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Design, synthesis, and preliminary biological evaluation of a novel triazole analogue of ceramide

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Abstract—The amide bond of ceramide was replaced by the non-hydrolyzable 1,2,3-triazole functionality. Click chemistry was employed for synthesis of the designed analogues. Our preliminary biological evaluation indicated that the amide moiety of ceramide is amenable to bioisosteric substitution with the triazole moiety. Some of the analogues were more potent than C2-ceramide as cytotoxic agents, and the observed cytotoxicity was possibly mediated through the induction of apoptosis.

Sphingolipid research has recently made significant strides.[1] The increased appreciation and understanding of the biological roles of sphingolipid raise the emerging concept of sphingolipid metabolites as therapeutics.[2] One of the remarkable recent findings in sphingolipid research is that sphingolipid metabolites, such as ceramide, sphingosine-1-phosphate (S1P), and sphingosine-1-phosphocholine, can act as second messengers that regulate diverse cellular processes. Another important finding is that different sphingolipid metabolites display different biological functions. For example, ceramide has pro-apoptotic and anti-proliferative activity, whereas its metabolite, S1P, exerts an opposite effect.[1,2] Thus, controlling the balance of sphingolipid metabolites, by the regulation of enzymes involved in sphingolipid metabolism or by local delivery of sphingolipid, could modulate the cellular processes.

Ceramide plays a central role in sphingolipid metabolism, both as a key precursor and as a penultimate degradation product of all major sphingolipids (Fig. 1).[3] The amide linkage of ceramide is hydrolyzed to fatty acid and sphingosine by the enzyme ceramidase. The resulting sphingosine is intrinsically bioactive, and it could serve as a substrate for sphingosine kinase to form S1P.

Due to the biological significance of ceramide, a number of analogues have been synthesized and evaluated.[4] However, to the best of our knowledge, modification of the amide moiety of ceramide to the non-hydrolyzable bioisosteric moiety has not been reported. We envisioned that hydrolytically stable ceramide analogues

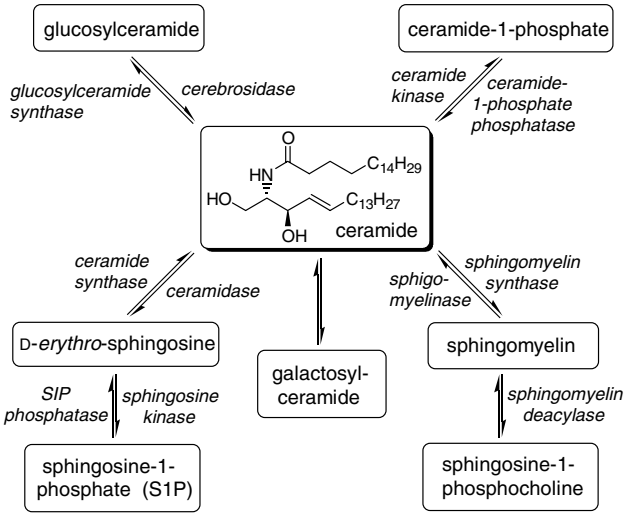

Figure 1. Pathways of ceramide metabolism.

Keywords: Ceramide; Click chemistry; 1,2,3-Triazole; Bioisosteric substitution; Apoptosis.
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might alter the balance of sphingolipid metabolites, either by mimicking the actions of endogenous ceramide or by regulating the related enzymes. Furthermore, their metabolic fate would be different from that of ceramide, due to the different physicochemical properties of the isosteres. As a consequence, the subsequent sphingolipid metabolism would be affected, thereby influencing the cellular processes.

Among a variety of bioisosteres of the amide moiety, 1,2,3-triazoles have gained increasing attention in drug discovery.[5] Triazoles can mimic the topological and electronic features of an amide bond, and they can actively participate in hydrogen bonding and dipole–dipole interactions. Indeed, we recently demonstrated that triazole could successfully serve as a linking unit that can mimic the atom placement of an amide bond of the immunostimulant α-GalCer.[6] However, unlike amides, triazoles are extremely stable to hydrolysis. Based on these considerations, we decided to replace an amide bond of ceramide by the 1,2,3-triazole functionality as a non-hydrolyzable bioisostere. We present here the synthesis and preliminary evaluation of a series of 1,2,3-triazole containing ceramide and phytoceramide analogues of general structures **1** and **2**, respectively (Fig. 2) in which the lipid-chain lengths have been incrementally varied.

For the divergent synthesis of the designed triazole analogues of ceramide, we employed the recently introduced click chemistry (Scheme 1). Sharpless and co-workers developed a copper(I)-catalyzed Huisgen reaction between azides and terminal alkynes, providing a regioselective construction of 1,4-disubstituted-1,2,3-triazoles.[7] Thus, the known azidophytosphingosine **4**, the requisite starting material for the synthesis of **2**, was prepared from D-*ribo*-phytosphingosine according to the published procedure.[8] Huisgen's 1,3-dipolar cycloaddition between the obtained azido-compound **4** and varied terminal alkynes in the presence of $CuSO_4$ and sodium-ascorbate in $^tBuOH/H_2O$ provided the desired 1,4-regioselective triazole products **2a–e** in high isolated yield (85–95%). For the synthesis of **1**, D-*erythro*-azidosphingosine **5** was prepared from azidophytosphingosine **4** according to our published four-step procedure.[9] Analogous to the preparation of **2**, we encountered no difficulties when converting azidosphingosine **5** into the corresponding triazoles **1a–e** through the copper(I)-catalyzed Huisgen reaction. The structure and regiochemistry of the obtained analogues were established by means of NMR analysis including HMBC.[10]

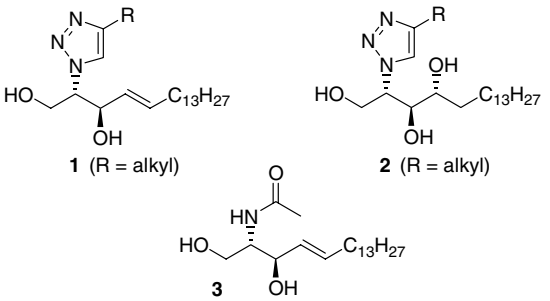
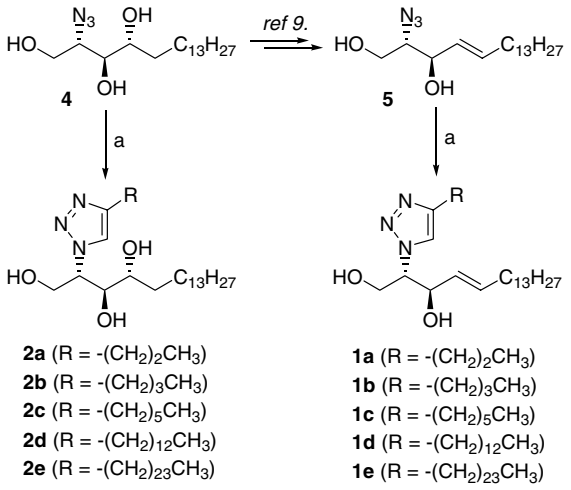


Figure 2. General structure of triazole containing ceramide analogues and structure of C2-ceramide.



Scheme 1. Reagents: (a) 1.5 equiv HC≡CR, $CuSO_4$, Na-ascorbate, $^tBuOH/H_2O$ (1:1), 3–12 h, 85–95%.

Among various fundamental biological functions of ceramides, it has long been known that they can induce apoptotic cell death.[11] Thus, as a preliminary evaluation of triazoles **1** and **2**, the cytotoxic activity in various cancer cells was determined by sulforhodamine B (SRB) or MTT assay as described previously.[12,13] In our experiments, the cell-permeable C2-ceramide **3** (Fig. 2) was employed as a positive control.[14] As shown in Table 1, the cytotoxic activities of triazole analogues **1** were highly influenced by the length of the attached chain. The short-chain triazole analogues **1a–c** were more effective than prototype C2-ceramide **3** in inhibiting cancer cell growth. On the other hand, the long-chain triazoles **1d–e** did not show any significant cytotoxicities against the cancer cells tested up to 50 μM. These results were not surprising in view of the generally accepted fact that synthetic short-chain ceramides, such as C2- and

Table 1. Cytotoxic activities for 1,2,3-triazole containing ceramide analogues **1** and **2**

Compound	EC_{50}[a] (μM)		
	HCT 116[b]	NCI-H358[c]	K-562[d]
C2-ceramide	24.5	19.6	35.1
1a	16.0	15.7	13.0
1b	12.9	13.6	12.3
1c	12.5	12.8	8.2
1d	>50	>50	>50
1e	>50	>50	>50
2a	13.9	13.2	14.9
2b	12.9	12.9	14.5
2c	12.0	13.7	12.6
2d	>50	>50	>50
2e	>50	>50	>50

[a] All values are means of a minimum of three experiments.
[b] Human colon carcinoma.
[c] Human bronchioalveolar carcinoma.
[d] Human chromic myelogenous leukemia.

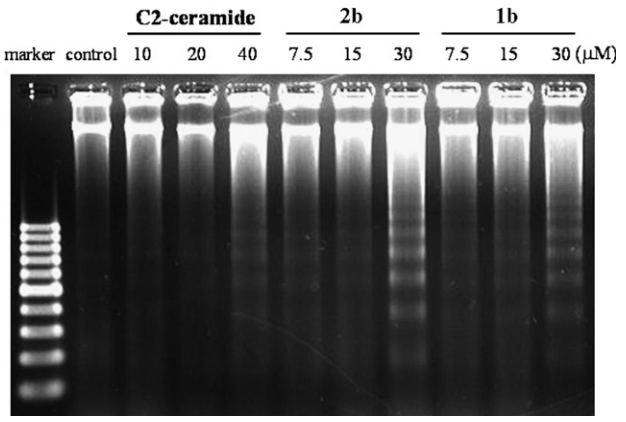


Figure 3. DNA fragmentation in K-562 cells treated with various concentrations of ceramide analogues.

C6-ceramide, are water soluble and membrane-permeable, whereas natural long-chain ceramide is not water-soluble and penetrates cellular membranes with difficulty.[15] Based on this fact, we could infer that the diminished cytotoxicities of long-chain triazoles **1d–e** could be attributed to their low aqueous solubility or poor membrane permeability, not to the intrinsic inactivity.

The phytoceramides, which contain a hydroxyl group at C-4 instead of double bond of ceramides, are slightly more cytotoxic compared with their corresponding ceramides.[16] These previous findings suggest that the cytotoxic activity could be also expected from the phytosphingosine derived triazoles **2**, if the triazole moiety successfully mimics the amide bond. As expected, triazoles **2** exhibited similar level of cytotoxic activities as the corresponding sphingosine derived triazoles **1**. Analogous to triazole **1**, only short-chain triazole analogues **2a–c** were more potent than C2-ceramide **3**. These results indicate that the amide moiety of ceramide is amenable to bioisosteric substitution with the triazole moiety.

To determine whether the cytotoxicity is mediated by the induction of apoptosis, DNA fragmentation was analyzed. K-562 cells were treated with the short-chain triazole analogues **1b** and **2b** for 18 h, and DNA samples were extracted and analyzed by electrophoresis on agarose gels.[17] In parallel, as an apoptosis-positive control, C2-ceramide **3** was used. The results showed that exposure to triazoles **1b** and **2b** exerted DNA cleavage that was characteristic of apoptosis (Fig. 3). Phytosphingosine derived triazole **2b** displayed a higher ability than sphingosine derived triazole **1b** to induce DNA fragmentation. C2-ceramide **3** causes much less fragmentation of K-562 DNA than triazoles **1b** and **2b** in our experiments. These observations suggest that triazole analogues possibly induce apoptosis in K-562 cells.

In conclusion, we have replaced an amide bond of ceramide with a non-hydrolyzable 1,2,3-triazole functionality. Click chemistry was employed to generate triazole containing ceramide analogues efficiently. As a preliminary evaluation of the obtained analogues, the cytotoxic activity in various cancer cells was measured. Our preliminary evaluation indicated that the amide moiety of ceramide is amenable to bioisosteric substitution with the triazole moiety, providing the basis for further development of triazole containing analogues. The short-chain triazole analogues displayed higher activities than C2-ceramide. Although the reason for these higher cytotoxic activities is not clear presently, the observed cytotoxicity is possibly mediated, in part, by the induction of apoptosis. Because of the numerous and complicated biological actions of ceramide, it is difficult and needs further studies to ascertain whether the triazole analogues simply mimic the actions of endogenous ceramide or exert different intracellular behavior. To this end, we are currently investigating this issue, and the results will be reported in due course.

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10. Spectroscopic data for the new typical products. Compound **1b**: White solid; mp 43–44 °C; $[\alpha]_D^{20}$ +5.86 (*c* 1.0, CH_3OH); ^1H NMR (300 MHz, CD_3OD) δ 0.89 (t, J = 7.2 Hz, 3H), 0.94 (t, J = 7.4 Hz, 3H), 1.12–1.44 (m, 24H), 1.64 (m, 2H), 1.88 (m, 2H), 2.68 (t, J = 7.7 Hz, 2H), 4.06 (dd, J = 4.5, 11.7 Hz, 1H), 4.12 (dd, J = 7.5, 11.7 Hz, 1H), 4.35 (app t, J = 7.2 Hz, 1H), 4.47 (ddd, J = 4.2, 7.2, 7.5 Hz, 1H), 5.27 (dd, J = 7.7, 15.3 Hz, 1H), 5.50 (td, J = 6.6, 15.6 Hz, 1H), 7.72 (s 1H); ^{13}C NMR (75 MHz, CD_3OD) δ 14.2, 14.5, 23.3, 23.8, 26.1, 30.2, 30.5, 30.65, 30.71, 30.80, 30.81, 30.83, 32.8, 33.1, 33.2, 62.0, 69.1, 73.2, 123.3, 129.8, 135.6, 148.6; HRMS (FAB) calcd for $C_{24}H_{46}N_3O_2$ 408.3590 ($[M+H]^+$), found 408.3592. Compound **2b**: white solid; mp 90–91 °C; $[\alpha]_D^{20}$ +4.93 (*c* 1.0, CH_3OH); ^1H NMR (500 MHz, CD_3OD) δ 0.89 (t, J = 6.9 Hz, 3H), 0.95 (t, J = 7.5 Hz, 3H), 1.20–1.35 (m, 24H), 1.39 (m, 2H), 1.51 (m, 1H), 1.58–1.69 (m, 3H), 2.70 (t, J = 7.7 Hz, 2H), 3.31–3.34 (m, 1H), 3.78 (dd, J = 5.1, 6.5 Hz, 1H), 4.12 (d, J = 6.2 Hz, 2H), 4.91 (dt, J = 5.1, 6.0 Hz, 1H), 7.81 (s 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 15.0, 15.3, 24.1, 24.5, 26.9, 27.6, 31.3, 31.52, 31.57, 31.59, 31.61, 33.6, 33.9, 34.5, 62.2, 66.5, 73.8, 77.1, 123.8, 149.5; HRMS (FAB) calcd for $C_{24}H_{48}N_3O_3$ 426.3696 ($[M+H]^+$), found 426.3693.
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13. Cells were plated in 96-well plates at the density of 5×10^3 cells/well (HCT 116 and NCI-H358) or 1×10^4 cells/well (K-562) and incubated for 24 h. Test compounds (dissolved in pure ethanol) were diluted in the medium and treated to cells for 48 h. Adherent cells (HCT 116 and NCI-H358) were fixed with 10% trichloroacetic acid for 30 min at 4 °C. Fixed cells were stained with 0.4% sulforhodamine B (SRB) for 30 min at room temperature. Stained cells were dissolved in 10 mM Tris (pH 10.0). Absorbance was measured at 515 nm. For floating cells (K-562), 5 mg/ml (final 500 μg/ml) MTT solution was added to cells and further incubated for 3 h at 37 °C. Media were discarded, and formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm. Cell survival (%) of each tested group was determined by comparison with solvent-treated control cells. EC_{50} value, the concentration of 50% cell survival, was estimated by non-linear regression analysis.
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17. K-562 cells (1×10^6 cells/dish in 100-mm dishes) were treated with various concentrations of test compounds for 18 h. Collected cells were lysed with lysis buffer (50 mM Tris–HCl (pH 7.5), 20 mM EDTA, and 1% NP-40) for 5 min at 4 °C. Lysates were incubated with 10% SDS (final 1%) for 1 h at 56 °C and then treated with proteinase K solution (final 2.5 μg/μl) for 2 h at 37 °C. DNA was isolated by adding 10 M ammonium acetate (final 5 M) and ice-cold pure ethanol. After incubation overnight and centrifugation, DNA was washed twice with ice-cold 80% ethanol, dried in the air, and dissolved in TE buffer (10 mM Tris–HCl (pH 8.0) and 1 mM EDTA). Five micrograms of DNA of each tested sample was subjected to 2% agarose gel electrophoresis. The gel was stained with SYBR Gold (Molecular Probes) for 1 h at room temperature and visualized under UV transillumination.