Inhibition of sphingolipid induced apoptosis by caspase inhibitors indicates that sphingosine acts in an earlier part of the apoptotic pathway than ceramide

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Abstract Caspases are specific proteases involved in apoptosis, and their inhibition by specific peptide inhibitors can inhibit apoptosis. With these inhibitors we examined the relationship of caspases and sphingolipids involved in the induction of apoptosis of human leukemic HL60 cells. We have previously shown that sphingosine (Sph) and its methylated derivative dimethylsphingosine (DMS) effectively induce apoptosis in HL60 cells. Using these lipids as well as ceramide analogues we found both similarities and differences in the caspase involvement in apoptosis induced by the two distinct lipid types. The wide-spectrum caspase inhibitor Z-VAD-FMK and Z-DEVD-FMK, an inhibitor of the downstream caspases 3 (CPP32, Yama) and 7, both inhibited apoptosis induced by all the lipids tested. Z-AAD-FMK which inhibits the serine protease Granzyme B, inhibited Sph/DMS induced apoptosis, but little or no effect on ceramide induced apoptosis. Granzyme B shares a substrate sequence preference with upstream caspases capable of activating themselves and other caspases downstream. Z-IETD-FMK, which inhibits caspase 8/FLICE also inhibited Sph/DMS induced apoptosis with no inhibition of apoptosis induced by either ceramide. Together, these data indicate that Sph/DMS act independently from ceramide in the apoptosis pathway and further suggest that Sph/DMS act earlier in the pathway than ceramide and are involved upstream of even the early proteases, whereas the point of action for ceramide is downstream of the early proteases but upstream from the late caspases.

Key words: Apoptosis; Caspase; Sphingosine; *N*,*N*-dimethylsphingosine; Ceramide

1. Introduction

The past few years have seen an explosion of information regarding the demise of cells by a specific set of self-programmed events termed apoptosis that we are now just beginning to understand. Apoptosis involves a distinct set of morphological and biochemical changes, including nuclear condensation, DNA fragmentation, and membrane blebbing, culminating in cell death and compact packaging of the cellular debris into apoptotic bodies [1]. One set of players found to be important in many steps of apoptosis is a group of enzymes known as cysteine-directed asparagine proteases (caspases). The first such protease identified as being involved in apoptosis was the product of the ced-3 gene in the nematode *C. elegans*, followed by its mammalian homologue interleukin 1 converting enzyme (ICE). Since then, ten mammalian homologues of ICE have been identified and designated as caspases 1–10 [2,3].

Recent discoveries have greatly increased our understanding of this family of enzymes and their preferred substrates. Caspases, which are normally present as zymogens and cleaved to form active heterodimers, can be divided into three groups based on preference for tetrapeptide sequences with the requisite asparagine in the first position (and a bias towards a glutamine in the third) [4,5]. Group I includes caspases 1 (ICE), 4 and 5, preferring the substrate sequence WEHD. Group II which includes caspases 3 (CPP32/Yama) and 7, must have asparagine in the 4th as well as the 1st position (DEXD). Finally, Group III caspases, which include caspases 8 (FLICE) and 6 (Mch 2) are more tolerant of various amino acids in position 4, but they prefer those with large aliphatic side chains (XEH/TD). Interestingly, this sequence specificity is shared by the serine protease Granzyme B (GraB). Furthermore, the optimal recognition sequences for Group III caspases (and GraB) are similar to cleavage sites within their own, or other, caspase prodomains, implicating them in autocatalytic and initial activation of an enzyme cascade [4,5]. Based on the known sequence preferences, tetrapeptide inhibitors have been synthesized and are now available commercially.

Apoptosis can be initiated extracellularly, as in receptor ligand binding, or triggered by internal factors. Short-chain ceramide analogues have been reported to induce apoptosis in hematopoietic cell lines such as HL60 [6]. We and others have recently shown that exogenously added sphingosine (Sph) and its dimethylated derivative dimethylsphingosine (DMS) can induce apoptosis in a number of cell lines including HL60 [7–10] and myocytes [11,12]. Evidence suggests that these two types of lipids work differently within the apoptotic pathway [13–15]. Still, the precise mechanisms involved in the induction of apoptosis by these sphingolipids are unknown [16]. In the present study we further investigate these mechanisms using tetrapeptide inhibitors to examine the relationship of caspases and sphingolipid induced apoptosis. We provide evidence here that suggests that Sph and DMS activate the apoptosis pathway early, prior to activation of early caspases, while the ceramide analogues trigger the pathway downstream from some caspases but upstream from executor caspases.

2. Materials and methods

2.1. Reagents

The protease inhibitors (Table 1) Ac-YVAD-CHO, Z-VAD-FMK, Z-DEVD-FMK, and Z-AAD-CMK were purchased from Oncogene Research Products (Cambridge, MA), and Z-IETD-FMK from Enzyme Systems Products (Livermore, CA). All inhibitors were kept as

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stock solutions of 20 mM in DMSO and added directly to cell cultures. Fumonisin B_1 was purchased from Sigma Chemical Co, St. Louis, MO.

Sph, C2-ceramide, and C6-ceramide were purchased from Sigma Chemical Co. DMS was prepared as previously described [17]. (1S,2R)-D-erythro-2-(*N*-Myristoylamino)-1-phenyl-1-propanol (MAPP) was prepared according to published reports [18].

2.2. Cell culture

HL60 cells were grown in RPMI/HEPES supplemented with sodium pyruvate, sodium glutamate, penicillin-streptomycin, and 10% heat inactivated (30 min, 55°C) fetal bovine serum (Hyclone, Logan, Utah). Assays were performed on 2×10^5 cells/ml in 24-well tissue culture plates.

For apoptosis studies, cells were pretreated for 1 h with protease inhibitors. Lipids were diluted in ethanol/water, 50/50, and added to cells (ethanol concentration was $<0.01\%$) for 5.5 h. Cells were harvested and stained as previously described [8]. Briefly, suspension cultures were centrifuged, washed in Hanks' salt solution with Mg^{2+} and Ca^{2+}, and resuspended in lysis buffer (100 mM Na citrate with 0.1% Triton X-100) containing 50 μg/ml propidium iodide then stored overnight at 4°C in the dark. Cells were analyzed using a Becton Dickinson FACScan (San Jose, CA). Apoptosis was confirmed by DNA fragmentation patterns using agarose gel electrophoresis [8].

3. Results and discussion

3.1. Sphingosine and ceramide induce apoptosis without interconversion of the lipids

Since sphingosine and ceramide are catabolites of each other, we tested the effect on apoptosis of blocking their conversion. As we previously reported [8], blocking ceramide synthesis from sphingosine by the addition of the fungal agent Fumonisin B_1 (25 μM) did not inhibit sphingosine from inducing apoptosis (Fig. 1A). Similarly, DMS, which cannot be metabolized to ceramide, causes apoptosis. This is in keeping with the ability of DMS to cause apoptosis since DMS cannot be metabolized to ceramide. Additionally, inhibiting the degradation of C6-ceramide to sphingosine by the ceramidase inhibitor MAPP (10 μM) did not inhibit C6-ceramide induced apoptosis (Fig. 1B). These data indicate that Sph/DMS and ceramide analogues act independently of conversion in inducing apoptosis.

3.2. Inhibition of sphingolipid induced apoptosis by inhibitors of execution phase caspases

The irreversible inhibitor Z-VAD-FMK inhibits a wide spectrum of caspases including the later caspases 3 and 7, and, to a lesser extent 1 and 4 [19], but also caspase 8 [20] but does not inhibit Granzyme B activity [21]. Z-VAD-FMK (25 μM) was found to strongly inhibit apoptosis induced by all four sphingolipids (Fig. 2). Apoptosis induced by Sph or DMS was inhibited to baseline levels, and that induced by C2- or C6-ceramide analogues by 60 and 75% respectively.

To further study the susceptibility of sphingolipid induced apoptosis to the different inhibitors, we compared dose curves of each inhibitor on the induction of apoptosis. In Z-VAD-FMK pretreated cells, Sph and DMS induced apoptosis was significantly inhibited at 5 μM and almost completely inhibited at 10 μM and 25 μM respectively, but C6-ceramide and, even more so, C2-ceramide induced apoptosis had much less dramatic decreases, needing 50 μM to inhibit completely (Fig. 3A). This increased sensitivity could be explained by the ability of Z-VAD to inhibit a broad range of caspases. Apoptosis triggered early in the pathway by Sph/DMS, as suggested below, would likely involve more caspases overall, giving Z-VAD more targets to inhibit, than in apoptosis triggered downstream by C2-ceramide.

Additionally, we tested sphingolipid induced apoptosis in the presence of the inhibitor Z-DEVD-FMK which mainly inhibits Group II caspases, especially the downstream execution phase caspase 3 and, to a lesser extent 7 [3]. Apoptosis induced by Sph/DMS and both ceramide analogues was inhibited by 75 μM Z-DEVD-FMK, with Sph and DMS induced apoptosis nearly completely inhibited, and that induced by the ceramides inhibited at 60% for C2-ceramide and 70% for C6-ceramide (Fig. 2). We also found this inhibition to be dose dependent. Apoptosis induced by Sph and DMS was

Table 1
Summary of caspase inhibitors used in this study and their effect on sphingolipid induced apoptosis

Inhibitor sequence	Target caspases	Caspase group	Optimal recognition sequence	Examples of substrates	Substrate sequence	Inhibition of apoptosis induced by[a]			
						Sph	DMS	C6Cer	C2Cer
Ac-YVAD-CHO	1 (ICE), 4	I	(W/L)EHD	Pro-IL1b	YVHD	−	−	−	−
Z-VAD-FMK	1 (ICE), 4	I	(W/L)EHD	Pro-IL1b	YVHD	++++	++++	+++	+++
	3, (CPP32, Yama), 7	II	DEVD	PARP	DEVD				
				DNA-dep kinase	DEVD				
			PKC	PKCd					
	8 (FLICE)	III	LETD	Caspase 3					
Z-DEVD-FMK	3, (CPP32, Yama), 7	II	DEVD	PARP	DEVD	++++	++++	+++	+++
	7			DNA-dep kinase	DEVD				
Z-AAD-CMK	Granzyme B	III-like	IEPD	Caspase 3 proenzyme	DEVD	+++	+++	+	−
				Caspase 7 proenzyme	DEVD				
Z-IETD-FMK	8 (MACH, FLICE)	III	LETD	Caspase 3 proenzyme	DEVD	++	+++	−	−
				Caspase 8 proenzyme	VETD				

[a]Determined by flow cytometry. ++++, completely inhibited; +++, strongly inhibited; ++, moderately inhibited; +, weakly inhibited; −, not inhibited.

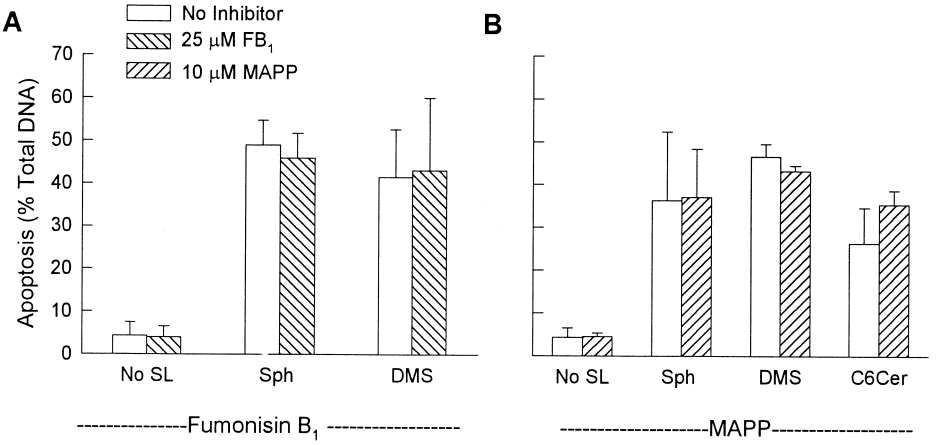

Fig. 1. Effect of lipid enzyme inhibitors on sphingolipid induced apoptosis in HL60 cells. Cells were preincubated for 1 h with (A) 25 μM Fumonisin B_1 or (B) 10 μM MAPP then treated for 5.5 h with 20 μM Sph or DMS or 50 μM C6-ceramide (C6Cer) then analyzed for apoptosis by flow cytometry as described in Section 2. Data are of two or three separate experiments, mean ± S.D.

inhibited identically at 60–65% inhibition in 20 μM Z-DEVD-FMK, but that of the ceramides was only 10–15% inhibited. However, at 75 μM all are inhibited more than 50% (Fig. 3B). This disparity might reflect either a difference in the involvement of caspases 3 and 7 in Sph/DMS vs. ceramide induced apoptosis, or, as with Z-VAD, may represent inhibition of multiple caspases in Sph/DMS induced apoptosis, as this irreversible inhibitor can inhibit other caspases weakly over longer periods of time [3]. Regardless, the abrogation of sphingolipid induced apoptosis by inhibition of these downstream, executor caspases indicates that all the sphingolipids tested act upstream from these caspases.

The inhibitor Ac-YVAD-CHO selectively inhibits Group I caspases, which are now believed to have little involvement in apoptosis and to be more associated with the pro-inflammatory cytokine processes. Sphingolipid induced apoptosis was not at all affected by the addition of this inhibitor even in high concentrations (Table 1). Together, these results confirm that caspases, specifically apoptosis associated caspases, are involved in sphingolipid induced apoptosis, and that these sphingolipids act upstream of the execution phase caspases.

3.3. *Inhibitors of upstream caspases inhibit Sph/DMS induced but not ceramide induced apoptosis*

To examine the roles of sphingolipids in the early apoptosis pathway, we used inhibitors of GraB and Group III caspases. GraB is a serine protease involved in NK cell killing which shares substrate specificity with the caspases in Group III, caspases which include caspase 8 (FLICE) and caspase 6. Granzyme B and Group III caspases act upstream in the apoptosis pathway and can cleave the zymogen form of other caspases (and in some cases their own) [4]. At 50 μM the GraB inhibitor Z-AAD-FMK inhibited Sph and DMS induced apoptosis by 65 and 70% respectively (Fig. 2). Likewise, the dose curve of Z-AAD-FMK showed a similar slope for inhibiting Sph and DMS induced apoptosis, but the Sph induced is more sensitive (Fig. 3C), a difference consistent with the derivative DMS causing more apoptosis in HL60 cells than its parent compound Sph, perhaps due to stronger binding or lack of metabolism of DMS. On the other hand, there was only a small amount of inhibition in C6-ceramide induced apoptosis and no inhibition in C2-ceramide induced apoptosis in the presence of even the highest concentration of Z-AAD-FMK (Figs. 2 and 3C). The slightly higher amount of apoptosis induced by C6-ceramide as opposed to C2-ceramide and the modest inhibition seen in C6-ceramide induced apoptosis may reflect the processing of some C6-ceramide by ceramidase to Sph. C6-ceramide has been shown to be more susceptible to cellular metabolism than C2-ceramide ([22], Yang, L., personal communication). Metabolism of C6-ceramide may, in fact, account for the repeated differences in the susceptibility to the inhibitors between the two analogues.

As there is presumably no GraB in this cell assay system, the peptide inhibitor is most likely inhibiting a member of the caspase family with similar substrate specificities, that is one in Group III. We therefore tested the effects of an inhibitor of the initiator caspase 8 [20] on sphingolipid induced apoptosis. The inhibitor Z-IETD-FMK inhibited both Sph and DMS induced apoptosis by up to 50%, but neither ceramide analogue was inhibited from inducing apoptosis (Fig. 2).

The inhibition of Sph/DMS induced apoptosis by Z-IETD-FMK was not as dramatic as that seen with the other inhibitors, but was linear and significant at each dose (Fig. 3D). This most likely represents the kinetics involved in blocking a

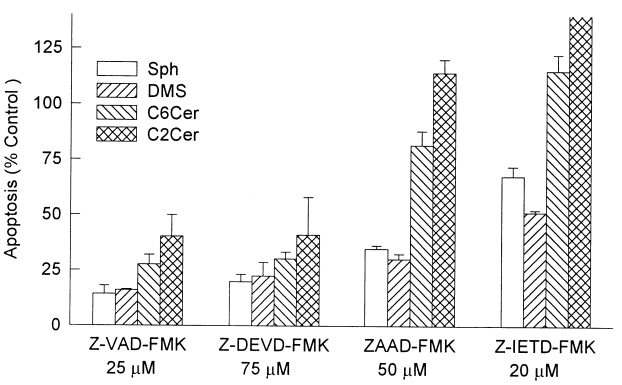

Fig. 2. Effect of caspase inhibitors on sphingolipid induced apoptosis in HL60 cells. Cells were preincubated for 1 h with the indicated inhibitor and treated for 5.5 h with 20 μM Sph or DMS, or 50 μM C2-ceramide (C2Cer) or C6-ceramide (C6Cer) then analyzed by flow cytometry for apoptosis as described in Section 2. Data are of two or three separate experiments, mean ± S.E.

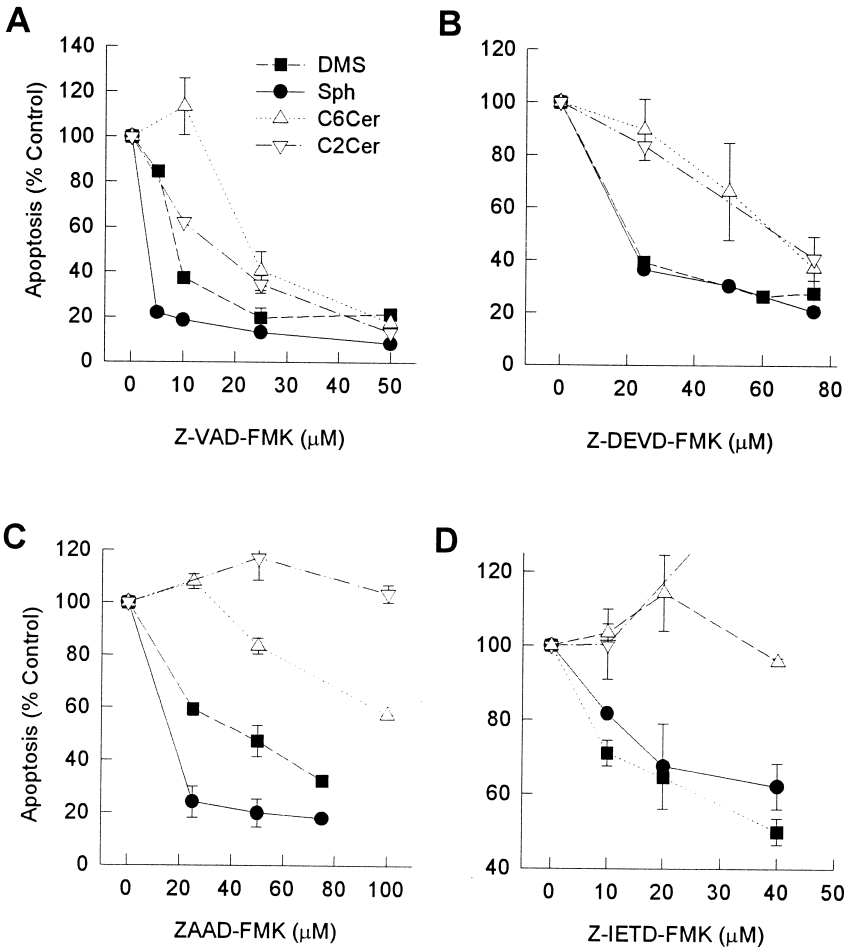

Fig. 3. Dose response curves for inhibition of sphingolipid induced apoptosis by caspase inhibitors in HL60 cells. Cells were preincubated with the indicated concentrations of each inhibitor for 1 h and then treated as in Fig. 2. Data are of two or three separate experiments, mean ± S.E., error bars not visible are within the data point.

single upstream protease in such a cascade rather than multiple enzymes. No inhibition was seen with either ceramide analog at even the highest concentrations, with some increased apoptosis. These data suggest that Sph/DMS act early in the apoptosis pathway whereas ceramide functions downstream of the early Group III caspases but upstream of those in Group II.

During receptor-generated apoptosis caspase 8/FLICE binds to FADD, a death associated molecule considered to be a common link in receptor-generated apoptosis [23], and then cleaves itself, initiating a caspase cascade. GraB also acts as an initiator caspase in the target cell, and is known to share some substrates with caspase 8, including caspase 8 itself as well as downstream caspases [2]. Thus, GraB (or some other Group III caspase) could act in parallel with, or cleave and activate caspase 8 to initiate the cascade. Other non-caspase proteases cannot be ruled as having a role in apoptosis, perhaps similar to that of GraB. Proteases have been shown to cleave caspase zymogens at alternative sites to give rise to active forms [2]. It is quite possible that Sph is initiating the apoptosis pathway through a non-caspase protease not yet identified which is able to cleave caspase 8.

Caspase 8 and GraB [24] are both inhibited by the viral protein CrmA, which shows surprising affinity for caspase 8 but not other death-related caspases [25], but ceramide induced apoptosis is not inhibited by CrmA [26,27]. On the other hand, the baculovirus protein p35 which inhibits a variety of caspases including caspase 3, reportedly inhibited ceramide analogue induced apoptosis [26,27]. Additionally, it has recently been reported that ceramide does not induce activation of caspase 8/FLICE, but does activate both caspase 3 and 7 [28]. Together, these studies support our conclusion that ceramides induce apoptosis downstream from the initiator phase, but upstream from the execution phase caspases.

The results presented here on the relationship between various caspase inhibitors and sphingolipid induced apoptosis are summarized in Table 1. In conclusion, these data strongly suggest that Sph/DMS act separately from ceramide in the apoptosis pathway and further imply that Sph/DMS act earlier than ceramide in the apoptotic pathway, upstream from even the early caspases, whereas the point of action for ceramide is downstream of the initiating caspases, but upstream from the executors.

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References

[1] Stellar, H. (1995) Science 267, 1445–1449.

[2] Salvesen, G.S. and Dixit, V.M. (1997) Cell 91, 443–446.
[3] Villa, P., Kaufmann, S.H. and Earnshaw, W.C. (1997) Trends Biochem. Sci. 22, 388–393.
[4] Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T. and Nicholson, D.W. (1997) J. Biol. Chem. 272, 179107–179110.
[5] Miller, D.K. (1997) Sem. Immun. 9, 35–49.
[6] Hannun, Y.A. and Obeid, L.M. (1995) Trends Biochem. Sci. 20, 73–77.
[7] Ohta, H., Sweeney, E.A., Masamune, A., Hakomori, S. and Igarashi, Y. (1995) Cancer Res. 55, 691–697.
[8] Sweeney, E.A., Shirahama, T., Sakakura, C., Hakomori, S. and Igarashi, Y. (1996) Int. J. Cancer 66, 358–366.
[9] Jarvis, W.D., Fornari, F.A., Traylor, R.S., Martin, H.A., Kramer, L.B., Erukula, R.K., Bittman, R. and Grant, S. (1996) J. Biol. Chem. 271, 8275–8284.
[10] Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P.G., Coso, O.A., Gutkind, J.S. and Spiegel, S. (1996) Nature 381, 800–803.
[11] Krown, K.A., Page, M.T., Nguyen, C., Zechner, D., Gutierrrez, V., Comstock, K.L., Glembotski, C.C., Quintana, P.J.E. and Sabadini, R.A. (1996) J. Clin. Invest. 98, 2854–2865.
[12] Oral, H., Dorn, G.W. and Mann, D.L. (1997) J. Biol. Chem. 272, 4836–4842.
[13] Sakakura, C., Sweeney, E.A., Shirahama, T., Solca, F., Kohno, M., Hakomori, S., Fisher, E.H. and Igarashi, Y. (1997) Int. J. Oncol. 11, 31–39.
[14] Jarvis, W.D., Fornari, F.A., Auer, K.L., Freemerman, A.J., Szabo, E., Birrer, M.J., Johnson, C.R., Barbour, S.E., Dent, P. and Grant, S. (1997) Mol. Pharmacol. 52, 935–947.
[15] Okazaki, T., Sawai, H., Tashima, M., Sawada, H. and Domae, N. (1995) Glycoconjug. J. 12, 579.
[16] Igarashi, Y. (1997) J. Biochem. 122, 1080–1087.
[17] Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K. and Racker, E. (1989) Biochemistry 28, 6796–6800.
[18] Bielawska, A., Greenberg, M.S., Perry, D., Jayadev, S., Shayman, J.A., McKay, C. and Hannun, Y.A. (1996) J. Biol. Chem. 271, 12646–12654.
[19] Margolin, N., Raybuck, S.A., Wilson, K.P., Chen, W., Fox, T., Gu, Y. and Livingston, D.J. (1997) J. Biol. Chem. 272, 7223–7228.
[20] Medema, J.P., Scaffidi, C., Kischkel, F.C., Shevchenko, A., Mann, M., Krammer, P.H. and Peter, M.E. (1997) EMBO J. 16, 2794–2804.
[21] Sarin, A., Wu, M. and Henkart, P.A. (1996) J. Exp. Med. 184, 2445–2450.
[22] Bernardo, K., Hurwitz, R., Zenk, T., Desnick, R.J., Ferlinz, K., Schuchman, E.H. and Sandhoff, K. (1995) J. Biol. Chem. 270, 11098–11102.
[23] Chinnaiyan, A.M. and Dixit, V.M. (1997) Sem. Immun. 9, 69–76.
[24] Quan, L.T., Caputo, A., Bleackly, R.C., Pickup, D.J. and Salvesen, G.S. (1995) J. Biol. Chem. 270, 10377–10379.
[25] Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V.M. and Salvesen, G.S. (1997) J. Biol. Chem. 272, 7797–7800.
[26] Kojima, H. and Datta, R. (1996) Oncol. Res. 8, 497–501.
[27] Geley, S., Hartmann, B. and Kofler, R. (1997) FEBS Lett. 400, 15–18.
[28] Cuvillier, O., Rosenthal, D.S., Smulson, M.E. and Spiegel, S. (1998) J. Biol. Chem. 273, 2910–2916.