Bcl-2 antagonizes apoptotic cell death induced by two new ceramide analogues

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Abstract **Ceramides which arise in part from the breakdown of sphingomyelin comprise a class of antiproliferative lipids and have been implicated in the regulation of programmed cell death better known as apoptosis. In the present study, two new synthetic ceramide analogues, *N*-thioacetylsphingosine and FS-5, were used in Molt 4 cells to induce cell death. Besides their cytotoxic effects at concentrations ≥14 μM the data obtained clearly show that both analogues induced apoptosis at concentrations below this critical concentration as assessed by trypan blue exclusion and cleavage of the death substrate poly-(ADP-ribose) polymerase (PARP). Additional experiments in bcl-2-transfected Molt 4 cells revealed that the apoptotic but not the lytic effects of the analogues were antagonized by the apoptosis inhibitor Bcl-2. Furthermore, neither *N*-thio-acetylsphingosine nor FS-5 induced PARP cleavage in bcl-2-transfected Molt 4 cells indicating that the induction of apoptotic cell death by cell permeable ceramides is not due to unspecific disturbance of the cell membrane.**

Key words: Apoptosis; Bcl-2; Sphingomyelin cycle; Synthetic ceramide

1. Introduction

Sphingolipids are well known structural components of the lipid bilayer of eukaryotic cell membranes. During the last few years, it has become increasingly obvious in different cell types that one member of this lipid class, sphingomyelin (SM), takes part in a novel pathway of cell signalling [1–5], and the term SM cycle has been coined for characterizing this function. 1α,25-Dihydroxyvitamin D_3 was the first compound identified as an inducer of SM hydrolysis in HL-60 human leukemia cells leading to the generation of intracellular ceramide [1]. Using cell-permeable ceramide analogues, it was possible to mimick the effects of 1α,25-dihydroxyvitamin D_3 on cell proliferation thereby confirming ceramide to be an important mediator of 1α,25-dihydroxyvitamin D_3-induced HL-60 cell differentiation [6]. In contrast to the mitogenic lipid mediator diacylglycerol (DAG) that arises from the hydrolysis of different glycerophospholipids [7,8] ceramide was described as a mediator of growth suppression and apoptosis [9].

Apoptosis, or programmed cell death, is a form of physiological cell death which can be distinguished from accidental destruction, or necrosis (for review, see [10]). Necrotic cell death occurs when a cell is severely injured, for example by a harsh physical insult, the cell begins to swell and the plasma membrane ruptures. On the other hand, cells undergoing apoptosis shrink and pull away from its neighbors. In apoptotic cells the plasma membrane remains intact and the chromatin frequently breaks into fragments that produce a ladder-like pattern. Recent experimental observations revealed that intracellular proteases might play a critical role in the initiation of apoptosis and the specific proteolytic cleavage of cellular proteins, such as poly-(ADP ribose) polymerase (PARP), has been identified during apoptosis in a wide variety of cells (for reviews, see [11,12]). Cleavage of PARP is catalyzed by a zymogen called Yama/CPP32β and leads to inactivation of this enzyme and generation of a 85 kDa apoptotic fragment [13]. Since ceramide was implicated in apoptotic cell death it is interesting that induction of the SM cycle or treatment of cells with cell-permeable ceramides also led to the cleavage of PARP and that this induction of PARP-cleavage was inhibited in bcl-2-transfected cells [14].

In the present study, the effects of two newly synthesized ceramide analogues, *N*-thio-acetylsphingosine (C_2-Cer = S) and FS-5 (for chemical structures, see Fig. 1), on the induction of apoptotic cell death and PARP-cleavage were investigated in Molt 4 and bcl-2-transfected Molt 4 cells. The results showed that treatment of Molt 4 cells with 11.2 μM C_2-Cer = S and 11.2–56.2 μM FS-5 induced cell death and cleavage of PARP after 13 h of incubation. Further experiments demonstrated that PARP-proteolysis was totally abolished in bcl-2-transfected Molt 4 cells.

2. Materials and methods

2.1. Materials

Polyclonal anti-PARP antibodies were a kind gift from Dr. Guy

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Abbreviations: C_2-Cer = O, *N*-acetylsphingosine; C_2-Cer = S, *N*-thio-acetylsphingosine; DAG, diacylglycerol; DETAPAc, diethylenetriaminepentaacetic acid; ECL, enhanced chemiluminescence; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FCS, fetal calf serum; FS-5, 4-dodecanoylamino-decan-5-ol; IC_{50}, half-inhibitory concentration; LD_{50}, half-lethal dosis; Molt 4, human leukemia cell line; PAGE, polyacrylamide gel electrophoresis; PARP, poly-(ADP-ribose)polymerase; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute; SDS, sodium dodecylsulfate; SM, sphingomyelin; TNFα, tumor necrosis factor α; TLC, thin layer chromatography

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Poirier (Quebec, Canada). DAG kinase was prepared as described [14,15]. Silica gel 60 TLC plates, solvents and reagents were from Whatman (Maidstone, UK). The ECL immunoblotting detection reagent was from Amersham (Cleveland, OH). [γ-^{32}P]ATP (110 TBq/mmol) was obtained from NEN-DuPont (Bad Homburg, Germany). Hygromycin B was from Calbiochem (San Diego, CA). Trypan blue solution (0.4%) was from Sigma (St. Louis, MO). C_2-Cer=O was synthesized as described [16]. C_2-Cer=O, C_2-Cer=S and FS-5 stock solutions were prepared in ethanol at concentrations of 10.0 mM, 28.0 mM and 28.1 mM, respectively. The purity of the used ceramide analogues was assessed by high-performance TLC using the solvent system chloroform/methanol (9:1, v/v) and compared with commercially available C_2-Cer=O (Alexis, Läufelfingen, Switzerland). In all cases the purity of the analogues was >98%. Ethanol (vehicle) was added to controls and was present at 0.2% or less in the experiments.

2.2. Synthesis of C_2-Cer=S and FS-5

C_2-Cer=S was synthesized from sphingosine and methyl dithioacetate [17,18]. The reaction product was fully characterized by NMR-spectroscopy and FAB MS. The detailed synthesis will be published elsewhere together with the preparation of additional analogues.

FS-5 (4-dodecanoylamino-decan-5-ol) was synthesized starting from 1-nitrobutane and *n*-hexanal according to [19], followed by acylation with dodecanoic acid. FS-5 was characterized by ^{1}H-NMR-spectroscopy and high-resolution MS.

2.3. Cell culture

Human leukemia Molt 4 vector control cells and a bcl-2-transfected clone [15] were grown in RPMI selection medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 250 μg/ml hygromycin B. For the experiments, vector control cells were used between passages 52 and 69, and bcl-2-transfected cells between passages 60 and 66.

2.4. Cytotoxicity assay

For this, Molt 4 vector control and bcl-2-transfected cells were seeded at a density of 5×10^5 cells/ml in RPMI medium supplemented with 2% FCS. The cells were equilibrated at 37°C for 30 min and then treated with different concentrations of C_2-Cer=S and FS-5. In these experiments, C_2-Cer=O and ethanol were used as positive and negative controls. After 6 and 13 h of incubation, 100 μl of a trypan blue solution (0.4%) were added to 500 μl cell suspension and blue (dead) and unstained (living) cells were counted in a hemacytometer as described previously [20].

2.5. Determination of PARP cleavage

Cleavage of the death substrate PARP was determined as described by Tewari et al. [13]. For this, Molt 4 vector control and bcl-2-transfected cells were seeded at a density of 5×10^5 cells/ml in RPMI medium supplemented with 2% FCS. The cells were equilibrated at 37°C for 30 min and then treated with different concentrations of C_2-Cer=S and FS-5. In these experiments, C_2-Cer=O and ethanol were used as positive and negative controls. After 6 and 13 h of incubation, 1 ml of each cell suspension was transferred to a reaction tube and the samples were centrifuged at $350\times g$, 4°C for 5 min. The supernatants were discarded and 20 μl reducing SDS sample buffer were added to the cell pellets. Proteins were separated by 6% SDS-PAGE [21] and transferred to nitrocellulose membrane. Then, PARP and the 85 kDa fragment of PARP were detected with a polyclonal rabbit anti-PARP antibody (1:2000) and the ECL immunoblotting detection kit from Amersham was used to visualize the proteins in question [22].

2.6. Determination of cellular ceramide

The ceramide assay was carried out by the method of Preiss et al. [23] using DAG kinase as described previously [24] with some modifications to simultaneously determine ceramide and diacylglycerol in the samples. In detail, Molt 4 vector control cells were seeded at a density of 5×10^5 cells/ml in RPMI containing 2% FCS and equilibrated at 37°C for 30 min. Then, cells were treated for 3 and 5 h with 20 μM etoposide, 11.2 μM C_2-Cer=S, 11.2 μM FS-5 or 0.2% ethanol. After incubation, cells were collected in a Beckmann CS-6 centrifuge at 1000 rpm for 5 min and lipids were extracted according to Bligh and Dyer [25]. The chloroform phase was transferred to glass tubes, dried under nitrogen and redissolved in 1 ml of chloroform/methanol (9:1, v/v). Subsequently, 800 μl were taken for ceramide measurements and 200 μl were taken for the determination of phospholipids by the phosphate assay of Ames and Dubin using ammonium molybdate and ascorbic acid [26]. For ceramide measurements, the dried lipid extract was solubilized in 20 μl of a detergent solution containing 7.5% octyl-β-D-glucoside and 25 mM dioleoyl phosphatidylglycerol in 1 mM diethylenetriaminepentaacetic acid (DETAPAc) by sonication. 70 μl of assay buffer containing 0.1 M imidazole-HCl, pH 6.6, 0.1 M NaCl, 25 mM $MgCl_2$, 2 mM [ethylene-bis(oxyethylenenitrilo)] tetraacetic acid (EGTA), 3 mM dithiothreitol and 5 μg *sn*1,2-DAG kinase from *E. coli* was added. The reaction was started by addition of 10 μl of a 2.5 mM [γ-^{32}P]ATP solution (116 000 dpm/nmol) in 0.1 M imidazole-HCl, pH 6.6, containing 1 mM DETAPAc. After incubation at 20°C for 30 min, the reaction was stopped by adding 2 ml of methanol, 1 ml of chloroform and 0.7 ml of water. The samples were stirred on a vortex mixer and phase separation was accomplished by addition of 1 ml of chloroform and 1 ml of water. After centrifugation, the upper phase was aspirated and 1.5 ml of the lower phase were transferred to another tube. The samples were dried under nitrogen and redissolved in 100 μl of chloroform/methanol (9:1, v/v). Then, 20 μl of each sample were spotted on silica gel 60 TLC plates. Plates were developed with chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, v/v) and subjected to autoradiography. Marked spots co-chromatographing with ceramide phosphate standards were scraped into scintillation vials and radioactivity in the silica gel scrapings was determined by liquid scintillation spectrometry. The amount of ceramide present in the samples was calculated from the sample volume and a ceramide standard curve. Determination of ceramide was linear over the range 20–640 pmol.

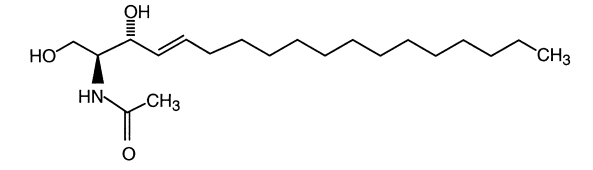
2.7. Other procedures

Statistical comparisons were made in these studies with Student's *t*-test.

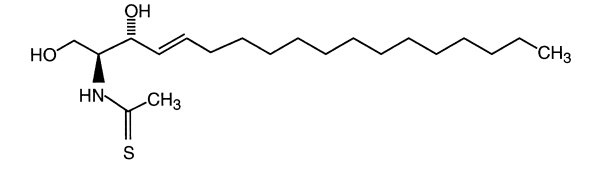
3. Results and discussion

3.1. Cytotoxic effects of C_2-Cer=S and FS-5 in Molt 4 cells

One possible mechanism mediating the biological properties of ceramide analogues might be a simple toxic effect, e.g. cellular lysis or unspecific disturbance of cellular membranes



N-Acetylsphingosine



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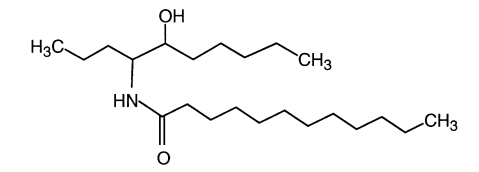

FS-5

Fig. 1. Chemical structures of C_2-Cer=O, C_2-Cer=S and FS-5.

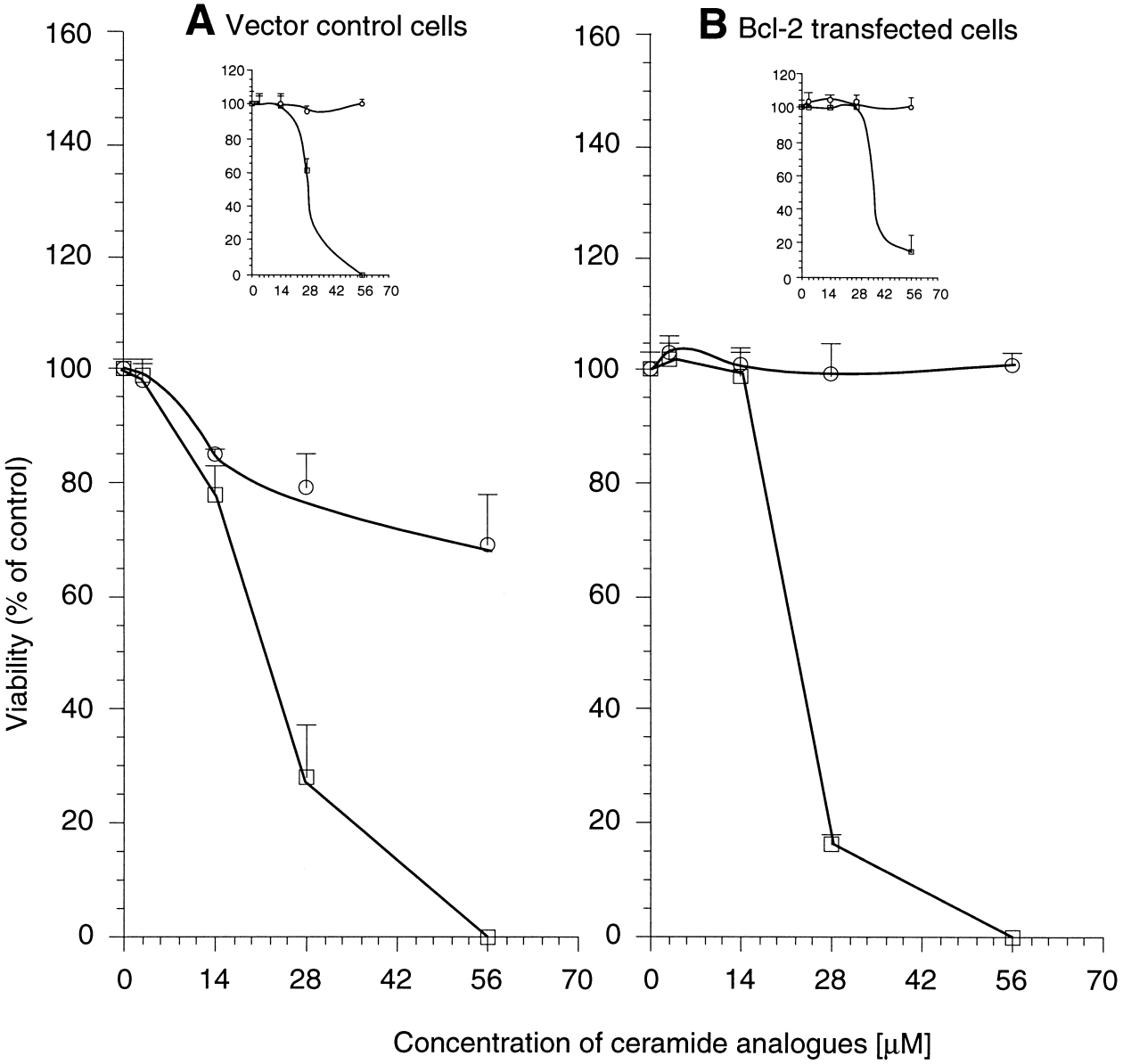

Fig. 2. Cytotoxicity of C_2-Cer=S and FS-5 in vector control and bcl-2 transfected Molt 4 cells. 5×10^5 vector control cells (A) and bcl-2-transfected cells (B) were treated with different concentrations of C_2-Cer=S (□) and FS-5 (○). After 6 h (insert) or 13 h, viability of the cells was determined by trypan blue exclusion as described in Section 2. Values are given in % of control ± SD ($n=4$).

as has been described at high concentrations of the synthetic sphingolipid, *N*-acetyl-*erythro*-sphingosine-1-phosphocholine [20]. On the other hand, elevation of intracellular ceramide levels has been observed in many different forms of apoptosis and treatment of cells with truncated ceramide analogues was sufficient to induce apoptosis [27]. In our experimental system, FS-5 did not have an effect on viability after 6 h of incubation whereas C_2-Cer=S showed acute toxic effects with a LD_{50} of 28.0 μM (Fig. 2A, insert). At longer incubation periods of 13 h, the cytotoxic effect of C_2-Cer=S was even more pronounced ($LD_{50}=22.4$ μM) and incubation of Molt 4 vector control cells with 56.2 μM FS-5 significantly decreased viability to about 70% of control (Fig. 2A). As compared with the well known ceramide analogue, *N*-acetylsphingosine (C_2-Cer=O) which decreased viability of Molt 4 cells to 11 ± 7% at 23.4 μM, C_2-Cer=S had a cytotoxic capacity of approximately 61% and FS-5 of 24%. These experiments revealed that both ceramide analogues are biologically active compounds in Molt 4 cells.

3.2. *Induction of PARP cleavage by C_2-Cer=S and FS-5 in Molt 4 cells*

Recently, it has been demonstrated that treatment of cells with truncated ceramides led to the proteolytic cleavage of the death substrate PARP [14] (see also Fig. 3). Therefore, the induction of PARP cleavage upon treatment with C_2-Cer=S and FS-5 was investigated in Molt 4 cells. As shown in Fig. 3, a weak signal of the 85 kDa fragment of PARP is seen after 6 h of incubation with 11.2 μM C_2-Cer=S and 11.2–56.2 μM FS-5 when the cell lysates were analyzed by Western blot. After 13 h of incubation, the 85 kDa fragment accumulated in C_2-Cer=S- and FS-5-treated cells thereby confirming that the cells were driven into apoptosis. In support of the data on viability described in the previous section only concentrations

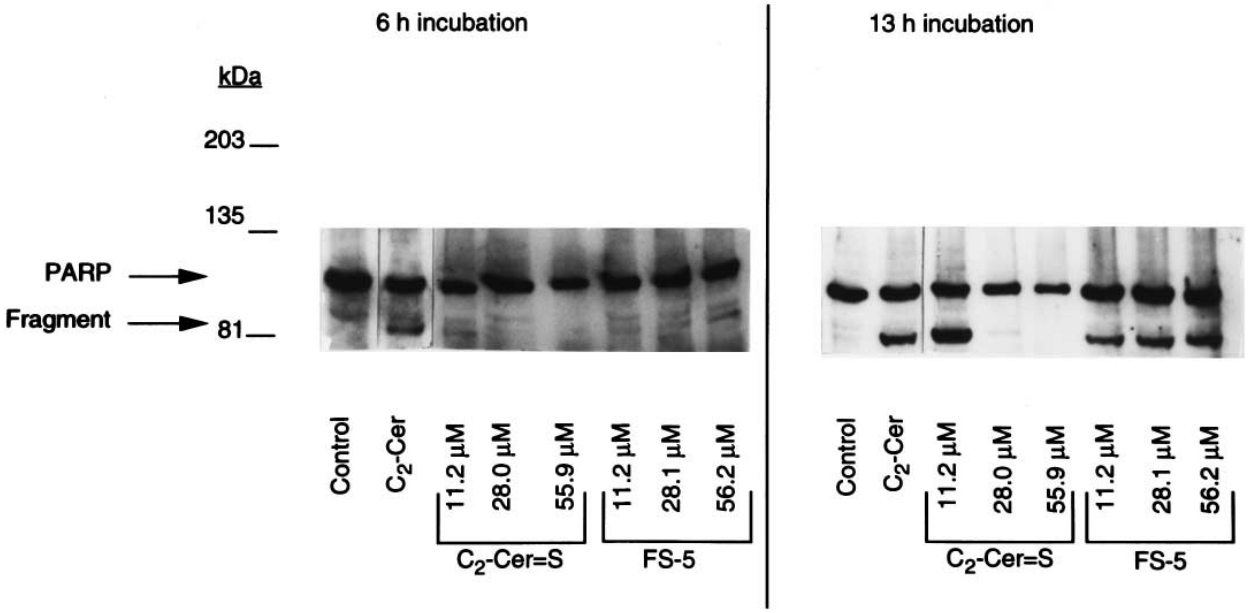

Fig. 3. Induction of PARP cleavage by C_2-Cer = S and FS-5 in Molt 4 cells. 5×10^5 vector control cells were treated with 11.7 µM C_2-Cer = O or with different concentrations of C_2-Cer = S or FS-5. After 6 h and 13 h of incubation, cleavage of the death substrate PARP was assessed by Western blot analysis as described in Section 2. The experiment was repeated and similar results were obtained.

< 14 µM C_2-Cer = S induced PARP-cleavage whereas the 85 kDa fragment was not observed in cells treated with C_2-Cer = S concentrations of 28.0 and 55.9 µM (Fig. 3). In additional experiments, we used C_2-Cer = S concentrations below 28.0 µM, and 11.2 µM was found to be the optimum concentration (data not shown). In contrast, FS-5 showed concentration-dependent effects on PARP-cleavage and 56.2 µM FS-5 was the most efficient concentration used. Interestingly, it was reported that only ceramides containing a 4,5-trans-double bond induce apoptosis [16]. Thus, FS-5 is the first compound with pro-apoptotic properties lacking this structural feature.

3.3. *Effects of C_2-Cer = S and FS-5 on endogeneous ceramide levels in Molt 4 cells*

Generation of ceramides has been reported as a common cellular response of different cell types after challenge with death-inducing agents, such as TNFα [28,29], interleukin-1β [30], ionizing radiation [31] and therapeutic agents, such as retinoic acid [3] and 1α,25-dihydroxyvitamin D_3 [32]. To exclude the possibility that the induction of PARP-cleavage by C_2-Cer = S and FS-5 is indirectly mediated by an increase in endogeneous ceramide levels, cellular ceramide was measured in extracts from Molt 4 cells which had been treated with 11.2 µM of the respective ceramide analogue for 3 and 5 h using DAG kinase. However, neither C_2-Cer = S nor FS-5 significantly increased ceramide levels in Molt 4 cells (Table 1).

Table 1
Effects of etoposide, C_2-Cer = S and FS-5 on endogenous ceramide levels in Molt 4 cells

Treatment	Ceramide (% of control)	
	Incubation (3 h)	(5 h)
Control	100 ± 5	100 ± 1320
µM etoposide	95 ± 23	149 ± 6*
11.2 µM C_2-Cer = S	88 ± 30	113 ± 18
11.2 µM FS-5	98 ± 21	104 ± 16

Molt 4 vector control cells were incubated for 3 and 5 h in medium containing 20 µM etoposide, 11.2 µM C_2-Cer = S, 11.2 µM FS-5 or 0.2% EtOH as control. After treatment, cells were collected and ceramide and lipid phosphate were analyzed as described in Section 2. Values of endogenous ceramide are given in % of control ± SD ($n = 3$). Ceramide levels in control cells were 4.0 ± 1.1 pmol/nmol phosphate.
*Significantly different from controls at $P < 0.01$.

3.4. *Bcl-2 antagonizes C_2-Cer = S- and FS-5-induced cytotoxicity and PARP cleavage*

The apoptosis inhibitors Bcl-2 and Bcl-XL have been shown to block ceramide-induced cell death in lymphoid cell lines [14,27,33,34]. To study the specificity of C_2-Cer = S and FS-5-induced cell death we used Molt 4 cells which had been transfected with bcl-2. As shown in Fig. 2B, bcl-2-transfected Molt 4 cells were less sensitive to treatment with C_2-Cer = S and FS-5. In the case of FS-5 the cytotoxic effect occurring after 13 h in control vector cells was totally blocked in bcl-2-transfected cells. On the other hand, only the cytotoxic effect induced by 14.0 µM C_2-Cer = S was antagonized in bcl-2-transfected cells whereas 28.0 and 55.9 µM C_2-Cer = S still killed the cells (Fig. 2B). This failure of anti-apoptotic proteins, such as Bcl-XL, to block the cytotoxic effect of truncated ceramides at high concentrations has also been observed in endothelial cells [34]. Further experiments revealed that C_2-Cer = S and FS-5-induced cleavage of PARP was totally abolished in bcl-2-transfected Molt 4 cells (Fig. 4) suggesting that overexpression of Bcl-2 interrupts the pro-apoptotic effects of both ceramide analogues. Since it was shown previously that bcl-2-transfection did not interfere with ceramide-production via the sphingomyelin cycle [15] these data are consistent with the hypothesis that Bcl-2 acts downstream of ceramide formation.

In summary, we provide data about two newly synthesized ceramide analogues with different biological activities. The sulfur-containing C_2-Cer = S induced apoptotic cell death at low concentrations as shown by PARP-cleavage. However, at

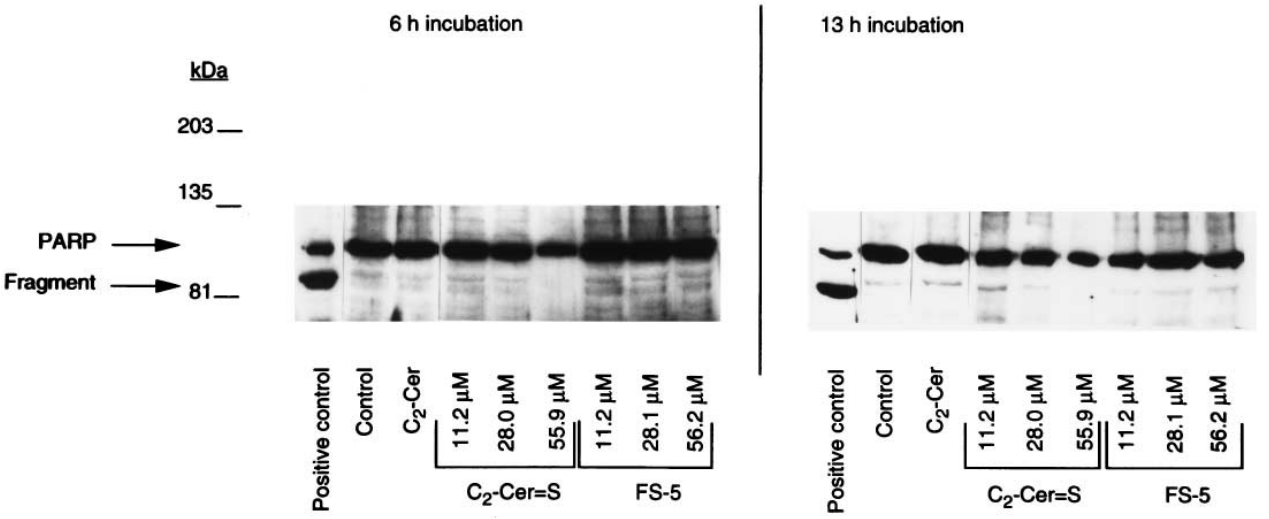

Fig. 4. Inhibition of C_2-Cer = S- and FS-5-induced PARP cleavage in bcl-2 transfected Molt 4 cells. 5×10^5 bcl-2-transfected cells were treated with 11.7 μM C_2-Cer = O or with different concentrations of C_2-Cer = S or FS-5. Additionally, vector control cells that had been treated with 11.7 μM C_2-Cer = O were used as positive controls. After 6 h and 13 h of incubation, cleavage of the death substrate PARP was assessed by Western blot analysis as described in Section 2. The experiment was repeated and similar results were obtained.

concentrations ≥28.0 μM the cell ghost-like morphology of the cells indicated membrane damage and the cytotoxic effects of C_2-Cer = S were no longer accompanied by an induction of PARP-cleavage and not inhibited by Bcl-2 thereby suggesting cell lysis. On the other hand, the ceramide analogue FS-5 which is blocked at the C1-position of the sphingosine backbone specifically induced apoptosis at concentrations ≤56.2 μM. From these data, we suggest that the concept of using synthetic ceramide analogues may lead to significant insights into ceramide-mediated signalling.

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