THE INDUCTION OF APOPTOSIS BY DAUNORUBICIN AND IDARUBICIN IN HUMAN TRISOMIC AND DIABETIC FIBROBLASTS

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Abstract: In this study, we investigated apoptosis induced in human trisomic and diabetic fibroblasts by daunorubicin (DNR) and its derivative, idarubicin (IDA). The cells were incubated with DNR or IDA for 2 h and then cultured in a drug-free medium for a further 2-48 h. The apoptosis in the cultured cell lines was assessed by biochemical analysis. We found that both drugs induced a time-dependent loss of mitochondrial membrane potential, and a significant increase in intracellular calcium and caspase-3 activity. Mitochondrial polarization and changes in the level of intracellular calcium were observed during the first 2-6 h after drug treatment. Caspase-3 activation occurred in the late stages of the apoptotic pathway. Our findings also demonstrated that idarubicin was more cytotoxic and more effective than daunorubicin in inducing apoptosis in trisomic and diabetic fibroblasts.

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INTRODUCTION

The anthracyclines, including daunorubicin (DNR) and idarubicin (IDA), are one of the most clinically useful groups of chemotherapeutic agents. These drugs are widely used in the treatment of hematological malignances. Idarubicin is a synthetic derivative of daunorubicin. It has a hydrogen atom in place of the methoxyl group in position C-4 [1]. A number of recently published clinical studies suggest that IDA is more potent and less cardiotoxic than DNR [2, 3]. The cytotoxic effect of most anti-cancer drugs *in vivo* and *in vitro* depends on the induction of apoptosis in tumor cells. Several studies demonstrated that DNR induces apoptosis in human leukemic cell lines [4, 5], epidermoid carcinoma A-431 cells [6], and pancreatic cell line L3.6 [7]. Little is still known about the induction of apoptosis by IDA in cancer cells and other types of cells. According to Liu *et al.* [8], IDA is able to induce apoptosis in human leukemia cells. Drug-induced cell death is mediated by caspase-3 activation. It was recently demonstrated that IDA induces the formation of DNA-topoisomerase II cleavable complexes and apoptosis in human K562 and CCRF-CEM leukemia cells [9, 10].

Accumulating evidence suggests that apoptosis could play an important role in the pathogenesis of many diseases. There are diseases linked with the suppression of apoptosis, including cancer and autoimmune disorders. Other diseases are linked with an increased level of apoptosis, for example Down's syndrome (DS, trisomy 21) and diabetes mellitus [11]. Increased susceptibility to apoptosis is displayed by the pancreatic β-cells of diabetic patients and the neurons of DS individuals. There is now experimental evidence that cell death in these diseases is mediated by an increase in oxidative damage [12, 13]. So far, the susceptibility of other diabetic and trisomic cells to apoptotic death, especially those cells exposed to anti-cancer agents, has yet to be elucidated.

In this study, we investigated the response of human trisomic and diabetic fibroblasts to anthracycline drugs, namely DNR and IDA treatment. As anthracycline drugs are metabolized to free radicals, it may be expected that the oxidative damage to cells will be different in the case of fibroblasts derived from diabetic and DS patients than the damage to normal cells. Therefore, we compared the effect of DNR and IDA on the induction of apoptosis in diabetic and DS fibroblasts with the cell death induced by the two drugs in normal cells. Apoptosis in all three cell lines was evaluated by measuring the mitochondrial membrane potential, the activation of caspase-3 and the level of intracellular calcium $[Ca^{2+}]_i$. We also determined the intracellular accumulation and distribution of anthracyclines in the investigated cell lines. We found that daunorubicin and idarubicin were less toxic for trisomic and diabetic fibroblasts than for normal cells.

MATERIALS AND METHODS

Cell lines

Human fibroblasts derived from the skin of a healthy donor (WA-1 cell line), a Down's syndrome patient (T-158 cell line) and a diabetic type II patient (C-5 cell line) were obtained from the tissue bank of the Centre of Child Health (Warsaw, Poland). All the cell lines were cultured as monolayers at 37ºC in a humidified atmosphere of 5% CO_2 and maintained in exponential growth in Eagle's Minimal Essential Medium supplemented with 10% newborn calf serum, 10% lactoalbumin hydrolysate and 5 μg/ml gentamycin. For the experiments, fibroblasts between the 5th and 15th passages were used.

Drug treatment

Normal, trisomic and diabetic fibroblasts at a density of $1x10^6$ cells/ml were exposed for 2 h to DNR or IDA at a final concentration of 3 μM. After incubation, the cells were washed twice with cold sodium phosphate buffered saline (PBS) to remove any free drug. Then, fresh culture medium was added and the cells were incubated for a further 2-48 h. At selected points, the cells were assessed for caspase-3 activity, mitochondrial membrane potential (ΔΨm) and intracellular calcium content.

Cytotoxicity studies

The sensitivity of the cell lines to the drugs was determined using the MTT assay as previously described [14]. Briefly, cells ($1x10^4$) seeded in a 96-well plate were treated with DNR or IDA (0.5-10 μM). After incubation for 2 h, the medium was replaced with drug-free medium, and the cells were cultured for a further 72 h. Then, MTT was added for 4 h. Formazan crystals, formed by mitochondrial reduction of MTT, were solubilized in dimethyl sulfoxide, and the absorbance was measured at 570 nm using a Microplate reader (Awareness Technology Inc, USA). The IC_{50} parameter, defined as the drug concentration that reduced cell growth to 50% of that of the control cells, was calculated from the linear transformation of the dose response curves.

Measurements of the intracellular accumulation of daunorubicin and idarubicin

Measurements of the accumulation of the drugs were performed according to Kiyomiya *et al.* [15]. Cells were incubated in the presence of 3 μM DNR or 3 μM IDA for 5-30 min at 37ºC. After incubation, the cells were washed twice with phosphate buffered saline by centrifugation at 1000 x g for 5 min. The cell pellet was homogenized in 5 mM Tris buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA. The homogenate was centrifuged at 600 x g to separate two fractions: the nuclear pellet and the post-nuclear supernatant. The drugs were extracted with 1 ml of 0.3 N HCl, 50% ethanol and 0.1% Triton X-100 solution. The analysis was done on a Perkin-Elmer LS-50B spectrofluorimeter, using excitation at a wavelength of 480 nm and emision at 585 nm.

Caspase-3 activity
The activity of caspase was determined using an EnzChek® Caspase-3 Fluorimetric Assay Kit (Molecular Probes, USA) according to the manufacturer's instructions. Briefly, following treatment with the drugs (3-9 μM), the cells were washed with ice-cold PBS and lysed with cell-lysis buffer. After centrifugation, the samples were incubated with the caspase-3 substrate, Z-DEVD-AMC. The fluorescence intensity was measured at an excitation wavelength of 350 nm and an emission wavelength of 450 nm with a Fluoroscan Ascent FL plate reader (Labsystems, Sweden). The fluorescence intensity of the studied samples increased with increasing enzyme activity. The degree of the increase in caspase activity was calculated by comparing the fluorescence of the drug-treated cells with that of the untreated controls, taken as 100%.

Determination of mitochondrial membrane potential
Changes in the mitochondrial membrane potential ($\Delta\Psi$m) were measured as described by Özgen *et al.* [16]. Following drug treatment (3 μM DNR or 3 μM IDA, 2 h at 37ºC), cells were cultured in drug-free medium for 2-48 h at 37ºC. At selected points, cells were incubated with $DiOC_6(3)$ (at a final concentration of 100 mM) for 15 min at 37ºC. The fluorescence intensity was recorded at an excitation wavelength of 460 nm and an emission wavelength of 515 nm using an LS-50B Perkin Elmer spectrofluorimeter (England). Depolarization of the mitochondrial membrane potential results in a reduction in $DiOC_6(3)$ fluorescence.

Measurement of intracellular calcium
Changes in the level of intracellular free calcium $[Ca^{2+}]_i$ were measured according to the procedure of Mulvaney *et al.* [17]. Cells were incubated with 5 μM indo 1-AM at 37ºC for 30 min in the dark. Then, the cells were washed twice with PBS and analyzed in a spectrofluorimeter (Perkin Elmer LS 50B) at an excitation wavelength of 330 nm and an emission wavelength of 401 nm. The level of Ca^{2+} was expressed as a ratio of the fluorescence intensity of drug-treated samples relative to the intensity for the corresponding control cells, taken as 100%.

Statistical analysis
Differences between the drug-treated and control cells were analyzed using Student's t-test. A value of $p < 0.05$ was considered to be significant.

RESULTS

Cytotoxicity assay
As shown in Tab. 1, the investigated cell lines exhibit a significantly different sensitivity to daunorubicin and idarubicin. Relative to normal cells, the trisomic and diabetic fibroblasts were respectively about 1.7-fold and 2.5-fold less

sensitive to DNR and about 2-fold and 3-fold less sensitive to IDA. In all the cell lines, IDA was evidently more potent than DNR.

Tab. 1. The mean IC_{50} concentrations of DNR and IDA in normal, diabetic and trisomic human fibroblasts. The IC_{50} values were determined at 72 h of the post-incubation time. The data is the mean $\pm$ SD of five independent experiments.

Cell line	IC_{50} (μM)	
	DNR	IDA
Normal WA-1	3.60 ± 0.17	$2.69 \pm 0.19^{\#}$
Trisomic T-158	$6.27 \pm 0.16^{*}$	$5.26 \pm 0.08^{\#*}$
Diabetic C-5	$9.11 \pm 0.12^{*}$	$7.69 \pm 0.14^{\#*}$

$^{*}p < 0.001$ as compared with normal cells, $^{\#}p < 0.05$ significant differences between DNR-treated and IDA-treated cell lines

Intracellular drug accumulation
To confirm whether the cytotoxic activity of anthracycline drugs was related to their intracellular accumulation, the uptake of DNR and IDA by human fibroblasts was determined. As shown in Fig. 1, both drugs were preferentially accumulated in the nuclear fraction of all the cell lines after a 10- and 30-min drug treatment.
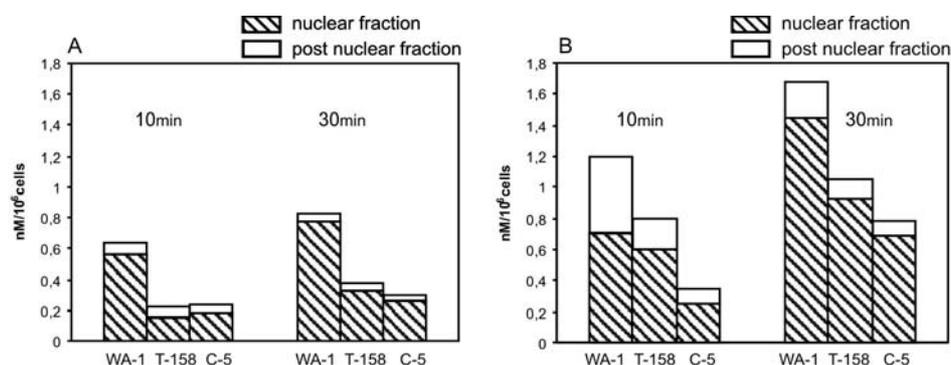

Fig. 1. Intracellular accumulation of daunorubicin (A) and idarubicin (B) in normal (WA-1), trisomic (T-158), and diabetic (C-5) cell lines. The data shown is the means from three independent experiments.

An analysis of drug accumulation revealed that in normal (WA-1) cells, the intracellular concentrations of DNR and IDA were much higher than in trisomic (T-158) and diabetic (C-5) cells. Under the same conditions, the intracellular accumulation of IDA was 2 times greater in normal cells and 3 times greater in trisomic and diabetic cells than that of DNR.

Mitochondrial membrane potential
To evaluate the mitochondrial alterations in the drug-treated cell lines, we measured the mitochondrial membrane potential using the fluorescent probe $DiOC_6(3)$. We found that changes in $\Delta\Psi$m were drug and time dependent (Fig. 2). During the first 2-6 h after drug treatment, we observed a significant decrease in the mitochondrial membrane potential. After 6 h, the $\Delta\Psi$m in daunorubicin- and idarubicin-treated normal cells was respectively lowered to about 40% and 27% of the value for the untreated control cells. At the same time, the $\Delta\Psi$m in the trisomic fibroblasts exposed to DNR and IDA was diminished to 34% and 45%, and in the diabetic cells, to 63% and 59%, respectively. After a prolonged post-incubation time (12-48 h), the $\Delta\Psi$m progressively increased to 75-90% of the control values in all the investigated cell lines. These results demonstrate that drug-induced mitochondrial depolarization was partly reversed during the prolonged post-treatment period. Furthermore, our findings show that both drugs induced considerably lesser diminution in the mitochondrial membrane potential in diabetic cells compared to the normal and trisomic cell lines.
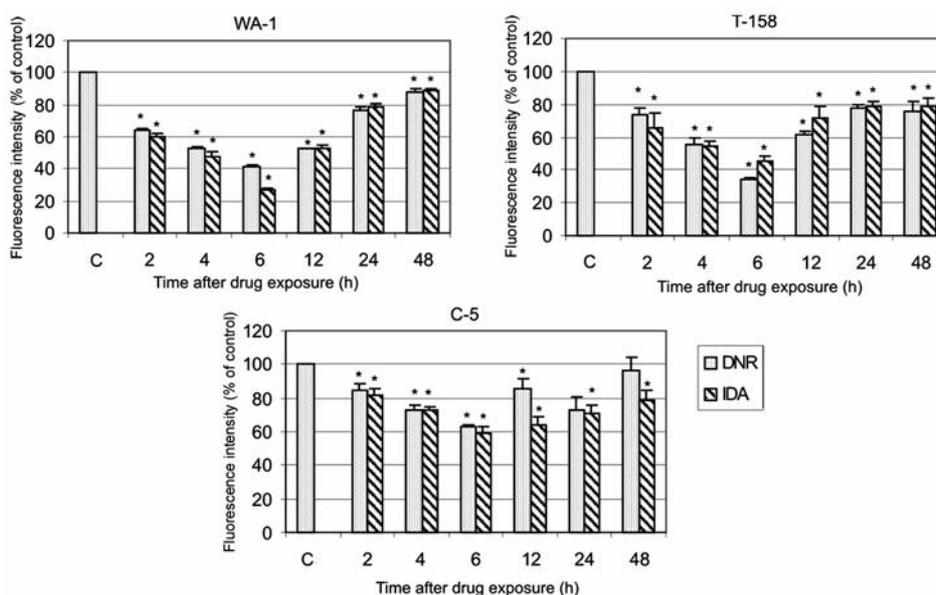

Fig. 2. The effects of daunorubicin and idarubicin on mitochondrial membrane potential in normal (WA-1), trisomic (T-158) and diabetic (C-5) cell lines. The data shown is the mean ± SD from five distinct experiments. *p < 0.05, statistically significant differences compared with untreated (control) cells taken as 100%.

Drug-induced changes in the level of calcium
We previously described that DNR and IDA induced time-dependent changes in the level of intracellular calcium in cultured normal (S-2) and trisomic (BB) cell lines [18]. In this study, we compared the effect of DNR and IDA on the calcium

content in normal (WA-1), trisomic (T-158) and diabetic (C-5) cell lines. As indicated in Tab. 2, the level of intracellular calcium, in all the types of cells preincubated with DNR or IDA reached maximal values at 2-4 h and declined after 12-48 h. The results from these experiments demonstrate that changes in the calcium content were significantly higher in the drug-treated normal and trisomic cell lines than in the diabetic cells.

Tab. 2. Changes in the intracellular calcium in the trisomic, diabetic and normal cells. Values are the means ± SD of four to six distinct experiments. The results are significantly different (*p < 0.05 and **p < 0.001) from the untreated, control cells.

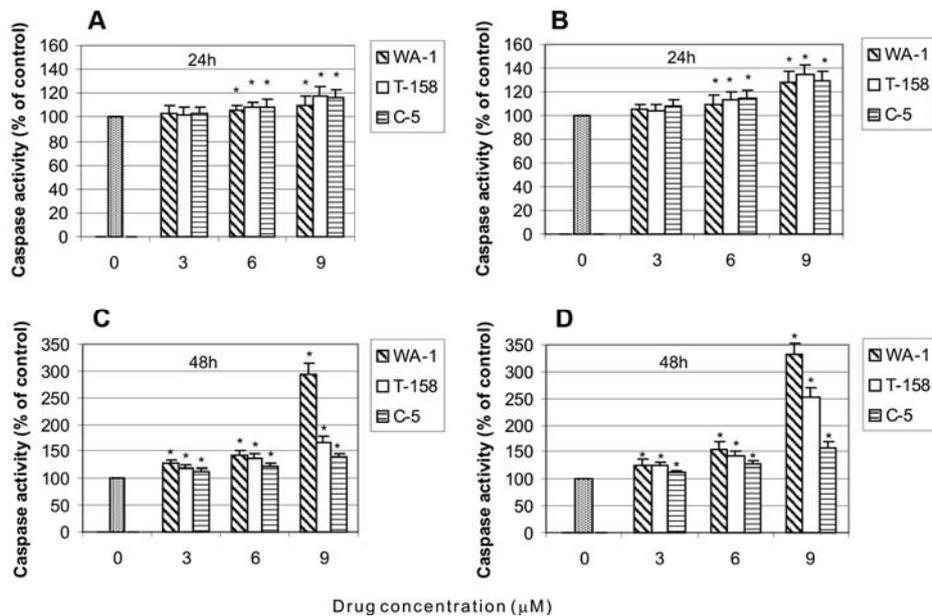| Time after drug treatment (h)	Normal (WA-1)		Trisomic (T-158)		Diabetic (C-5)	
	DNR	IDA	DNR	IDA	DNR	IDA
0	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
2	182.1 ± 6.7**	195.4 ± 7.7**	138.6 ± 5.7**	159.2 ± 6.5**	119.7 ± 5.9**	139.8 ± 3.9**
4	171.9 ± 5.5**	178.8 ± 6.7**	131.2 ± 4.5**	141.4 ± 3.2**	131.2 ± 7.3**	128.2 ± 9.7**
12	138.4 ± 9.5**	159.3 ± 4.6**	122.3 ± 3.1**	135.2 ± 4.3**	119.6 ± 6.2**	125.5 ± 8.6**
24	125.8 ± 9.3**	136.2 ± 4.2**	116.3 ± 4.7**	128.6 ± 3.3**	115.4 ± 3.5**	126.2 ± 6.4**
48	118.6 ± 4.6**	128.6 ± 4.3**	109.0 ± 3.2*	115.6 ± 4.2**	109.2 ± 3.7*	113.5 ± 7.2*

Fig. 3. The activation of caspase-3 in normal (WA-1), trisomic (T-158) and diabetic (C-5) cells. The activity of the enzyme was measured 24 h and 48 h after exposure of the cells to daunorubicin (A and C) or idarubicin (B and D). The data represents the mean ± SD of five separate experiments. *p < 0.05 indicating statistically significant differences compared with the control cells, taken as 100%.

The activation of caspase-3 by anthracyclines
Another feature of apoptosis is the activation of caspases. In this study, we measured the activity of the effector caspase-3. Fig. 3 shows the alterations in caspase-3 activity that occurred 24 h and 48 h after the exposure of human fibroblasts to DNR and IDA. Both drugs induced a dose- and time-dependent increase in the enzyme activity in all cell types. Maximal caspase-3 activity was observed 48 h after treatment with the highest concentration (9 µM) of DNR and IDA. Under the same conditions, caspase-3 activation was considerably greater in normal and trisomic fibroblasts than in diabetic cells. In the investigated cell lines, idarubicin was more effective than daunorubicin.

DISCUSSION

There is considerable evidence that patients with DS have a significantly increased risk of developing hematological disorders and diabetes [19]. In this context, the elucidation of the response of cells derived from DS individuals and diabetic patients to anti-cancer drugs is of special importance. In this study, we demonstrated that both trisomic and diabetic fibroblasts were significantly less sensitive to daunorubicin and idarubicin than normal cells. An analysis of the intracellular drug distribution indicated that DNR and IDA were localized preferentially in the nuclear fraction. 30 min after the treatment, only slight drug fluorescence was observed outside of the nucleus. At this point, the content of DNR in the nuclear fraction was 2.3-fold lower in the trisomic and about 3-fold lower in the diabetic cells than in the normal fibroblasts. During the exposure of the trisomic and diabetic cell lines to IDA, the drug accumulation in the nucleus was about 1.5-2 times less than in the normal cell line. The observed differences in the cytotoxicity and intracellular content of DNR and IDA might be related to their chemical structure and the fluidity of the cell membranes. The deletion of the methoxy group at position 4 makes idarubicin more lipophilic [1]. This results in a more rapid cellular uptake and higher intracellular drug accumulation than with daunorubicin [3]. Our previous study revealed that the untreated human trisomic fibroblasts had lower fluidity of the plasma membrane than normal cells. DNR caused a further decrease in the membrane fluidity in trisomic cells [20]. Preliminary studies performed in our laboratory with cultured diabetic fibroblasts indicated that their plasma membrane was also markedly less fluid than normal and trisomic cell lines. Like DNR, IDA predominantly affected the hydrophobic regions of the lipid bilayer inducing a marked decrease in their fluidity (data not shown). Similar results were demonstrated in immortal rodent fibroblasts exposed to doxorubicin, aclarubicin and mitoxantrone. All three drugs at concentrations of 5-20 µM induced increased rigidity in the membranes of B14 and NIH3T3 cells [21]. These findings clearly show that the intracellular accumulation of DNR and IDA is dependent on plasma membrane fluidity. The trisomic and diabetic cells with greater membrane lipid rigidity

exhibited a significantly lower level of both drugs in the nuclear and post-nuclear fractions in comparison with normal fibroblasts.

A substantial part of our study was focused on the biochemical changes induced during apoptosis by anthracycline drugs in human fibroblasts. In this study, we investigated mitochondrial membrane potential, caspase-activation and the level of intracellular Ca^{2+}. The dissipation of $\Delta\Psi m$ is one of the markers for mitochondrial involvement in apoptosis. Our data shows that a drop in mitochondrial membrane potential occurred during the first 6 h following the drug treatment, and that it was the most prominent in normal fibroblasts. After a prolonged time of incubation (12-48 h), independent of the cell type, the $\Delta\Psi m$ gradually recovered. These observations suggest that a loss of $\Delta\Psi m$ was an early event in apoptosis induced by both drugs in human fibroblasts. We also found that, of the two tested drugs, IDA was more effective in the depolarization of the mitochondria. A decrease in the mitochondrial potential was also observed in doxorubicin-induced apoptosis in mammary adenocarcinoma (MTLn3) cells, porcine renal proximal tubular cell line LLC-PK1 [22], and in human tumor cells after amrubicin treatment [23].

Increasing evidence suggests that Ca^{2+} is also one of the most important signalling agents in mammalian cells [24]. A rise in intracellular Ca^{2+} may be one of the triggering events that leads to cell injury or apoptosis in various cell models [25, 26]. The experiments performed in this study demonstrate that $[Ca^{2+}]_i$ increased early after DNR and IDA treatment and was accompanied by $\Delta\Psi m$ reduction in all the cell types. Another pivotal event in the course of apoptosis is the activation of specific cysteine proteases, especially caspase-3. As shown by many authors, anthracycline drugs induce apoptosis in cancer cells by activating caspase-3 [27-29]. In this study, both DNR and IDA caused the activation of caspase-3 in trisomic and diabetic cell lines. The enzyme activation was maximal 48 h after drug exposure. It was followed by a loss of $\Delta\Psi m$ and an increase in $[Ca^{2+}]_i$. As expected, a significant elevation in caspase-3 activity was observed when the cells were exposed to the drugs at the highest concentration (9 μM). These results indicated that at 48 h, the level of enzyme activation was markedly lower in trisomic and diabetic cell lines than in normal cells.

The differences in the response of trisomic, diabetic and normal fibroblasts to daunorubicin and idarubicin treatment may result not only from the different fluidity of the plasma membranes but also from changes in the content of cellular antioxidants. An analysis of antioxidant enzymes in trisomic fibroblasts revealed an increased activity of superoxide dismutase and glutathione reductase [30, 31]. Our previous studies showed that trisomic and diabetic fibroblasts also contain a higher level of GSH and glutathione peroxidase [14, 31]. However, the effects of anthracycline drugs on the investigated cell lines are related to oxidative stress; the trisomic and diabetic fibroblasts, containing more efficient antioxidant systems, are better protected against drug-induced apoptosis than the normal cells.

Several recent studies demonstrated the activation of the apoptotic pathway in patients with Down's Syndrome and diabetes. The apoptotic mechanism of cell death in DS was presented in human cortical neurons [32], the brain [33], and neuronal cultures and thymocytes [34, 35]. Apoptosis in DS was shown to be mediated by caspases and the cytochrome c-independent pathway [36]. The role of apoptosis in the pathogenesis of diabetes mellitus is poorly understood. It has been recently reported that patients with type 2 diabetes exhibit increased activities of caspase 3, 4 and 6 [37]. According to other authors, apoptosis induced by hyperglycemia in several types of cells is mediated by caspase-3 activation and the formation of reactive oxygen species [38, 39]. Whether anthracycline drugs promote *in vivo* apoptosis in people with Down's syndrome and diabetes is not known. Previous studies from our laboratory have shown that DNR and IDA induce apoptotic changes in the morphology of the nucleus in cultured trisomic and diabetic fibroblasts [14, 18]. Changes in the cell morphology of diabetic fibroblasts were accompanied by the depletion of GSH and a significant increase in the activity of glutathione peroxidase and glutathione transferase [14]. Recently, Roat *et al.* [40] found that DNR-treated peripheral blood cells from DS and normal children have a similar tendency to undergo apoptosis.

In conclusion, the findings of this study provide evidence that daunorubicin and idarubicin induced *in vitro* the characteristic biochemical changes for apoptosis in human trisomic and diabetic fibroblasts. Daunorubicin and idarubicin were slowly incorporated into the trisomic and diabetic cell lines, and caused lower changes in the mitochondrial membrane potential, and in the level of calcium and caspase-3 activity compared to normal fibroblasts. Under the investigated conditions, the diabetic cell line was the most resistant to both drugs. Our results also demonstrate that idarubicin was more cytotoxic than daunorubicin, and seems to be a more effective modulator of biochemical events involved in the regulation of apoptotic pathways in trisomic and diabetic cells. A more detailed study on the effect of anti-cancer drugs on the apoptotic pathway of diabetic and DS fibroblasts cell death is currently being undertaken in our laboratory.

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