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Role of antioxidants in the *O*-hydroxyethyl-D-(Ser)^8-cyclosporine A (SDZ IMM125)-induced apoptosis in rat hepatocytes

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Abstract

The mechanisms underlying the apoptotic activity of the immunosuppressive drug cyclosporine A and its *O*-hydroxyethyl-D-(Ser)^8-derivative SDZ IMM125 in rat hepatocytes are not yet fully understood. It was the purpose of the present study to investigate the role of anti- and pro-oxidants and of caspase-3 and intracellular Ca^{2+} in SDZ IMM125-induced apoptosis in rat hepatocytes. SDZ IMM125 induced an increase in chromatin condensation and fragmentation, and the activation of caspase-3. Supplementing the cell cultures with the antioxidants, D,L-α-tocopherol-polyethylene-glycol-1000-succinate, ascorbic acid, and the reducing agent, dithiothreitol, significantly inhibited the SDZ IMM125-mediated increase in chromatin condensation and fragmentation, and caspase-3 activity. D,L-α-tocopherol-polyethylene-glycol-1000-succinate and dithiothreitol caused significant inhibition on SDZ IMM125-mediated cellular Ca^{2+} uptake. The glutathione synthetase inhibitor, buthionine sulfoximine, increased SDZ IMM125-mediated caspase-3 action in parallel to chromatin condensation and fragmentation as well as Ca^{2+} influx. Supplementation the culture medium with the intracellular Ca^{2+} chelator bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid as well as omission of calcium in the medium reduced SDZ IMM125-induced apoptosis whereas the calcium supplementation of the culture medium elevated SDZ IMM125-induced apoptosis. Calcium antagonists inhibited SDZ IMM125-induced caspase-3 activation. Our data indicate that SDZ IMM125-mediated apoptosis in rat hepatocytes can be inhibited by antioxidants, and that the intracellular redox-state can act as a modulator of cytotoxicity and apoptosis. Further, the results suggest that SDZ IMM125-induced uptake of extracellular calcium is also a redox-sensitive process and that the increased intracellular calcium might directly cause apoptosis by increasing the caspase-3 activity as a central event in the cyclosporine-induced apoptotic mechanism.

Keywords: Cyclosporine A; SDZ IMM125; Apoptosis; Antioxidants; Prooxidants; Ca^{2+}

1. Introduction

The immunosuppressive drug Cyclosporine A (CsA, Sandimmun®) is successfully used in clinical application for the treatment of organ transplant recipients and for the treatment of autoimmune disorders. The *O*-hydroxyethyl-D-(Ser)^8-cyclosporine A derivative SDZ IMM125 is almost equipotent to CsA with regards to its immunosuppressive activity, deriving from its inhibition of interleukin-2 expression in T-helper cells at the mRNA level, thus preventing them from orchestrating a response to foreign antigens [1]. However, the usage of both cyclosporines, CsA and SDZ IMM125, is accompanied by reversible side effects occurring mainly in the kidney but also in the liver [2–4]. In preclinical studies, SDZ IMM125 was superior compared to CsA causing less renal dysfunction in the rat and having a wider therapeutic window [5,6]. In man SDZ IMM125 was well tolerated in healthy volunteers and in psoriatic patients, in whom the compound showed a dose-related beneficial effect in clearing psoriasis [7]. Clinical adverse effects were similar to those reported for CsA, i.e. transient impairment of liver function, which manifests itself by elevated, serum bile-acid levels, together with hyperbilirubinemia. There was clear evidence for liver

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Abbreviations: AA, ascorbic acid; ATP, adenosine triphosphate; BAPTA, bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAPTA/AM, bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid/acetomethyl ester; BSO, buthionine sulfoximine; CsA, Cyclosporine A; DEVD, Asp-Glu-Val-Asp; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GSSG, oxidized glutathione; LDH, lactate dehydrogenase; PBS, phosphate buffered saline; ROS, reactive oxygen species; SDZ IMM125, *O*-hydroxyethyl-D-(Ser)^8-cyclosporine A; TPGS, D,L-α-tocopherol-polyethylene-glycol-1000-succinate.

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intolerance of SDZ IMM125, which resulted in significant dose-dependent increases in serum transaminases. Elevation of aminotransferases was found more frequently after SDZ IMM125 treatment than after treatment with CsA [7]. In hepatocyte primary cultures and in the isolated perfused liver, SDZ IMM125 caused the release of lactate dehydrogenase. The results obtained in the rat hepatocyte primary culture reflect the *in vivo* situation in human quite well. Since the ranking of increased transaminases after CsA and SDZ IMM125 treatment was in the same order as the release of lactate dehydrogenase (LDH) *in vitro* in rat hepatocytes, it appears that the applied model is a suitable tool to investigate the mechanisms leading to the cyclosporine hepatotoxicity [8].

We have recently shown that, in rat hepatocytes, SDZ IMM125 induced apoptosis, which was accompanied by proteolytic activation of caspase-3, chromatin condensation and fragmentation, DNA fragmentation, and phosphatidylserine translocation to the outer surface of the plasma membrane [9]. Under the same experimental conditions both cyclosporines induce in parallel to apoptosis oxidative stress by disturbing the balance between the antioxidant and prooxidant cellular steady state [10,11].

Reactive oxygen species (ROS) are supposed to be inducers of apoptosis in different cellular *in vitro* systems. At low concentrations, ROS are known to induce apoptosis, whereas, at higher concentrations, cytotoxicity (LDH leakage) is observed [12,13]. Reinforcement of antioxidative capacity by supplementation with antioxidants resulted in a significant decrease in apoptosis [14].

Caspases appear to be redox sensitive. Mild oxidative stress has been shown to activate caspases, such as caspase-3 and caspase-8; however, the mechanism by which oxidative stimuli activates the caspase cascade is not yet clear [15,16].

Disturbances in calcium homeostasis might also be involved in triggering apoptosis. Increased, intracellular, free calcium can serve as a mediator of apoptosis by activating Ca^{2+}-dependent enzymes, such as phosphatases, proteases and endonucleases, which degrade important cellular macromolecules [17–21]. In addition to caspase activation, augmentation of Ca^{2+}-dependent endonuclease activity can be regarded as a further mechanism responsible for DNA fragmentation in apoptosis [22]. Ca^{2+} may play a role in modifying chromatin conformation, thus making chromatin regions accessible to enzymes such as DNase I, or other endonucleases [23]. ROS might trigger an increase in intracellular Ca^{2+} concentrations, leading to the induction of apoptosis. Antioxidants are known to interfere with free-radical processes within cells by the direct interaction with free radicals, thus preventing the influx of Ca^{2+} in the cells [18].

As oxidative stress and increased intracellular Ca^{2+} concentrations have been described as inducers of apoptosis, the aim of present investigation was to examine the effect of the non-enzymatic antioxidants, ascorbic acid (AA) and D,L-α-tocopherol-polyethylene-glycol-1000-succinate (TPGS), and the role of changes in cellular glutathione caused by buthionine sulfoximine (BSO) and dithiothreitol (DTT), on SDZ IMM125-induced chromatin condensation and fragmentation, caspase-3 activation, and calcium uptake.

2. Materials and methods

2.1. Animals

Permission for the animal studies was obtained from the Veterinäramt Basel-Landschaft, CH-4410 Liestal, and all study protocols were in compliance with the institutional guidelines. Male Wistar rats were obtained from Biological Research Laboratories (CH-4414). They were kept in Macrolon® cages with wood shavings as bedding, under optimal hygienic conditions, at a temperature of 22–23°, a relative humidity of 50–74%, and fluorescent light for a 12-hr day:12-hr night cycle. They were given water and rodent pellets *ad libitum*.

2.2. Hepatocyte isolation and cell-culture conditions

Rat hepatocytes (rats 180–220 g) were isolated according to the two-step liver perfusion method [24]. The cells were seeded in 35 mm, six-well culture dishes (Primaria; Falcon) at a density of 0.7×10^6 cells in 2 mL William's medium E, or in 60 mm culture dishes (Primaria; Falcon) at a density of 2×10^6 cells in 5 mL William's medium E (Gibco, BRL Life Technologies AG). The culture medium contained 10% fetal calf serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL), insulin (10^{-7} M) and dexamethasone (10^{-7} M). After an attachment period of 2 hr at 37° in a 5% CO_2/95% air atmosphere, the medium was changed. The test compound was added together with the new medium. SDZ IMM125 (Novartis) was dissolved in dimethyl sulfoxide (DMSO), and this solution was added to the culture medium, resulting in a final concentration of 1% DMSO in the culture medium. Control plates received the DMSO-containing medium without SDZ IMM125. The maximum soluble SDZ IMM125 concentration was 50 μM. In all experiments, the absence of SDZ IMM125 precipitation was checked.

Coincubation experiments with SDZ IMM125 were carried out 100 and 500 μM TPGS (Kodak, UK), 2 mM AA (Sigma), 1 mM DTT (Sigma) and 2 mM BSO (Sigma). All anti- and pro-oxidant supplements were added 1 hr prior to the addition of SDZ IMM125, and maintained during the incubation time indicated.

2.3. Determination of cytotoxicity

Lactate dehydrogenase (LDH) activity in the culture media was measured spectrophotometrically as an index of

plasma membrane damage and loss of membrane integrity [25]. Enzyme activity was expressed as the percentage of extracellular LDH activity of the total LDH activity on the plates.

2.4. Determination of chromatin condensation and degradation

Chromatin condensation and fragmentation were determined by Feulgen staining, and the use of light microscopy to count the percentage of cells containing alterations in nuclear structure [26].

Hepatocyte samples were treated as follows: after the cell culture medium has been removed, the cells were fixed overnight with 4% formaldehyde in phosphate buffered saline (PBS). After hydrolyzing in 5 N HCl for 90 min at room temperature, the cells were rinsed in distilled water and stained for 30 min in Schiff reagent (Merck). After this, the hepatocytes were rinsed in 0.05 M $Na_2S_2O_3$ solution for 2 min, washed first in tap water, and then washed in distilled water, and mounted by Crystal Mount (Biomeda). After staining, hepatocyte nuclei were violet in color.

The following criteria were used: normal nuclei were those in which the chromatin was unaltered and uniformly spread over the whole nucleus. Condensed chromatin was located at the nuclear membrane periphery and appeared in a half-moon form. Fragmented chromatin was identifiable by its scattered, drop-like structure, which was located on the area of the original nucleus. The total size of apoptotic nuclei appeared to be smaller and more shrunken when compared with intact cells. For each sample, 1000–1500 nuclei were counted.

2.5. Determination of caspase-3 activity

Caspase activity was determined according to the method of Rodriguez *et al*. [27].

After incubation, 2×10^6 cells were washed once in ice-cold PBS and lysed in 1 mL buffer A [10 mM Hepes, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and protease inhibitors (Complete; Roche)]. After three thaw–freeze cycles, the lysate was centrifuged for 20 min at 13,000 *g* at 4°. The supernatant (lysate) was removed and stored at −80° until the assay was performed.

Lysates (70 μg protein) were assayed in 0.1% CHAPS, 100 mM Hepes, 10% sucrose and 10 mM DTT, pH 7.5, with or without 100 μM protease inhibitor for caspase-3 (Ac-DEVD-CHO) (Bachem) added in DMSO. The reaction was started with 20 μM of the substrate for caspase-3 (Ac-DEVD-AMC), which was labeled with the fluorochrome, 7-amino-4-methyl coumarin (Bachem), and the reaction was followed for 60 min. Fluorescence was measured at excitation 360 nm and emission 460 nm in a fluorescence plate reader.

Fluorescence intensity was calibrated with standard concentrations of 7-amino-4-methyl coumarin. Protease activity was calculated from the slope of the recorder trace and expressed as pmol/mg protein/min.

The difference between the substrate cleavage activity levels in the presence and absence of a selective inhibitor reflected the contribution of caspase-3 activity.

2.6. Determination of calcium uptake

Calcium uptake was determined according to the method of Wallnöfer *et al*. [28]. Hepatocytes were preincubated for 4 or 20 hr with different doses of SDZ IMM125. After this preincubation time, the medium was removed, and 1 μCi/mL $^{45}Ca^{2+}$ (Amersham) was added together with SDZ IMM125 1–50 μM. After 0, 5, 10, 20 and 30 min, aliquots of the supernatant were sampled. In order to avoid unspecific binding to the cells, the attached cells were washed three times with PBS and finally solubilized with Triton-100. Aliquots of the supernatant and the cell pellet were counted for radioactivity in a liquid scintillation counter. The zero-time values were subtracted from the individual values obtained after different time points of measurement (5, 10, 20, 30 min). The results were expressed as pmol/mg protein.

2.7. Determination of protein concentration

Protein content was determined according to Bradford [29]. Bovine serum albumin served as a standard.

2.8. Statistics

A two-way ANOVA was performed (group and animal; each of them was regarded as qualitative). If the effect of the animal number was not significant, it was omitted. A quantile plot was used to judge visually the normality of the residuals. If the residuals were not normally distributed, we tried to achieve (approximate) normality by transforming the response or by omitting of outliners.

A multiple comparison method was applied using the three methods of Hayter [30], Sidak [31] and Dunnett [32]. The Dunnett test compares every treated group with the control group, while the other two methods can be used to compare each group with other group.

The multiple comparison method delivers an estimation of the difference in the response expected between the two groups compared, the standard error of the response, and a lower and upper confidence limit for the difference. If the two limits did not include zero, the difference was significantly different from zero, on the level of 5%. By repeating the method for 1 and 0.1%, we could analyze how big the significance was.

The S-Plus software (version 5) was used for the computations; the three methods of Hayter [30], Sidak [31] and Dunnett [32] were used adaptively, i.e. in every case, the most sensitive method was used.

3. Results

3.1. Chromatin condensation and fragmentation

Treatment of hepatocyte cultures for 4 and 20 hr with SDZ IMM125 at concentrations of 0, 10, 25 or 50 μM showed significant increases of nuclear condensation and fragmentation (Figs. 1 and 2).

After 4 hr, 50 μM SDZ IMM125 was not cytotoxic, as determined by LDH-release (data not shown), but a 3-fold increase in chromatin condensation and an 8.8-fold increase in chromatin fragmentation were already observed. Supplementation of the cell cultures with the antioxidant vitamins, TPGS and AA resulted in a statistically significant protection against SDZ IMM125-induced chromatin condensation and fragmentation. DTT inhibited SDZ IMM125-induced fragmentation and significantly inhibited chromatin condensation. BSO increased SDZ IMM125-induced nuclear condensation and fragmentation by mean value (Fig. 1A and B).

After 20 hr of treatment, SDZ IMM125 effects on the nuclei became more pronounced. SDZ IMM125, at concentrations of 10 and 25 μM resulted in an increase in chromatin condensation and fragmentation. Coincubation

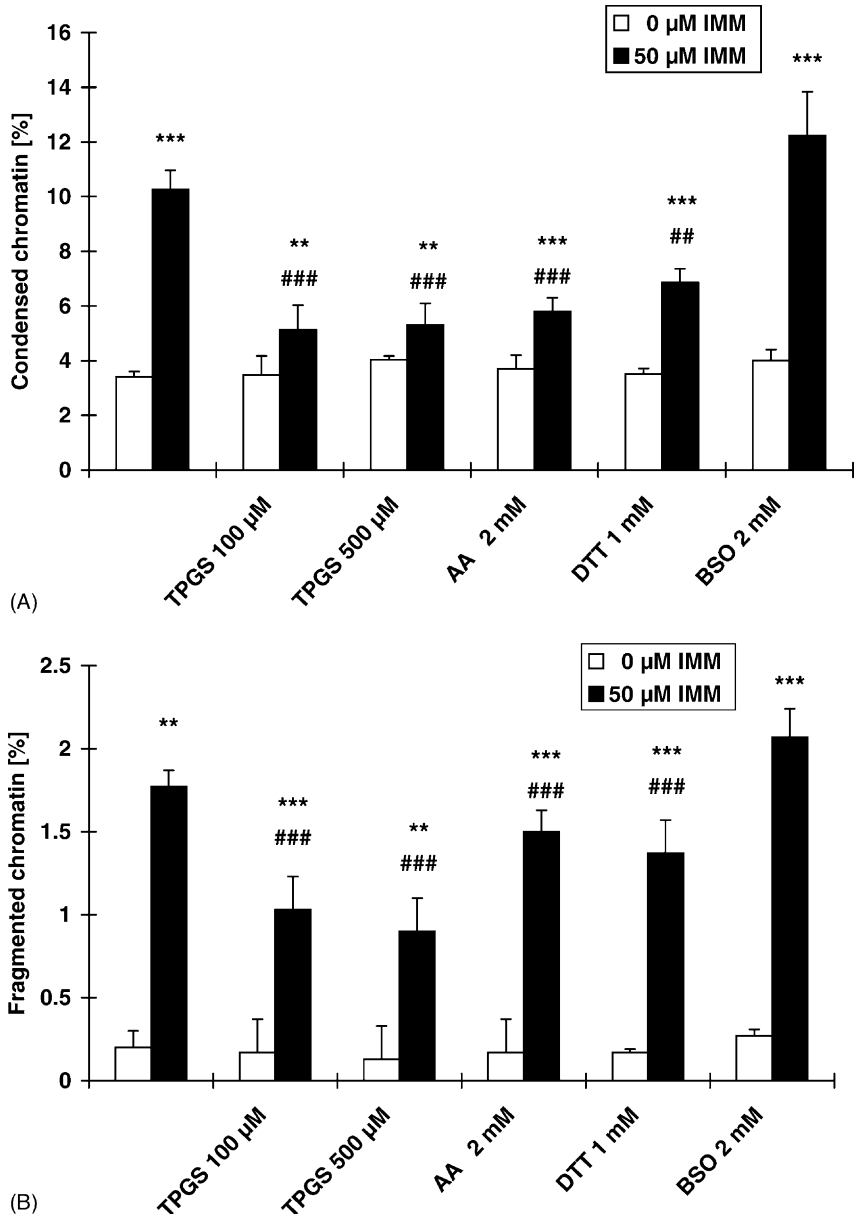

Fig. 1. (A) Effect of TPGS, AA, DTT and BSO on SDZ IMM125-induced chromatin condensation. Chromatin condensation was measured after 4 hr incubation. Data are expressed as mean ± SD (N = 3). Statistically significant differences vs. the control group are expressed as $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistically significant differences in comparison with the 50 μM SDZ IMM125 group are indicated by $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$. (B) Effect of TPGS, AA, DTT and BSO on SDZ IMM125-induced chromatin fragmentation. Chromatin fragmentation was measured after 4 hr incubation. Data are expressed as mean ± SD (N = 3). Statistically significant differences vs. the control group are expressed as $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistically significant differences in comparison with the 50 μM SDZ IMM125 group are indicated by $^{\#\#\#}P < 0.001$.

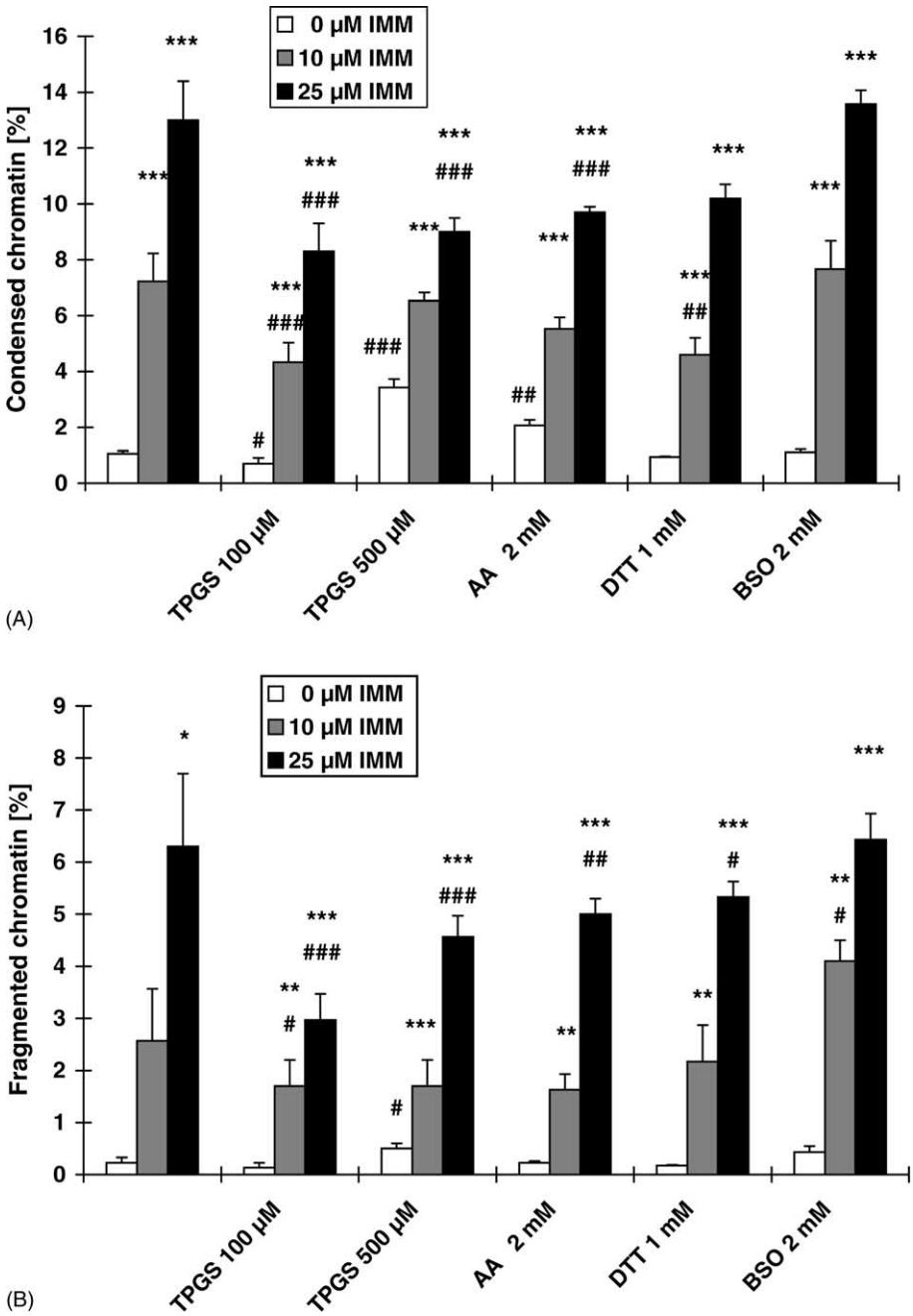

Fig. 2. (A) Effect of TPGS, AA, DTT and BSO on SDZ IMM125-induced chromatin condensation. Chromatin condensation was measured after 20 hr incubation. Data are expressed as mean ± SD (N = 3). Statistically significant differences vs. the control group are expressed as $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistically significant differences in comparison with the respective SDZ IMM125 group are indicated by $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$. (B) Effect of TPGS, AA, DTT and BSO on SDZ IMM125-induced chromatin fragmentation. Chromatin fragmentation was measured after 20 hr incubation. Data are expressed as mean ± SD (N = 3). Statistically significant differences vs. the control group are expressed as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistically significant differences in comparison with the respective SDZ IMM125 group are indicated by $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

of the hepatocytes with SDZ IMM125 and TPGS, AA or DTT inhibited SDZ IMM125 induced chromatin condensation and fragmentation. The GSH synthetase inhibitor BSO raised SDZ IMM125-induced chromatin condensation by mean values (Fig. 2A and B).

After 20 hr, TPGS at 500 µM was toxic by itself. Since 500 µM TPGS was toxic, we did not use this high concentrations in follow-up experiments. Therefore we regarded the concentration of 100 µM TPGS as more appropriate.

3.2. Caspase-3 activity

Caspase-3 activity was tested in hepatocytes incubated with SDZ IMM125 at the concentration 50 µM for 4 hr and at 25 and 50 µM for 20 hr. The addition of TPGS, AA and DTT significantly inhibited SDZ IMM125-induced caspase-3 activation at both time points at concentrations of 25 and 50 µM. SDZ IMM125 caused greater caspase-3 activation at 25 µM than at 50 µM.

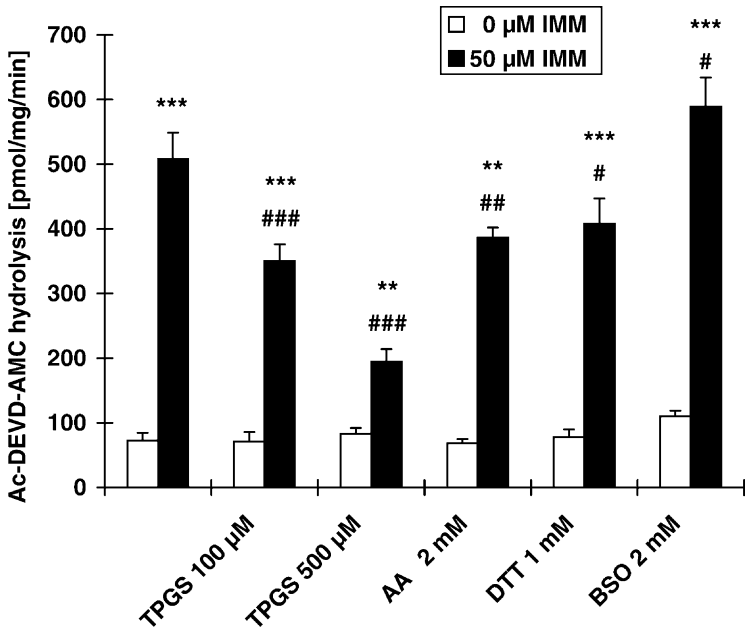

Fig. 3. Effect of TPGS, AA, DTT and BSO on SDZ IMM125-induced caspase-3 activity after 4 hr incubation. Data are expressed as mean ± SD (N = 3). Statistically significant differences vs. the control group are expressed as $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistically significant differences in comparison with the respective SDZ IMM125 group are indicated by $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

After 4 hr the strongest inhibitory effect was found with TPGS whereas AA and DTT significantly reduced SDZ IMM125 caspase-3 activity. The prooxidant BSO significantly increased SDZ IMM125-mediated caspase-3 activity (Fig. 3).

After 20 hr of coincubation TPGS, DTT and AA reduced SDZ IMM125-induced caspase-3 activity; AA had the weakest effect. Contrary, coadministration of BSO statistically significantly increased caspase-3 activity (Fig. 4).

3.3. LDH release

In the 20-hr hepatocyte culture, coincubation with TPGS, AA and DTT protected against SDZ IMM125

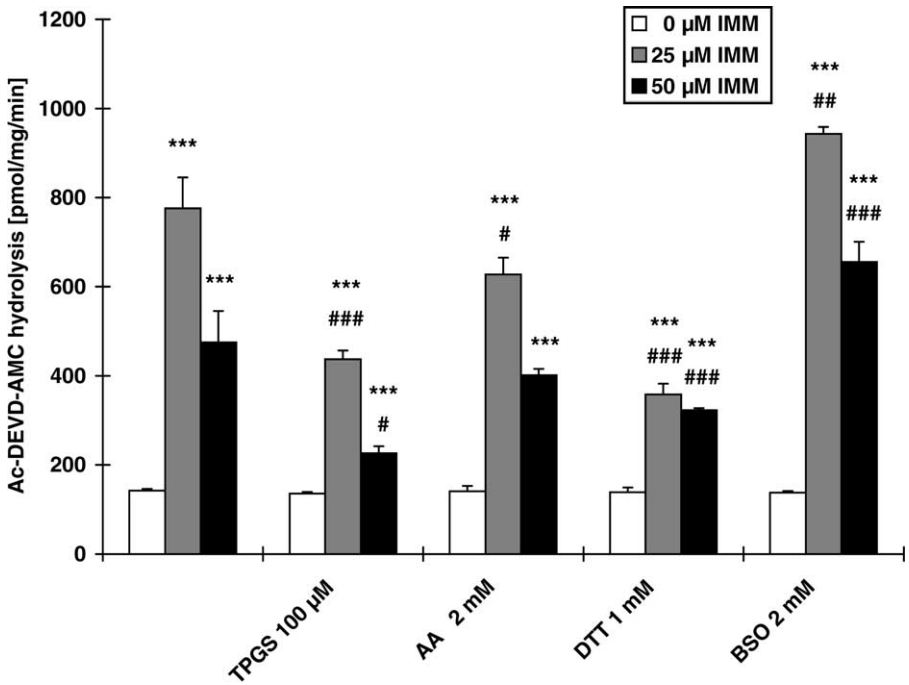

Fig. 4. Effect of TPGS, AA, DTT and BSO on SDZ IMM125-induced caspase-3 activity after 20 hr incubation. Data are expressed as mean ± SD (N = 3). Statistically significant differences vs. the control group are expressed as $^{***}P < 0.001$. Statistically significant differences in comparison with the respective SDZ IMM125 group are indicated by $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

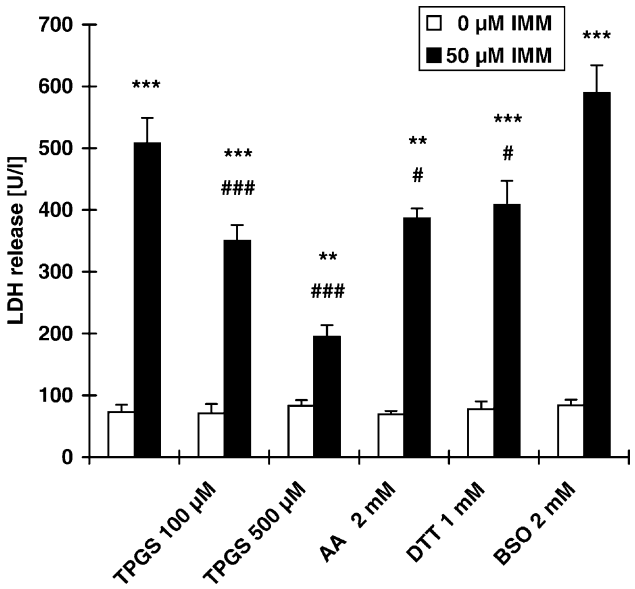

Fig. 5. Effect of AA, TPGS, DTT and BSO on SDZ IMM125-induced LDH-release after 20 hr. The results are mean values ± SD (N = 3). Statistically significant differences vs. the control group are expressed as $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistically significant differences in comparison with the 50 µM SDZ IMM125 group are indicated by $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$.

toxicity; the decrease in LDH release was significant with all antioxidants. The prooxidant BSO tended to increase SDZ IMM125-induced LDH release (Fig. 5).

3.4. Calcium uptake

SDZ IMM125, when incubated with rat hepatocytes for 4 and 20 hr in the concentration range between 1 and 10 µM, had the strongest effect on extracellular Ca^{2+} uptake at the concentration of 2.5 µM. After 4 hr of incubation with SDZ IMM125 in combination with the antioxidant vitamins, TPGS, AA and DTT inhibited extracellular Ca^{2+} uptake in the hepatocytes; this was statistically significant in the case of 500 µM TPGS and DTT (Table 1). After 20 hr of incubation, the inhibition of Ca^{2+} uptake was statistically significant in the case of 100 and 500 µM TPGS and DTT (Table 2). BSO treatment resulted in an increase in mean values of SDZ IMM125-mediated Ca^{2+} uptake over the observation period of 30 min (Tables 1 and 2).

3.5. Effect of calcium on SDZ IMM125-induced apoptosis

Rat hepatocytes were cultured with SDZ IMM125 in culture medium containing 0 mM (no Ca^{2+} added), 1.8 mM (physiological calcium concentration) and 7 mM Ca^{2+}. The co-incubation experiments with the intracellular Ca^{2+}chelator bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) were performed in the normal medium containing 1.8 mM Ca^{2+}.

Table 1
Calcium uptake in hepatocyte cultures treated for 4 hr with 2.5 µM SDZ IMM125 or in combination with antioxidants (2 mM AA, 100 and 500 µM TPGS or 1 mM DTT) and the prooxidant BSO (2 mM)

Treatment	Cellular $^{45}Ca^{2+}$ (pmol/mg)			
	Time			
	5 min	10 min	20 min	30 min
Control	385 ± 7	307 ± 9	299 ± 14	295 ± 22
AA	370 ± 10	325 ± 8	332 ± 22	319 ± 28
TPGS, 100 µM	331 ± 7	282 ± 18	260 ± 17	240 ± 26
TPGS, 500 µM	353 ± 21	305 ± 34	290 ± 15	285 ± 8
DTT	373 ± 23	306 ± 32	323 ± 36	309 ± 36
BSO	375 ± 42	341 ± 57	356 ± 56	475 ± 67
IMM	551 ± 17	456 ± 21	451 ± 78	384 ± 34
IMM + AA	543 ± 13	440 ± 66	441 ± 81	397 ± 79
IMM + TPGS, 100 µM	531 ± 42	433 ± 36	412 ± 35	365 ± 29
IMM + TPGS, 500 µM	496 ± 16	428 ± 9$^{\#\#\#}$	381 ± 10$^{\#\#\#}$	312 ± 4
IMM + DTT	485 ± 65$^{\#\#}$	389 ± 50$^{\#\#}$	362 ± 40$^{\#\#\#}$	305 ± 34
IMM + BSO	618 ± 44	566 ± 57	570 ± 55	516 ± 97

The results are expressed mean values ± SD (N = 3). In SDZ IMM125 treated cells intracellular $^{45}Ca^{2+}$ concentrations were statistically increased in comparison to controls at all time points of $^{45}Ca^{2+}$ incubation ($P < 0.05$). Statistically significant differences in comparison with the SDZ IMM125 group are expressed as $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

After 4 and 20 hr (data shown for 20 hr), SDZ IMM125-induced chromatin condensation and fragmentation were enhanced by supplementation the culture medium with 7 mM Ca^{2+}, whereas omission of Ca^{2+} in the medium reduced both effects. BAPTA, by depleting intracellular Ca^{2+}, was able to statistically significantly suppress

Table 2
Calcium uptake in hepatocyte cultures treated for 20 hr with 2.5 µM SDZ IMM125 or in combination with antioxidants (2 mM AA, 100 and 500 µM TPGS or 1 mM DTT) and the prooxidant BSO (2 mM)

Treatment	Cellular $^{45}Ca^{2+}$ (pmol/mg)			
	Time			
	5 min	10 min	20 min	30 min
Control	405 ± 33	344 ± 27	337 ± 35	323 ± 36
AA	432 ± 41	440 ± 94	402 ± 64	385 ± 65
TPGS, 100 µM	342 ± 23	348 ± 31	325 ± 26	251 ± 16
TPGS, 500 µM	418 ± 9	366 ± 7	328 ± 6	332 ± 20
DTT	373 ± 23	306 ± 32	323 ± 36	309 ± 36
BSO	463 ± 35	468 ± 40	425 ± 38	356 ± 32
IMM	618 ± 44	607 ± 82	544 ± 61	364 ± 16
IMM + AA	610 ± 44	575 ± 85	531 ± 65	359 ± 31
IMM + TPGS, 100 µM	557 ± 35	468 ± 37$^{\#}$	425 ± 39	321 ± 23
IMM + TPGS, 500 µM	430 ± 18	355 ± 24$^{\#\#\#}$	337 ± 23$^{\#\#\#}$	300 ± 35
IMM + DTT	492 ± 52$^{\#\#\#}$	472 ± 36$^{\#\#\#}$	409 ± 35$^{\#\#\#}$	336 ± 39
IMM + BSO	658 ± 40	644 ± 32	570 ± 42	408 ± 33

The results are expressed mean values ± SD (N = 3). In SDZ IMM125 treated cells intracellular $^{45}Ca^{2+}$ concentrations were statistically increased in comparison to controls after 5, 10 and 20 min of $^{45}Ca^{2+}$ incubation ($P < 0.05$). Statistically significant differences in comparison with the SDZ IMM125 group are expressed as $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$.

cyclosporine-induced chromatin condensation, whereas its effect on chromatin fragmentation was less pronounced. Incubation of hepatocytes with BAPTA alone, without SDZ IMM125, had no effect on the nucleus (Fig. 6A and B).

SDZ IMM125 induced caspase-3 activity was enhanced in the presence of extracellular calcium (7 mM Ca^{2+}) in the cell culture medium. The omission of calcium in the incubation medium as well as co-incubation with BAPTA significantly inhibited SDZ IMM125-mediated caspase-3 activity (Fig. 6C).

Co-incubation of SDZ IMM125 (25 μM) with the calcium channel blockers verapamil (10 μM), diltiazem (50 μM) and nifedipine (50 μM) resulted in significant decreases of caspase-3 activity (Fig. 6D).

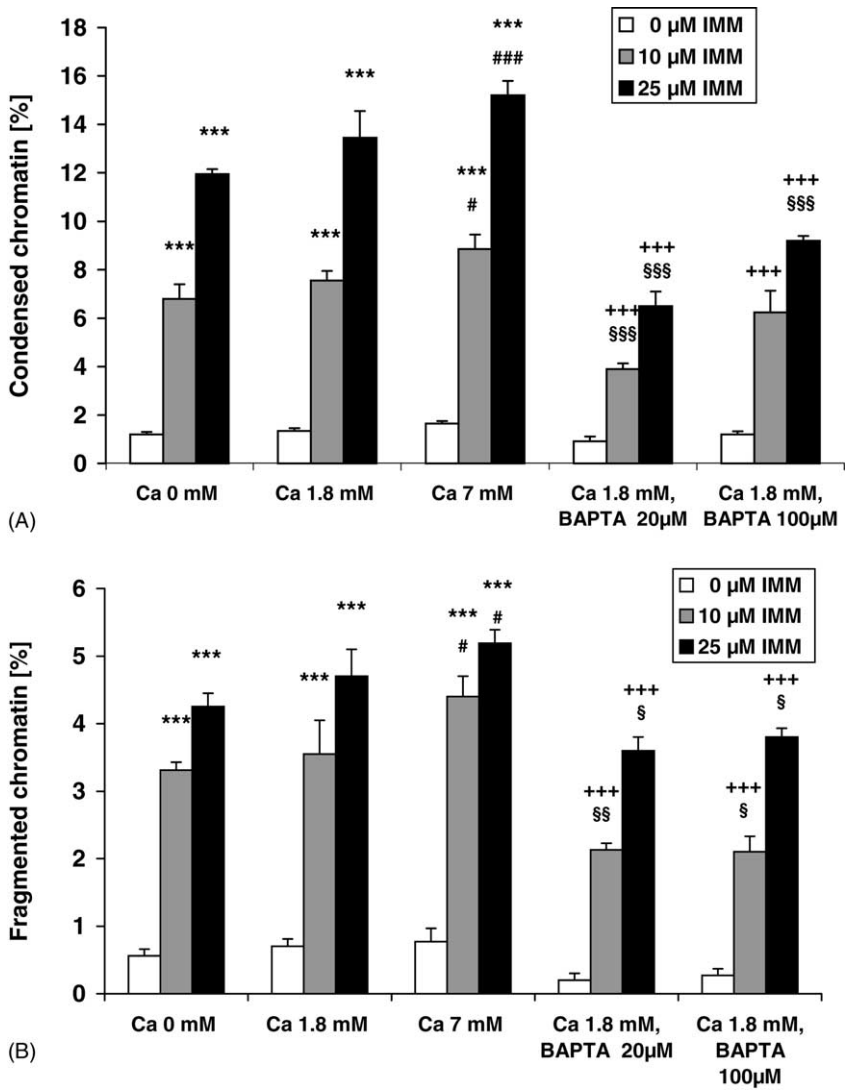

Fig. 6. (A) Effect of extracellular calcium and intracellular calcium chelation on SDZ IMM125-induced chromatin condensation after 20 hr. The results are mean values ± SD from three independent experiments (N = 3). Statistically significant differences vs. the control group are expressed as $^{***}P < 0.001$. Statistically significant differences in comparison with the respective 125 group containing 1.8 mM Ca^{2+} are indicated by $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$. Statistically significant differences vs. the control group with 1.8 mM Ca^{2+} are expressed as $^{+++}P < 0.001$. Statistically significant differences vs. the respective SDZ IMM125 group containing 1.8 mM Ca^{2+} are indicated by $^{\S\S\S}P < 0.001$. (B) Effect of extracellular calcium and intracellular calcium chelation on SDZ IMM125-induced chromatin fragmentation after 20 hr. The results are mean values ± SD from three independent experiments (N = 3). Statistically significant differences vs. the control group are expressed as $^{***}P < 0.001$. Statistically significant differences in comparison with the respective SDZ IMM125 group containing 1.8 mM Ca^{2+} are indicated by $^{\#}P < 0.05$. Statistically significant differences vs. the control group with 1.8 mM Ca^{2+} are expressed as $^{+++}P < 0.001$. Statistically significant differences vs. the respective SDZ IMM125 group containing 1.8 mM Ca^{2+} are indicated by $^{\S}P < 0.05$ and $^{\S\S}P < 0.01$. (C) Effect of extracellular calcium and intracellular calcium chelation on SDZ IMM125-induced caspase-3 activity after 20 hr. The results are mean values ± SD from three independent experiments (N = 3). Statistically significant differences vs. the control group are expressed as $^{***}P < 0.001$. Statistically significant differences in comparison with the respective SDZ IMM125 group containing 1.8 mM Ca^{2+} are indicated by $^{\#}P < 0.05$. Statistically significant differences vs. the control group with 1.8 mM Ca^{2+} are expressed as $^{+++}P < 0.001$ and vs. the respective SDZ IMM125 group containing 1.8 mM Ca^{2+} are indicated by $^{\S}P < 0.05$ and $^{\S\S\S}P < 0.001$. (D) Effect of verapamil, diltiazem and nifedipine on SDZ IMM125-induced caspase-3 activity after 20 hr. The results are mean values ± SD from three experiments (N = 3). Statistically significant differences vs. the control group are expressed as $^{***}P < 0.001$. Statistically significant differences in comparison with the 25 μM SDZ IMM125 group are indicated by $^{\#\#\#}P < 0.001$.

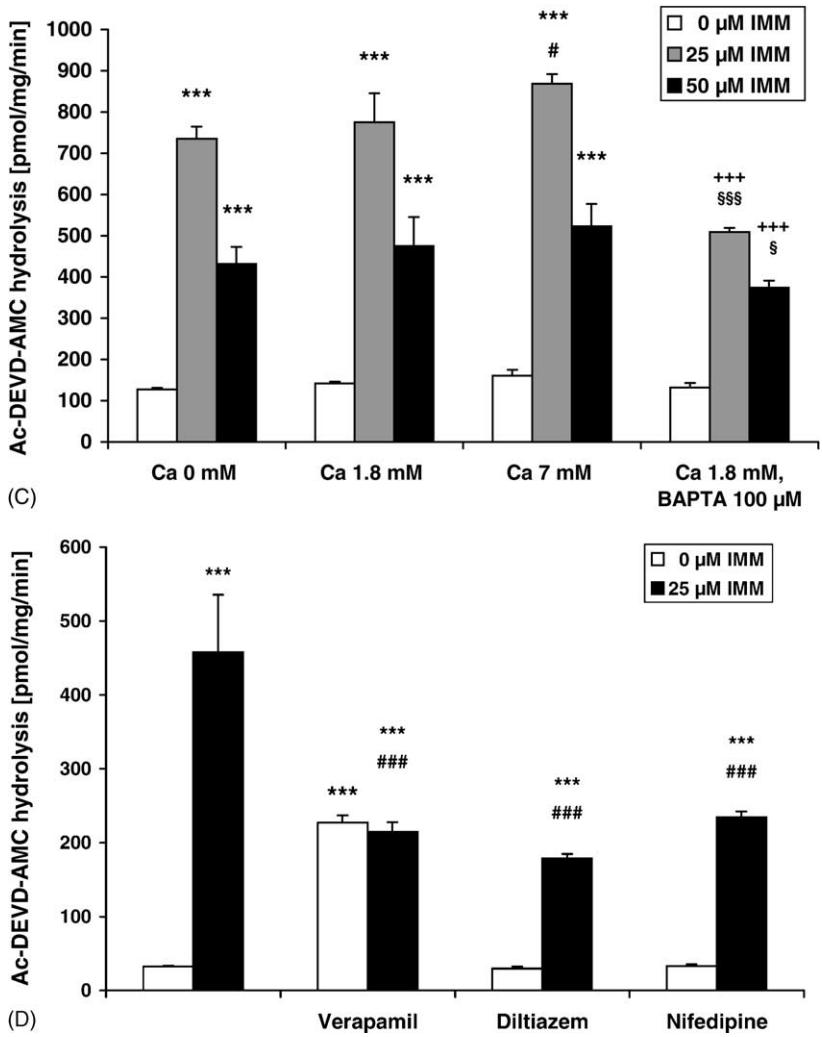

Fig. 6. (*Continued*).

4. Discussion

Although SDZ IMM125 was found to be superior to CsA in preclinical toxicological evaluations [5], there was clear evidence of liver intolerance of SDZ IMM125 after application in man, mainly by significant increases in the serum transaminases ALAT and ASAT [7]. The magnitude and the incidence of such effects have never been observed after administration of CsA, either in healthy volunteers or in transplant patients. As compares the toxicity of CsA and SDZ IMM125 in man and the results obtained in rat hepatocytes, the *in vitro* model mimics the *in vivo* situation in man quite well. In rat hepatocytes, SDZ IMM125 also has a stronger effect compared to CsA concerning the release of the cytosolic enzyme LDH. Similar results were obtained concerning apoptotic parameters. CsA and SDZ IMM125 were found to induce apoptosis in primary rat hepatocytes, whereby SDZ IMM125 always caused stronger apoptotic effects than CsA [9,33,34].

Previously we could show that oxidative stress is part of the mechanism by which CsA and SDZ IMM125 can cause toxicity in rat liver cells [10,11]. In a previous study, the antioxidants AA and TPGS protected against cyclosporine toxicity and decreased the level of endogenous ROS [10,11]. The weakening or enforcement of the cellular glutathione state by the glutathione synthesis inhibitor, BSO, or the oxidized glutathione (GSSG) reducing agent, DTT, either increased or inhibited cyclosporine cytotoxicity [10,11]. Under the same conditions, we were able to demonstrate that SDZ IMM125, in addition to its cytotoxic action, induced apoptosis in rat hepatocyte cultures. SDZ IMM125-mediated apoptosis was accompanied by a decrease in the mitochondrial membrane potential, cytochrome c release from the mitochondria into the cytosol, caspase-3 activation, enhanced uptake of Ca^{2+}, chromatin condensation and fragmentation and DNA fragmentation [9].

Furthermore, the present study, for the first time, presents strong evidence that SDZ IMM125-mediated apoptosis is dependent on the intracellular redox-state and the antioxidant supply. The antioxidant vitamins, TPGS and AA, and the GSSG-reducing agent, DTT, significantly inhibited both chromatin condensation and fragmentation.

4.1. On the contrary, BSO enhanced both effects

Active caspase-3 is a mediator of SDZ IMM125-induced apoptosis. Treatment with the antioxidants, TPGS, AA and DTT, resulted in a statistically significant inhibition of SDZ IMM125-induced caspase-3 activity, whereas the prooxidant, BSO, significantly increased caspase-3 activity. This finding is in agreement with the current literature, which shows the redox-sensitivity of caspase-3 [15,16].

The non-dose-linear caspase-3 activation after 20 hr could be the result of the strong cytotoxicity observed at 50 μM. In general, cytotoxicty goes in parallel to decreased ATP levels, which could be responsible for the lower caspase-3 activities. The role of ATP in caspase-3 activation is well described in the current literature [35,36].

The general role of calcium in the enhancement of DNA fragmentation has been shown in the literature [20]. Calcium might also be involved in the SDZ IMM125-induced apoptosis. This can be concluded indirectly from the ability of cyclosporines to increase the intracellular calcium levels. Indications on a CsA-mediated increase in cellular Ca^{2+} can be taken from the studies of Ellouk-Achard *et al.* [37] in rat hepatocytes using a fluorescence probe. First direct evidence that cyclosporines potentially could enhance the Ca^{2+} uptake comes from experiments with liposomal vesicles [10] and in other cell types different from hepatocytes [38,39]. To make sure that extracellular calcium is taken up by the hepatocytes and not an intracellular redistribution of calcium has taken place, we measured calcium uptake using labeled $^{45}Ca^{2+}$. We have found that the intracellular uptake of Ca^{2+} is stongly enhanced in hepatocytes by SDZ IMM125 and that the increase is about twice as much as after treatment with CsA (Wolf, data not shown). SDZ IMM125-mediated oxidative stress might serve as a trigger for increased intracellular Ca^{2+} concentrations. The antioxidants, TPGS and DDT, inhibited, whereas the prooxidant, BSO, slightly enhanced SDZ IMM125-induced calcium uptake. As it has also been shown that TPGS can prevent protein oxidation after cyclosporine treatment [11], the role of specific redox-sensitive calcium membrane pores in Ca^{2+}-uptake might be indicated. Similar effects are discussed in the current literature for redox-active compounds. For example, *tert*-butylhydroperoxide, menadione, ethanol, and 1-methyl-4-phenyl-1,2,3,6-tetra-hydropyridine are reported to impair the Ca^{2+}-ATPase activity [40–44]. In rat hepatocytes, the thiol reagent, DTT, reversed the inhibition of the Ca^{2+}-ATPase activity caused by oxidizing quinones [40]. Parola was able to show that carbon tetrachloride induced damage to the Ca^{2+}-ATPase, which could be inhibited by Vitamin E [45].

Direct indication of the involvement of calcium in the SDZ IMM125-induced apoptosis can be taken from the experiments after changing the intra- and extracellular calcium concentrations. We showed that modulation of the concentration of extracellular calcium in the medium could influence SDZ IMM125-mediated apoptosis. Elevation of the calcium medium concentration to 7 mM increased apoptosis, compared with the normal medium calcium concentration of 1.8 mM, whereas, omission of calcium in the medium significantly decreased apoptosis.

Co-incubation of SDZ IMM125 with the intracellular Ca^{2+} chelator, BAPTA, also resulted in protection against apoptosis. The role of BAPTA as an intracellular Ca^{2+} chelator is well described in the current literature [46,47]. The calcium chelator bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid/acetomethyl ester (BAPTA/AM) is inactive until it passes through the cell membrane, where it is enzymatically activated by esterases to become BAPTA. During loading, the chelator is sequestered into the nucleus, where it can buffer intranuclear Ca^{2+} accumulation and inhibit Ca^{2+}-activated nuclear processes. Thus, it is possible that nuclear processes are involved in apoptosis, however the chelator is also able to sequester cytosolic Ca^{2+} [40]. Although BAPTA, by its Ca^{2+} buffering capacity is close to the Ca^{2+} levels in resting cells (K_d of 1.1×10^{-7} M), it is able to chelate also other cations such as iron ions (K_d for Fe^{2+} and Fe^{3+} of 1×10^{-6} and 5×10^{-9} M) [46], however, the results from own experiments with the iron chelator deferrioxamine do not suggest the involvement of iron in the SDZ IMM125 induced apoptosis (unpublished observation). By modulating the calcium concentration in the medium and by supplementation the intracellular calcium chelator, BAPTA, strong inhibitory effects on caspase-3 activity were also found. We have also shown that verapamil, nifedipine and diltiazem are able to inhibit SDZ IMM125-mediated caspase-3 activity demonstrating the association between intracellular calcium concentration and caspase-3 activity. In the current literature, similar events were observed showing that calcium channel blockers such as verapamil and nifedipine inhibited calcium influx and, thus, apoptosis [47–49].

In conclusion, our data indicate that SDZ IMM125-mediated apoptosis in rat hepatocytes can be inhibited by antioxidants, and that the intracellular redox-state can act as a modulator of cytotoxicity and apoptosis. The results suggest SDZ IMM125 can cause increased uptake of extracellular calcium, which is a redox sensitive process and that the increased calcium may directly cause apoptosis by increasing the caspase-3 activity, which might finally be responsible for the execution of irreversible apoptotic processes.

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