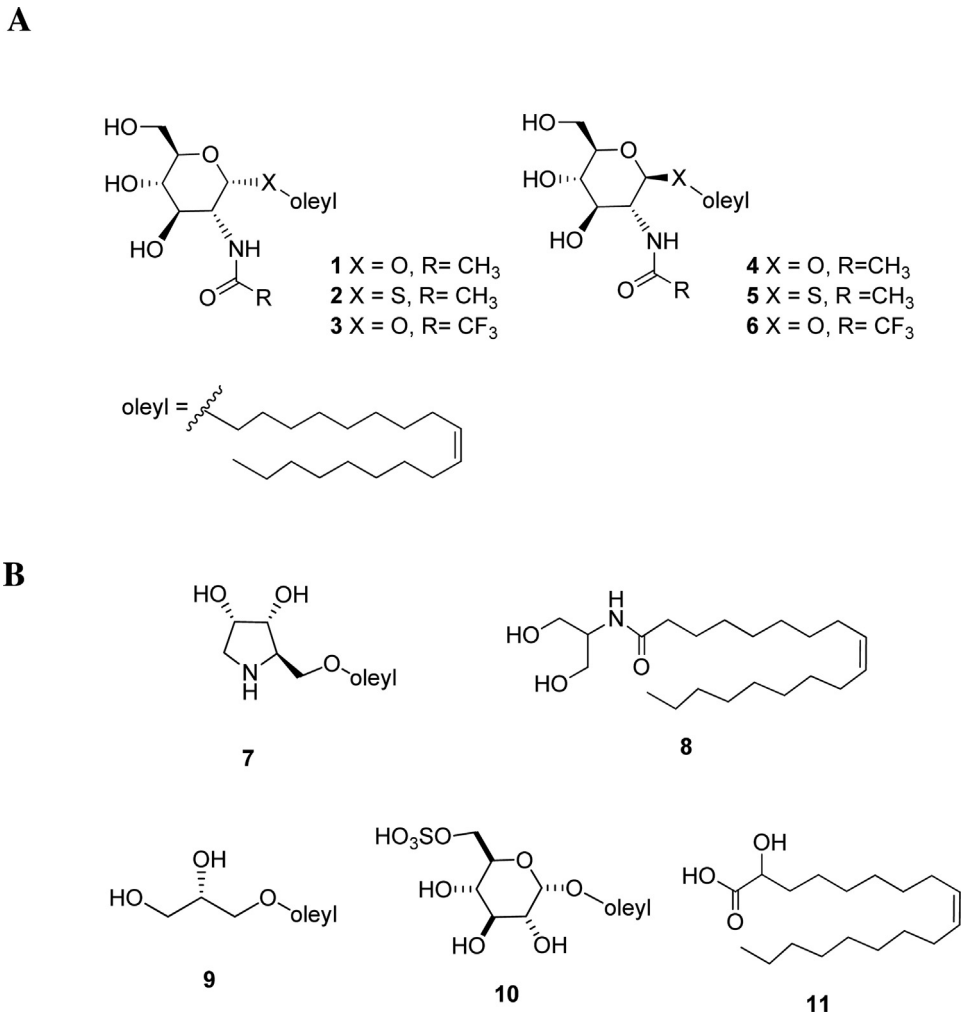


**Fig. 1.** (A) Chemical structures of glycosides **1–6**, and (B) oleyl derivatives **7–11** with anti-cancer properties.

altered drastically the lipid profile in the cells at concentrations above 30 μM, causing a significant increase in sphingolipid and glycosphingolipid levels that led eventually to cell death. These metabolite changes were attributed to activation of ER stress pathways, caused by treatment with **1** [9]. Due to the susceptibility of **1** to hexosaminidase-catalyzed hydrolysis, which precludes its use in vivo, the anticancer activity was confirmed in animal models by using an analog compound, the enzyme resistant thio-glycoside **2** (Fig. 1), which, when assayed in tumor bearing mice, reduced significantly the tumor, compared to controls [10]. The analysis by $^{1}$H HR-MAS NMR spectroscopy of C6 cells and tumor tissues treated with **2** showed a significant increase in choline and phosphocholine [11], that was hypothetically attributed to activation of plasma membrane sphingomyelinases. Nevertheless, the use of the thio-derivative **2** presented disadvantages compared to **1**, such as its more complicated synthesis and reduced water solubility.

With the aim of improving the synthesis and water solubility of the previous glycosides and of carrying out further studies on their mode of action, we have now prepared the new oleyl glucosaminide derivative **3** (Fig. 1A). Compound **3** presents a trifluoroacetyl group instead of the acetyl group at the N atom in position 2. It was designed reasoning that the presence of the electronegative fluorine substituent would significantly decrease the rate of hydrolysis catalyzed by hexosaminidases, as compared with the acetylated derivative **1** [12]. In addition, the polar trifluoroacetyl group should make the glycoside more water soluble than **1** and **2,** and should facilitate drug monitoring in biological samples by $^{19}$F-NMR spectroscopy. We report here the synthesis and antitumoral activity of **3** and its β-anomer **6** (Fig. 1A), confirm the stability of **6** against a commercially available β-*N*-acetylhexosaminidase, and report the effect of **3** on C6 cells sphingolipidome. We have also evaluated the induction of ER stress and cell death in C6 cells treated with compound **3**. By using specific enzyme inhibitors we have obtained information about how glycolipid **3** targets on particular pathways of sphingolipid and glycosphingolipid metabolism. These results provide a better understanding of the mechanism by which this family of compounds exerts their antitumoral action.

## 2. Materials and Methods

### 2.1. Reagents and synthetic methods

All chemicals were of reagent grade or higher and were purchased from commercial suppliers or purified by standard techniques. *N*-trifluoroacetyl-D-glucosamine was prepared following a described procedure [13]. Thin-layer chromatography (TLC) was performed on aluminum sheets 60 F254 Merck silica gel (Merck, Darmstadt, Germany) and compounds were visualized by irradiation with UV light and/or by treatment with a solution of $Ce_2MoO_4$ or 5% $H_2SO_4$ in EtOH, followed by heating. Flash column