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Synergism between staurosporine and drugs inducing endoplasmic reticulum stress

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Abbreviations:
Ac-LEVD-AMC, Ac-L-leucyl-L-glutamyl-L-valyl-L-aspartyl-7-amino-4-methylcoumarin
BiP, binding protein
CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
DTT, dithiothreitol
CHOP, C/EBP homologous protein
FITC, fluorescein 5-isothiocyanate
PIPES, piperazine-1,4-bis(2-ethanesulfonic acid)
PMSF, phenylmethanesulfonyl fluoride
Z-DEVD-R110, rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide)

ABSTRACT

Drugs causing endoplasmic reticulum or mitochondrial dysfunction may trigger apoptosis in eukaryotic cells. The thiol reagent dithiothreitol (DTT) belongs to the first group whereas the protein kinases inhibitor staurosporine acts on mitochondria. Since the endoplasmic reticulum and the mitochondrial pathways of apoptosis may converge in common steps, we examined the possibility of synergism between these two drugs. Using the activation of caspase-3 as indicator of apoptosis, we found that in two cell lines, Jurkat and Mono-Mac 6, staurosporine and DTT elicited apoptosis with a different pattern: staurosporine acted rapidly and at nanomolar concentrations while DTT acted slowly and at higher concentrations (1 mM). When staurosporine and DTT were combined, the proapoptotic action was increased. This was confirmed examining late apoptotic events such as the translocation of phosphatidylserine across the plasma membrane and the cleavage of the antiapoptotic protein Mcl-1. The use of subthreshold DTT concentrations and isobologram analysis demonstrated the synergic nature of the interaction. Tunicamycin, a drug that, like DTT, inhibits protein folding in the endoplasmic reticulum also increased the proapoptotic effect of staurosporine. In agreement with the interplay between the mitochondrial and the endoplasmic reticulum pathways it was found that both staurosporine and DTT induced cytochrome c release. Furthermore, 90 min incubation with DTT did not induce caspase-4 activation while staurosporine alone or in combination with DTT stimulated caspase-4 activity. We conclude that staurosporine is more active in cells undergoing endoplasmic reticulum stress. This synergism may warrant evaluation to establish whether the anticancer activity of staurosporine is also enhanced.

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1. Introduction

In eukaryotic cells a wide variety of stimuli such as nutrient deprivation, diseases, chemotherapeutic drugs may activate the programmed cell death, a process called apoptosis. Apoptosis requires the sequential activation of initiator and effector intracellular proteases (procaspases) that trigger the disassembly of cell components [1,2]. The major initiator procaspases are procaspase-8 and -10, associated with the extrinsic pathway, and procaspase-9, associated with the intrinsic (mitochondrial) pathway [2]. In the extrinsic pathway, the triggering event is the cross-linking of the plasma membrane death receptors (Fas/CD95, TNFαR) by extracellular ligands. The mitochondrial pathway, together with providing an amplification mechanism for the other major apoptotic pathways of the cell, is activated in response to a wide array of stimuli including growth factor deprivation, DNA damage or drugs acting inside the cells. A strict regulation is provided by a family of proteins, collectively named Bcl-2 and comprising pro- and anti-apoptotic members [3]. Disturbances of the function of the endoplasmic reticulum (ER stress) induced by stimuli such as hypoxia and glucose starvation trigger a signaling pathway known as the unfolded protein response [4]. Prolonged ER stress leads to the induction of cell death cascade whose mechanisms are not fully understood. In all these pathways the initiator procaspases, after their activation, transmit the signal to the downstream effector procaspases (procaspases-3, -6, -7) whose activity becomes a test of ongoing apoptotic programme.

The three major apoptotic pathways of eukaryotic cells are amenable to pharmacological influence. The most specific effect is obtained with antibodies triggering the extrinsic pathway by cross-linking the death receptors in the plasma membrane. Several conventional drugs such as the antineoplastic agents activate the mitochondrial pathway. Among these, the inhibitor of protein kinases staurosporine shows remarkable activity in inducing apoptosis in a wide variety of mammalian cells [5]. Although its effect may not be entirely confined to mitochondria, the staurosporine-induced release of cytochrome *c* is well documented [6]. Recent studies disclosed several drugs impairing the function of ER. Well characterized are thapsigargin, an inhibitor of Ca^{2+}-ATPase [7], brefeldin A, an inhibitor of ER/Golgi transport [8], tunicamycin an inhibitor of protein glycosylation [9,10] and dithiothreitol (DTT) which prevents disulfide bond formation [11]. All these drugs cause the accumulation of unfolded proteins in the ER, giving rise to the unfolded protein response, a reversible condition that, if not compensated, may lead to apoptosis [12]. The other known pathways, namely the ASK/JNK and the Bap31 pathways, converge with the mitochondrial pathway causing the release of cytochrome *c* and the subsequent activation of caspase-9 [13].

The ER-mitochondrial crosstalk suggests that drugs acting in their respective proapoptotic pathways may potentiate each other when added together. To test this possibility we selected staurosporine and DTT as their interaction may have pharmacological interest. The first is a prototype of potent antineoplastic drugs [14,15] and the second is a member of the thiol-containing compounds, sometime used to avoid unwanted effects in anticancer therapy. A potentiation by DTT of the proapoptotic action of staurosporine may at the same time decrease the toxic effect of this drug and reduce the resistance of neoplastic cells. These experiments were subsequently extended to tunicamycin, an additional compound inducing ER stress.

2. Materials and methods

2.1. Reagents and cell cultures

RPMI 1640, fetal bovine serum, staurosporine, DTT and tunicamycin were purchased from Sigma. Caspase-3 assay kit was from Molecular Probes. Caspase-4 substrate (Ac-LEVD-AMC) was from Alexis. Annexin V-FITC kit was from Immunotech. Mouse monoclonal antibodies to human Mcl-1 and to human CHOP and rabbit polyclonal antibody to human BiP were from Santa Cruz Biotechnology Inc. Mouse monoclonal to human β-actin was from Sigma. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Amersham. Stock solution of staurosporine in methanol (0.5 mM) and tunicamycin in DMSO (10 mg/ml) were maintained at −20 °C.

The human lymphocytic leukemic T cell line Jurkat was grown in RPMI medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine and antibiotics. The human monocytic leukemic cell line Mono-Mac 6 was grown in the same medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, antibiotics, 1 mM sodium pyruvate and MEM non-essential aminoacids.

2.2. Cell incubation and drug addition

The cells were collected by centrifugation, resuspended at 10^6 cells/ml in serum-free RPMI supplemented with 5 mg/ml of BSA and incubated at 37 °C for 90 or 180 min in the presence and in the absence of staurosporine. DTT was added with staurosporine whereas tunicamycin was added together with staurosporine in a first series of experiments with 90 min of incubation. In a second series, cells were incubated with tunicamycin 12 h at 37 °C and staurosporine was added in the last 90 min.

2.3. Determination of caspase-3 and caspase-4 activity

After the incubation, 5×10^5 cells (for the determination of caspase-3) or 2.5×10^6 cells (for the determination of caspase-4) were pelleted and resuspended respectively in 50 or 125 μl of lysis buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.01% w/v Triton X-100, pH 7.5). The samples were frozen in liquid nitrogen, thawed and kept in ice for 15 min. After 20 min centrifugation at 17000 × *g*, the supernatant was used for the assay. Caspase activity was measured by the addition of triplicate samples of cell extract (10 μl each) to an equal volume of caspase-3 reaction buffer (20 mM PIPES, 10 mM EDTA, 0.2% CHAPS, 10 mM DTT, pH 7.4) containing 10 μM of caspase-3 substrate (Z-DEVD-R110) or caspase-4 reaction buffer (0,1 M HEPES, 20% glycerol, 5 mM DTT, 0,5 mM EDTA, pH 7,4) containing 1 mM of caspase-4 substrate (Ac-LEVD-AMC). The samples were then incubated 30 min at room temperature. The fluorescence was measured at 496 nm of

Table 1 – Combined effect of staurosporine and DTT on caspase-3 activity of mononuclear cells

Cells	Caspase-3 activity/10^5 cells (fluorescence units $\times$ 10^{-3})				
	Time of incubation (min)	None	250 nM staurosporine	1 mM DTT	Staurosporine plus DTT
Jurkat (7)	90	117.5 ± 14.7	444.1 ± 46.8[a]	217.1 ± 14.2[a]	1502.0 ± 207.2[b]
Jurkat (5)	180	198.2 ± 10.3	984.4 ± 164.3[a]	548.3 ± 69.4[a]	1543.0 ± 210.9[c]
Mono-Mac 6 (3)	90	477.3 ± 71.7	1891.7 ± 333.2[a]	1255.0 ± 112.0[a]	3925.1 ± 630.8[c]
Mono-Mac 6 (3)	180	292.6 ± 176.4	3217.3 ± 97.6[a]	719.5 ± 388.8	4257.7 ± 336.2[c]

Cells (5×10^{-5}) in 0.5 ml of RPMI containing 5 mg/ml of BSA were incubated for the indicated time with staurosporine and DTT. At the end of incubation, the cells were pelletted, lysed and used for the determination of caspase-3 activity. Data are means ± S.E.M., number of experiments in parentheses. Student's *t*-tests (unpaired, one tailed).

[a] $P < 0.01$ vs. none.

[b] $P < 0.01$ vs. none, DTT and staurosporine alone.

[c] $P < 0.01$ vs. none and DTT alone, <0.05 vs. staurosporine alone.

excitation and 520 nm of emission for the caspase-3 and at 380 nm of excitation and 460 of emission for caspase-4 with the fluorescence microplate reader Victor^2 (Wallac).

2.4. Analysis of synergism

The traditional isobologram method was used to analyse the effect of the staurosporine-DTT combinations [16]. Drug concentrations producing the same activation of the caspase-3 activity were selected to obtain the line of additivity. In our conditions, equivalent concentrations of staurosporine and DTT were 100 nM and 1.25 mM, respectively. Since below these concentrations the action of the two drugs was linear, fractions of these concentrations were combined to observe at which combination they yielded the same effect of 100 nM staurosporine or 1.25 mM DTT. If the line joining all dose pairs inducing this effect was coincident with the line of additivity, the action of the two drugs was considered to be additive. If it was located above the line of additivity the combination was considered to be antagonistic, if it was located below, the interaction was considered to be synergic.

2.5. Binding of annexin V

After the incubation with the drugs, the cells were centrifuged and resuspended in 100 μl of a binding buffer containing 0.125 μg/ml of annexin V-FITC and 10 μM of propidium iodide. The samples were incubated 10 min at room temperature in the dark, diluted to 500 μl with binding buffer and analysed by flow cytometry (EPICS XL, Beckman Coulter). When phosphatidylserine was translocated to the external surface of plasma membrane as a consequence of apoptosis, the cells appeared annexin V positive and propidium iodide negative.

2.6. Western blot analysis

After 90 min of incubation with staurosporine and/or DTT the expression of Mcl-1, CHOP and BiP were analysed in Jurkat cells. 4×10^6 cells were lysed in 200 μl of Laemmli buffer (62.5 mM Tris, 2% (w/v) of sodium dodecyl sulfate, 10% (v/v) of glycerol, 100 mM β-mercaptoethanol, pH 6.8) and heated 5 min at 100 °C. After 15 min centrifugation at 10000 × *g*, an aliquot of the supernatant containing 40 μg of protein was subjected to 10% (w/v) SDS-PAGE. The protein bands were transferred to a PVDF membrane (Hybond-P, Amersham) and exposed overnight at 4 °C to the primary antibody. After 1 h incubation with the secondary antibody at room temperature, the signal was detected by chemiluminescence using the ECL-Advance western blotting detection kit (Amersham). Anti β-actin antibody was used as loading control.

2.7. Release of cytochrome c from mitochondria

After 90 min of incubation with staurosporine and/or DTT, 1.5×10^6 cells were pelleted, resuspended in 1 ml of 250 mM sucrose, 1 mM EGTA, 5 mM Tris, 0.1 mM of the protease inhibitor PMSF (pH 7.4) and disrupted by 15 strokes of a Potter homogenizer equipped with a teflon pestle. The homogenate was then centrifuged 20 min at 15000 × *g* and the supernatant was immediately tested for the presence of cytochrome *c* using a human cytochrome c ELISA kit (Bender Medsystems). A

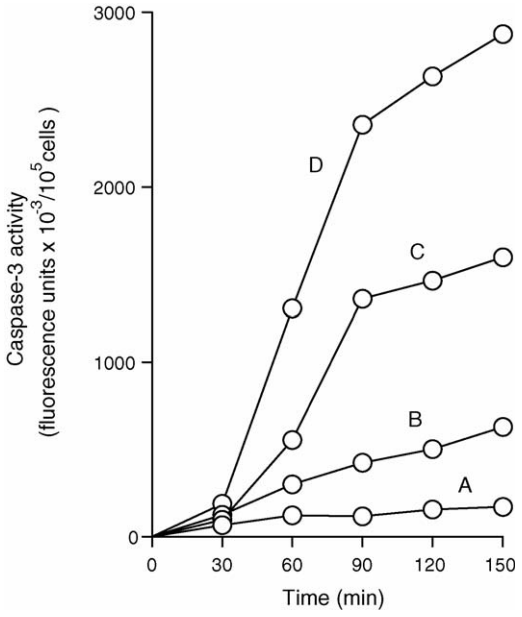

Fig. 1 – Time course of the proapoptotic effect of staurosporine and DTT in Jurkat cells. The activity of caspase-3 at the end of the indicated time of incubation is expressed as arbitrary fluorescent units/10^5 cells × 10^{-3}. Experimental conditions described in the legend to Table 1. (A) Activity without drugs; (B) 1 mM DTT; (C) 250 nM staurosporine; (D) staurosporine plus DTT.

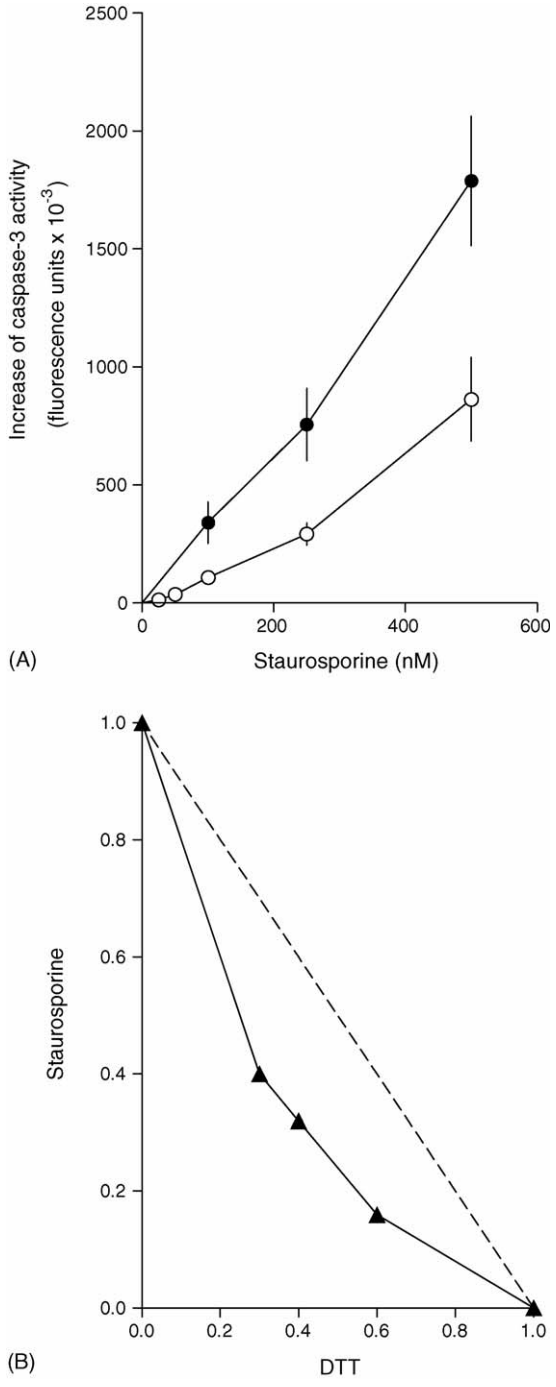

Fig. 2 – Synergism between staurosporine and DTT in Jurkat cells. In (A) cells were incubated 90 min at 37 °C with the indicated concentrations of staurosporine as described in Table 1. White symbols, staurosporine alone (n = 7); black symbols, staurosporine plus 0.35 mM DTT (n = 4). The data are expressed as the increase of caspase-3 activity with respect to the basal value (means ± S.E.M.); $P < 0.01$ for the difference between the two curves. The isobologram of binary drug combinations is reported in (B). Concentrations of staurosporine and DTT producing equivalent effect were made equal to 1 and were used to trace the line of additivity (broken line). These were 100 nM staurosporine and 1.25 mM DTT, that yielded a mean value of 100% increase of caspase-3 activity in three separate experiments. Then three binary fractional concentrations of the two drugs producing the same effect were plotted. In case of additivity they should have been coincident with the line of additivity. In case of synergism, they should have been below this line.

calibration curve for the concentration of cytochrome c was obtained using the purified preparation included in the kit. The data were expressed as concentration of cytochrome c in the supernatant (ng/ml).

2.8. *Statistical analysis*

Statistical analysis was performed using GraphPad Prism version 3.02 for Windows, GraphPad software.

3. Results

A number of previous studies demonstrated that Jurkat cells are sensitive to the apoptotic effect of staurosporine [15,17]. In agreement, we found a significant activation of caspase-3 activity upon 90 min incubation of these cells with nanomolar concentration of staurosporine (Table 1). Prolonging the treatment to 180 min increased the activation. The same sensitivity to staurosporine was found in the monocytic cell line Mono-Mac 6. Although with lower potency, a significant activation of caspase-3 activity was also observed with DTT, a drug causing ER stress [11]. When staurosporine and DTT were combined, the increase of caspase-3 activity was far greater than that expected from a simple additive effect.

The kinetics of caspase-3 activation induced by staurosporine and DTT reflected their different mechanism of action (Fig. 1). While the action of DTT slowly progressed with the time, in agreement with a gradual accumulation of unfolded proteins in the ER, the rapid action of staurosporine reflected an acute toxic effect. When the two drugs were combined the resulting activation of caspase-3 had a kinetic similar to that of staurosporine alone, suggesting that the potentiation of the staurosporine effect was the dominant event. To confirm this result and to have a direct evidence of a synergic staurosporine–DTT interaction a titration curve for staurosporine was obtained in the presence of a subthreshold DTT concentration. As expected, a twofold increase in the potency of staurosporine was observed in the presence of DTT used at 0.35 mM, a concentration unable to activate caspase-3 (Fig. 2A). In accord, the traditional isobologram analysis based on isoeffective binary drug combinations indicated a synergistic interaction between the two agents (Fig. 2B).

To test other signals of apoptosis we first measured the translocation of phosphatidylserine to the external surface of the plasma membrane using the binding of annexin V. When used separately at 180 min of incubation staurosporine and DTT both increased the binding of annexin V. However, when the two drugs were combined the action was significantly greater than a simple additive effects (Fig. 3A). Reducing the time of incubation to 90 min the two drugs had no effect when used alone but their combination gave a clear increase of annexin V binding (Fig. 3B). Similar results were obtained measuring the expression of the protein Mcl-1 (Fig. 4). This

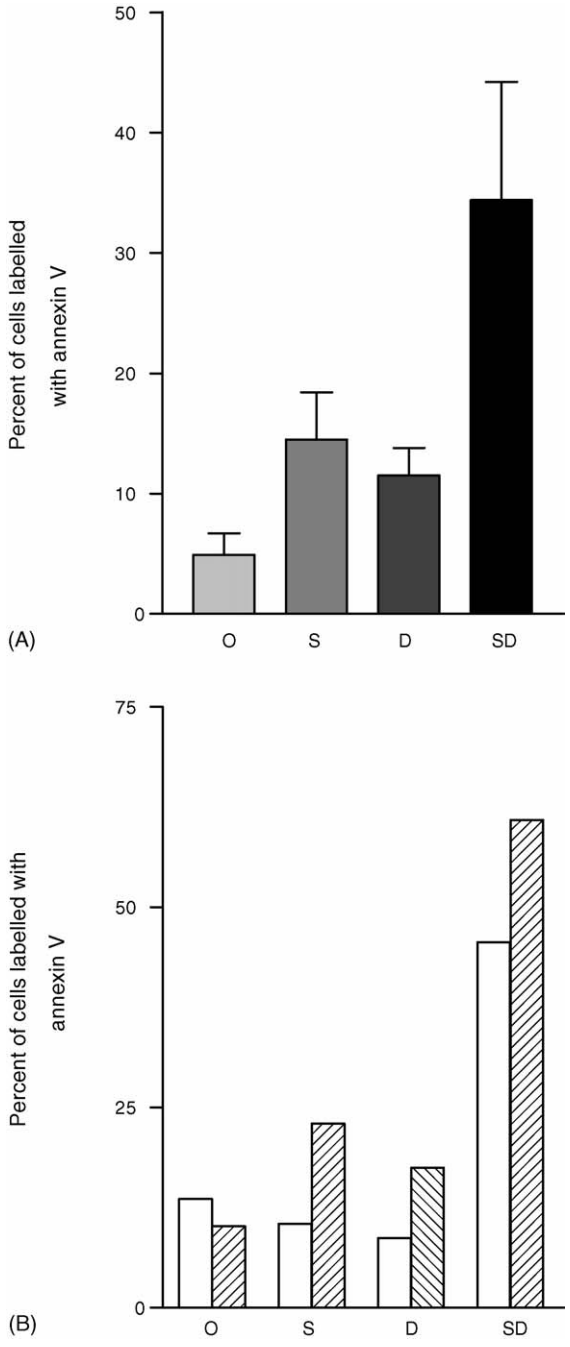

Fig. 3 – Binding of annexin V. Jurkat cells (5×10^5) in 0.5 ml of RPMI containing 5 mg/ml of BSA were incubated with staurosporine, DTT or their combination. Cells were then collected, labelled with annexin V and propidium iodide and analysed by flow cytometry. (A) Annexin V binding after 180 min treatment, data are mean ± S.E.M. of four experiments; Student's tests (paired, two tailed): $P < 0.05$ staurosporine vs. control, DTT vs. control, staurosporine plus DTT vs. control, staurosporine alone and DTT alone. (B) Representative experiment showing the effect of staurosporine and DTT at 90 min (white bars) and at 180 min (hatched bars) on annexin V binding. O, none; S, 250 nM staurosporine; D, 1 mM DTT; SD, 250 nM staurosporine plus 1 mM DTT.

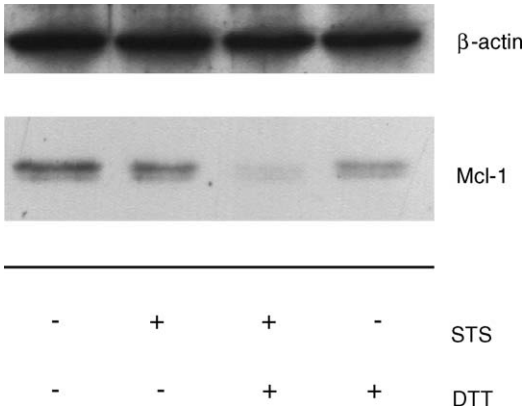

Fig. 4 – Expression of Mcl-1 protein. Jurkat cells (4×10^6) in 4 ml of RPMI containing 5 mg/ml of BSA, were incubated 90 min at 37 °C with or without 1 mM DTT and/or 250 nM staurosporine (STS). Cells were then pelletted, lysed in 200 μl of Laemmli buffer and heated 5 min at 100 °C. After centrifugation an aliquot of the supernatant containing 40 μg of protein was subjected to Western blotting analysis as described in Section 2.

protein is an anti-apoptotic member of the Bcl-2 family of proteins, also required for the development and maintenance of B and T lymphocytes [18]. Recently, it has been shown that Mcl-1 is cleaved by activated caspase-3 in Jurkat cells undergoing apoptosis [19]. In agreement with these results it was found that 90 min of incubation with staurosporine caused a decrease of Mcl-1 expression. The same effect was produced by DTT in agreement with its activating effect on caspase-3. When the two drugs were combined the cleavage of Mcl-1 was almost complete.

To gain information on the apoptotic pathways used by DTT and staurosporine and to define the possible point of the synergic interaction, we studied the effect of the two drugs on the expression of three markers of ER stress (CHOP, BiP, caspase-4 activation) and on the release of cytochrome c from mitochondria, indicative of the mitochondrial pathway of apoptosis. As shown in Fig. 5, after 90 min incubation we found a slight increase in CHOP expression in the cells treated with DTT 1 mM or DTT 1 mM plus staurosporine 250 nM. BiP expression was not modified by the two drugs or by their combination after 90 min treatment. Interestingly, 90 min treatment with DTT did not stimulate caspase-4 activity whereas staurosporine induced a significant increase of the activity that was further enhanced by the addition of DTT (Fig. 6). These results are in agreement with the previous observations showing that the activation of caspase-4 (and caspase-12 in rodents) is subsequent to the activation of mitochondrial pathway of apoptosis [20,21]. Further support of this possibility is given by the results on cytochrome *c* release. As shown previously on Jurkat [17] and melanoma cells [6], we found that 1 μM staurosporine caused an increase in cytosolic concentration of cytochrome *c* (276 ± 50%, $n = 8$). DTT was also active: at 1 mM the increase was 239 ± 42% ($n = 5$). Combination of a subthreshold concentrations of DTT and staurosporine yielded a large increase of cytochrome *c* release (6.1 times increase) (Fig. 7).

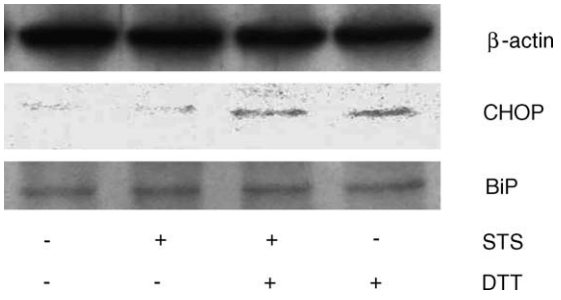

Fig. 5 – Expression of CHOP and BIP proteins. Jurkat cells (4×10^6) in 4 ml of RPMI containing 5 mg/ml of BSA, were incubated 90 min at 37 °C with or without 1 mM DTT and/or 250 nM staurosporine (STS). Cells were then pelletted, lysed in 200 μl of Laemmli buffer and heated 5 min at 100 °C. After centrifugation an aliquot of the supernatant containing 40 μg of protein was subjected to Western blotting analysis as described in Section 2.

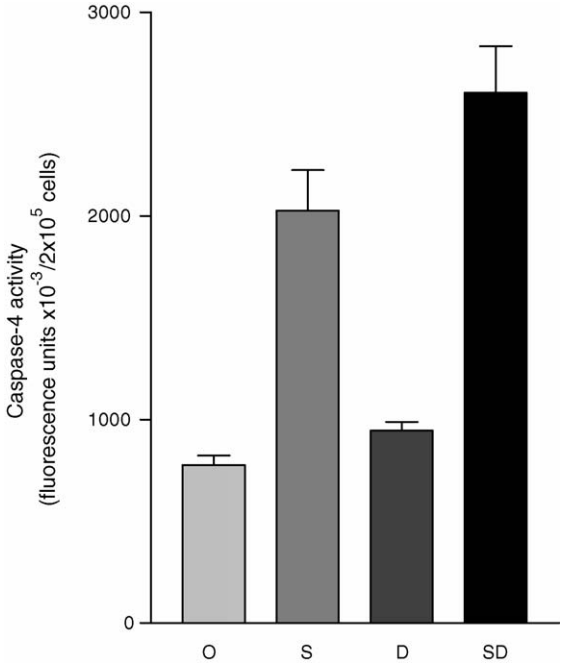

Fig. 6 – Measurement of caspase-4 activity. 2.5×10^6 Jurkat cells were suspended at 10^6 cell/ml in RPMI containing 5 mg/ml bovine serum albumin. 250 nM staurosporine, 1 mM DTT or their combination were added and the cells were incubated for 90 min at 37 °C. At the end of the incubation, the cells were pelleted, lysed and used for the determination of caspase-4 activity as indicated in Section 2. O, none; S, staurosporine; D, DTT; SD, staurosporine plus DTT. Data are expressed as arbitrary fluorescence units $\times 10^{-3}/2 \times 10^5$ cells (means ± S.E.M., $n = 6$). Student's *t*-test: $P < 0.01$ for the effect of staurosporine vs. none, staurosporine plus DTT vs. none, $P < 0.05$ for the effect of staurosporine plus DTT vs. staurosporine.

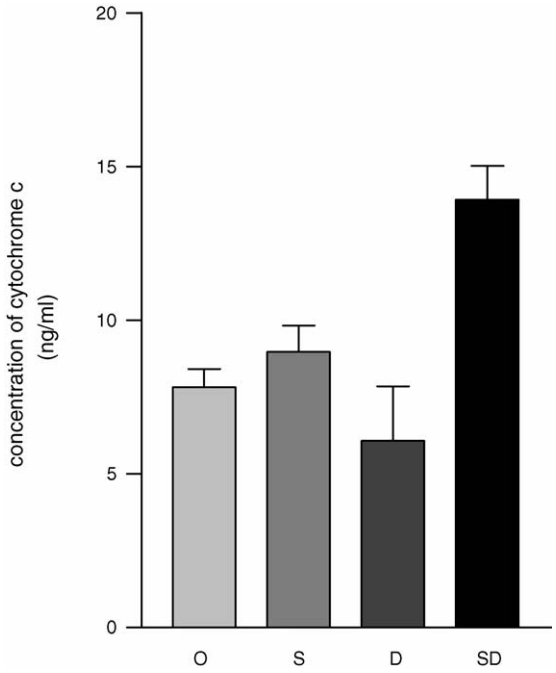

Fig. 7 – Release of cytochrome *c* from mitochondria. Jurkat cells (1.5×10^6) were incubated 90 min at 37 °C in 1.5 ml of RPMI supplemented with 0.5% bovine serum albumin. 250 nM staurosporine, 350 μM DTT or their combination were added and the release of cytochrome *c* was measured as indicated in Section 2. O, none; S, staurosporine; D, DTT; SD, staurosporine plus DTT. Data are expressed as concentration (ng/ml) of cytochrome *c* in the supernatant (means ± S.E.M., $n = 6$). Student's *t*-test: $P < 0.01$ for the effect of staurosporine plus DTT vs. none.

In addition to DTT we investigated whether other drugs causing ER stress interacted with staurosporine. Tunicamycin was selected since it causes ER disfunction by inhibiting protein folding. The subsequent unfolded protein response gives rise to apoptosis in a treatment of several hours [22]. Like DTT, tunicamycin had a slow onset of action. At 90 min a treatment with 10 μg/ml of tunicamycin was not sufficient to increase the caspase-3 activity of Jurkat cells, but the action was manifest through a significant increase of the staurosporine-induced apoptosis. Prolonging the incubation of tunicamycin to 12 h caused a large enhancement of staurosporine effect. At this time of incubation it was possible to detect a significant increase in caspase-3 activity also when tunicamycin was added alone (Table 2).

4. Discussion

Apoptosis is a physiological event regulating cell homeostasis in tissues. Dysregulation of this process forms the basis of several diseases. Cell loss due to ER stress is implicated in dilated cardiomyopathy [23], in neurodegenerative disorders such as the Alzheimer disease [24] and in the paracetamol-induced renal tubular injury [25]. Also, the apoptosis driven by

Table 2 – Combined effect of staurosporine and tunicamycin on caspase-3 activity of Jurkat cells

Additions	Caspase-3 activity/10^5 cells (fluorescence units $\times 10^{-3}$)	
	90 min ($n = 7$)	12 h ($n = 3$)
None	154.2 ± 19.6	134.0 ± 18.1
250 nM staurisporine	848.5 ± 94.8[a]	903.1 ± 113.9[a]
5 μg/ml tunicamycin	171.6 ± 33.9	196.8 ± 6.7[c]
10 μg/ml tunicamycin	169.3 ± 17.9	210.0 ± 32.7[c]
Staurosporine, 5 μg/ml tunicamycin	925.6 ± 170.0[a]	1855.0 ± 28.3[d]
Staurosporine, 10 μg/ml tunicamycin	1147.0 ± 135.2[b]	1665.0 ± 195.4[d]

Jurkat cells (5×10^5) in 0.5 ml of RPMI containing 5 mg/ml of BSA, were incubated 90 min with the indicated concentration of staurosporine and tunicamycin. Alternatively, cells were incubated 12 h with tunicamycin and staurosporine was added in the last 90 min. At the end of incubation, the cells were collected, lysed and used for the determination of caspase-3 activity. Data are means ± S.E.M. Student's *t* tests (unpaired, one tailed).

[a] $P < 0.01$ vs. none.

[b] $P < 0.01$ vs. none and <0.05 vs. staurosporine alone.

[c] $P < 0.05$ vs. none.

[d] $P < 0.01$ vs. none and vs. staurosporine alone.

the extrinsic and the mitochondrial pathway may depress the immune response. Conversely, the repression of apoptosis contributes to the onset and the rapid development of a neoplastic growth. To develop effective therapies in these diseases, much effort is currently devoted to the possibility of achieving a pharmacological control of apoptosis. Progress in this field shows that antineoplastic drugs act through the stimulation of apoptosis. On the basis of their effect, tumor cells are classified in type I or type II, depending on whether their death is only produced by the activation of plasma membrane death receptors or it requires the assistance of the mitochondrial pathway [26].

In the present study we examine the possibility to strengthen the pharmacological control on apoptosis, combining drugs acting at different sites in the cell. To this end we selected staurosporine and DTT. The first is a known apoptotic agent, prototype of potent antineoplastic drugs [14]. Its major site of action is the mitochondrial pathway, although the components of the extrinsic pathway are also implicated [6,15]. DTT is a thiol reagent, preventing the formation of disulfide bonds in nascent proteins. Due to their reducing effect, DTT and other thiol reagents may show anti-apoptotic action when an oxidative cell injury is produced. However, their general effect is the unfolded protein response leading to the ER-driven apoptosis programme [11]. In our tests the different mechanism of action between staurosporine and DTT is shown by the kinetics of the induced caspase-3 activation. While staurosporine induced an acute response, the effect of DTT slowly progressed with the time, as predicted by its action on protein folding and indirect pro-apoptotic effect. Direct evidence of a difference between the staurosporine and DTT effect is provided by the activation of CHOP, a marker of apoptosis driven by ER stress. In contrast to staurosporine, DTT elicited the expression of this protein. Interestingly, both drugs induce the release of cytochrome *c*, indicating that mitochondria could be a point of convergence of the pathways elicited by DTT and staurosporine. This result is in agreement with other observations reporting that ER stress-induced apoptosis uses the mitochondrial pathway [24].

It has been reported that human procaspase-4 shows similar characteristics to mouse procaspase-12, an initiator procaspase specific for ER stress [27]. However recent studies indicate that in several cell lines either caspase-4 or caspase-12 operate downstream of caspase-9, suggesting that these two caspases could act as effector caspases activated after cytochrome *c* release [20,21,27]. Our results on caspase-4 activation in Jurkat cells by staurosporine or by staurosporine plus DTT are in agreement with these observations.

When staurosporine and DTT are added together we observe a severe apoptotic reaction, greater than that expected from a simple additive effect. Traditional isobolograms analysis confirmed the synergic nature of staurosporine–DTT interaction. The proapoptotic action of the combination has been documented by the release of cytochrome *c* and by the activation of caspase-3 and caspase-4 that are early manifestations of the apoptotic sequence. Two downstream events such as the translocation of phosphatidylserine to the external side of plasma membrane and the cleavage of the antiapoptotic protein Mcl-1 confirmed the synergism.

The data on DTT were in agreement with those obtained with tunicamycin, a drug belonging to a complex family of nucleosides with potent antibiotic and antiviral activities [28]. Due to the induced inhibition of *N*-glycosylation, this drug causes protein unfolding and their accumulation in the ER [9,10]. In agreement with its indirect proapoptotic effect, tunicamycin has not a rapid effect. To observe a significant activation of caspase-3, Jurkat cells had to be incubated with the drug at least 12 h. In spite of slow proapoptotic effect, tunicamycin required only 90 min of incubation to potentiate the action of staurosporine.

Taken together, the data on DTT and tunicamycin suggest that staurosporine is more active in cells undergoing ER stress. When a cell is under apoptotic stimuli, its fate depends on the balance between death and survival signals. Survival signals are provided by the phosphatidylinositol 3-kinase/Akt and by the MEK/ERK kinase pathways, and by the antiapoptotic members of the Bcl-2 family of proteins. In agreement, Bcl-2 proteins have been shown to prevent the release of cytochrome *c* induced by the ER stress [29] and the activation of PI 3-kinase and of ERK facilitates the survival function of unfolded protein response [22]. The survival signals that may rescue the cell under the apoptotic influence of DTT and tunicamycin could be neutralized by staurosporine that inhibits several protein kinases including Akt [30,31]. On these basis it may be concluded that the combination of staurosporine with drugs inducing the unfolded protein response results in the enhancement of death signals whereas the survival signals are inhibited. This conclusion may be of value in therapeutic measures based on the activation of apoptosis. For example, it would be of interest to study whether the antineoplastic activity of staurosporine and its analogues is increased in the presence of agents acting on protein folding in the ER.

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