# Thymidylate synthase inhibition triggers glucose-dependent apoptosis in p53-negative leukemic cells

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**Abstract Chemotherapeutic drugs that inhibit the synthesis of DNA precursor thymidine triphosphate cause apoptosis, although the mechanism underlying this process remains rather unknown. Here, we describe thymineless death of human myeloid leukemia U937 cells treated with the thymidylate-synthase inhibitor 5′-fluoro-2′-deoxyuridine (FUdR). This apoptotic process was shown to be independent of p53, reactive oxygen species generation and CD95 activation. Caspases were activated downstream of cytochrome *c* but upstream of mitochondrial depolarization. Furthermore, FUdR-induced apoptosis required the presence of glucose in the culture medium at a step upstream of the release of cytochrome *c* from mitochondria.**


*Keywords:* Thymineless death; p53; Leukemia; Floxuridine; Glucose; Apoptosis

## 1. Introduction

A balanced supply of deoxyribonucleoside triphosphates (dNTP) is essential for cell viability and replication. Perturbations of the dNTP pools affect genetic stability [1] and cause cell death [2,3]. Several human diseases like anemia caused by folate deficiency or immunodeficiency due to the lack of adenosine-deaminase occur as a consequence of a non-balanced supply of dNTPs in hematopoietic cells. Several evidences link metabolism of dNTP and apoptosis. First, antineoplastic agents that inhibit dNTP synthesis have been shown to kill tumor cells by induction of apoptosis [4]. Second, dATP may be essential for activation of the apoptosome [5], and the mitochondrial dATP/ATP transporter adenine nucleotide translocator (ANT) is a component of the mitochondrial Permeability Transition Pore Complex, that may be involved in the onset of apoptosis in several systems [6]. Third, changes in dNTP levels have been found to precede DNA fragmentation in apoptosis driven by starvation of the survival factor interleukin-3 [7].

"Thymineless death" is observed in mammalian cells when severe deoxythymidine-triphosphate (dTTP) depletion takes place due to mutations or drug treatments. In this respect, dTTP-depleting drugs such as methotrexate or 5-fluorouracil (5-FU) are currently being used in chemotherapy [8], although the mechanism of dTTP depletion-induced apoptosis remains to be elucidated. These drugs cause a decrease in dTTP levels and a concomitant increase in dUTP, which can get incorporated into DNA. This would lead to extensive DNA damage as a result of the active process of excision repair at the many uracil-containing sites in DNA [9,10], and thus trigger a DNA-damage induced pathway of apoptosis. Alternatively, perturbation of dNTP pools might induce a mitochondrial pathway of apoptosis by a direct effect on mitochondrial nucleotide translocators such as ANT, or by perturbation of mitochondrial nucleotide metabolism. This hypothesis is supported by observations of a direct effect of nucleotide analogs on mitochondria [11]. Several groups, including ours [4,12,13], have demonstrated that over-expression of the anti-apoptotic protein Bcl-2 prevents the induction of apoptosis by inhibitors of thymidylate synthesis. Bcl-2 inhibits apoptosis without affecting the depletion of dTTP, thus indicating a role of mitochondria in the execution of apoptosis [12].

Recent studies show that apoptosis induced by chemotherapeutic drugs is blocked in ATP-depleted cells, both in vitro [14] and in vivo [15]. The ATP-dependent step remains to been determined. Although DNA damage-induced caspase activation does not occur under conditions of ATP depletion, the initial steps of sensing DNA damage are unaffected [16]. It has been suggested that the requirement of ATP may be due to its stimulatory effect of Apaf-1 in the apoptosome [14]. It is not known whether apoptosis triggered by inhibition of thymidylate-synthase requires ATP-generating systems.

In this report, we analyzed the mechanism of apoptosis induced by the thymidylate-synthase inhibitor 5′-fluoro-2′-deoxyuridine (FUdR) in human leukemic p53-negative U937 cells. Requirement of caspase-8 and effector caspases, ordering of caspase activation and mitochondrial events, production of reactive oxygen species (ROS), and requirement of exogenous glucose as supplier of ATP for the induction of apoptosis were examined.

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*Abbreviations:* ANT, adenine nucleotide translocator; dNTP, deoxyribonucleoside triphosphates; Z-VAD-fmk, benzyloxycarbonyl-Val–Ala–Asp-(OMe) fluoromethyl ketone; FUdR, 5′-fluoro-2′-deoxyuridine; PBS, phosphate-buffered saline; dTTP, deoxythymidine-triphosphate; $\Delta\Psi_m$, mitochondrial membrane potential; FBS, fetal bovine serum; $H_2$DCFDA, dichlorodihydrofluorescein diacetate; BSO, L-buthionine-(*S*,*R*)-sulfoximine; ROS, reactive oxygen species; PI, propidium iodide

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## 2. Materials and methods

### 2.1. Reagents

Caspase inhibitor benzyloxycarbonyl-Val–Ala–Asp-(OMe) fluoromethyl ketone (Z-VAD-FMK) was purchased from Bachem. IETD-FMK was from Enzyme System Inc. L-Buthionine-(*S*,*R*)-sulfoximine (BSO) was a generous gift of Dr. Isabel Fabregat, Universidad Complutense (Madrid). All other reagents were purchased from Sigma–Aldrich Quimica, Spain.

### 2.2. Cell cultures

Human myeloid leukemic U937, THP-1 and HL-60 cells were maintained in RPMI medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco), 1 mM glutamine and gentamycin, at 37 °C in a humidified 5% CO2/95% air incubator. Incubation under glucose-free conditions was performed by washing cells twice in glucose-free RPMI 1640 medium and incubating them in the same medium with 1 mM glutamine, 2 mM pyruvate and 1% dialyzed fetal bovine serum. Control cultures were incubated with 2 g/l glucose instead of pyruvate.

### 2.3. Analysis of viability and apoptosis

Hypodiploid apoptotic cells were detected by flow cytometry according to published procedures [17]. For analysis of membrane permeability in Table 1, cells were incubated with 5 μM propidium iodide (PI) in PBS for 15 min and fluorescence was detected by flow cytometry.

### 2.4. Measurements of mitochondrial depolarization and reactive oxygen species (ROS)

For mitochondrial depolarization determinations, cells were collected by centrifugation and resuspended in PBS with 40 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, Molecular Probes) and 5 μM PI, and incubated for 15 min at room temperature. Generation of ROS was quantified by adding 5 μm of $H_2$DCFDA (dichlorodihydrofluorescein diacetate, Molecular Probes) to the culture media for the last 30 min of treatment. Quantitative analysis of low $\Delta\Psi_m$ (mitochondrial membrane potential) population and ROS production was carried out in a FACScan cytometer using the Cell Quest software.

### 2.5. Immunoblot analysis of proteins

Preparation of cell lysates and analysis of proteins were performed as described [18]. Antibodies used were: Mouse anti-β-tubulin mAb (Sigma–Aldrich), mouse anti-human caspase-8 mAb (Cell Diagnostica, Münster, Germany); rabbit anti-cleaved caspase-3 polyclonal antibody (New England BioLabs Inc), goat polyclonal anti-caspase-3 p20 (Santa Cruz Biotech), mouse monoclonal antibodies to cytochrome *c* and Bax (clone 6A7) (Pharmingen). For measurement of Cytochrome *c* release and Bax translocation, cells were lysed and cytosolic fractions were separated from mitochondria and nuclei as previously described [17]. Equal protein loading in each lane was verified by incubating membranes with β-tubulin antibody.

### 2.6. Statistical analysis

Potential differences between treatments were evaluated using the two tailed unpaired Student's *t* test. Changes were considered significant when $P < 0.05$.

## 3. Results and discussion

### 3.1. 5′-Fluoro-2′-deoxyuridine induces caspase-dependent apoptosis in human leukemic p53-negative cells

Despite the fact that drugs that inhibit dTTP metabolism are currently used in chemotherapy, the mechanism involved in dTTP depletion-induced apoptosis (thymineless death) is rather unknown. Tumor suppressor p53 has been often associated with death induced by inhibitors of dTTP synthesis, both in vitro [19,20] and in vivo [21]. In contrast, p53-independent apoptosis has been reported in colon cancer cell lines [22], and we have previously shown that in a bone marrow-derived hematopoietic cell line, prevention of FUdR-induced p53 accumulation does not lead to inhibition of death [23]. We observed that several human myeloid leukemic cells (HL-60, THP-1 and U937), which do not express p53 [24], undergo apoptosis when treated with the thymidylate synthase inhibitor FUdR, as measured by subG1 peak generation (Fig. 1(a)). Differential requirement for p53 in response to dTTP-depleting drugs may be cell-type specific or reflect different mechanisms of action of each drug. In this respect, disruption of p53 in colon cancer cell lines has been shown to inhibit death induced by 5-FU but not by two other inhibitors of thymidylate synthase, methotrexate and Tomudex [25]. This indicates that some of these drugs such as 5-FU may have other cellular targets than thymidylate synthase, and that these other effects, such as perturbation of RNA metabolism, may be responsible for the p53-dependence. Indeed, death induced by 5-FU can be prevented by the addition of exogenous uridine but not by thymidine [21,25], while apoptosis induced by specific inhibition of thymidylate-synthase is prevented by exogenous thymidine [26]. As shown in Fig. 1(a), apoptosis induced by FUdR in leukemic cells was prevented by addition of thymidine (20–50 μM) to the culture medium. In contrast, addition of uridine did not protect U937 cells from apoptosis, indicating that FUdR acts directly by inhibiting thymidilate-synthase activity in p53-negative cells.

Several reports have described the pair CD95–CD95L as critical mediators of thymineless death. CD95L messenger RNA is upregulated in response to dTTP depletion in colon carcinoma cells and a CD95-blocking mAb completely prevents thymineless death in these cells [27]. However, we and others have reported that apoptosis induced by inhibition of thymidylate-synthase is not prevented by inhibiting ligation of CD95 receptors by CD95L [22,23,28,29]. In U937 cells, we could not find any induction of CD95-ligand after treatment with FUdR (not shown), suggesting that in this case, the pair

Table 1
Production of ROS and FUdR-induced death in cells depleted of glutathione

| | ROS (fold induction of mean fluorescence)[a] | PI incorporation (%) (average ± S.D.)[b] | Apoptosis (% subG1) (average ± S.D.)[b] |
|---|---|---|---|
| Untreated | 1 | 1.2 ± 0.2 | 4.5 ± 0.7 |
| FUdR | 1.8 ± 0.7 | 23.0 ± 4.2 | 46.5 ± 4.9 |
| BSO | 2.3 ± 0.8 | 1.4 ± 0.3 | 5.8 ± 3.2 |
| BSO + FUdR | 4.1 ± 0.5 | 22.3 ± 1.1 | 47.5 ± 7 |

Cells were treated as described in legends for Fig. 3(b).
[a] ROS production is expressed as the fold increase in the fluorescence of cells incubated with $H_2$DCFDA, compared to the mean fluorescence of the untreated cells incubated with $H_2$DCFDA in each experiment. Data show average and range of two experiments.
[b] Cells were collected and subjected to analysis of PI incorporation and subG1 peak as described under Section 2. Averages and standard deviation of three experiments are shown.

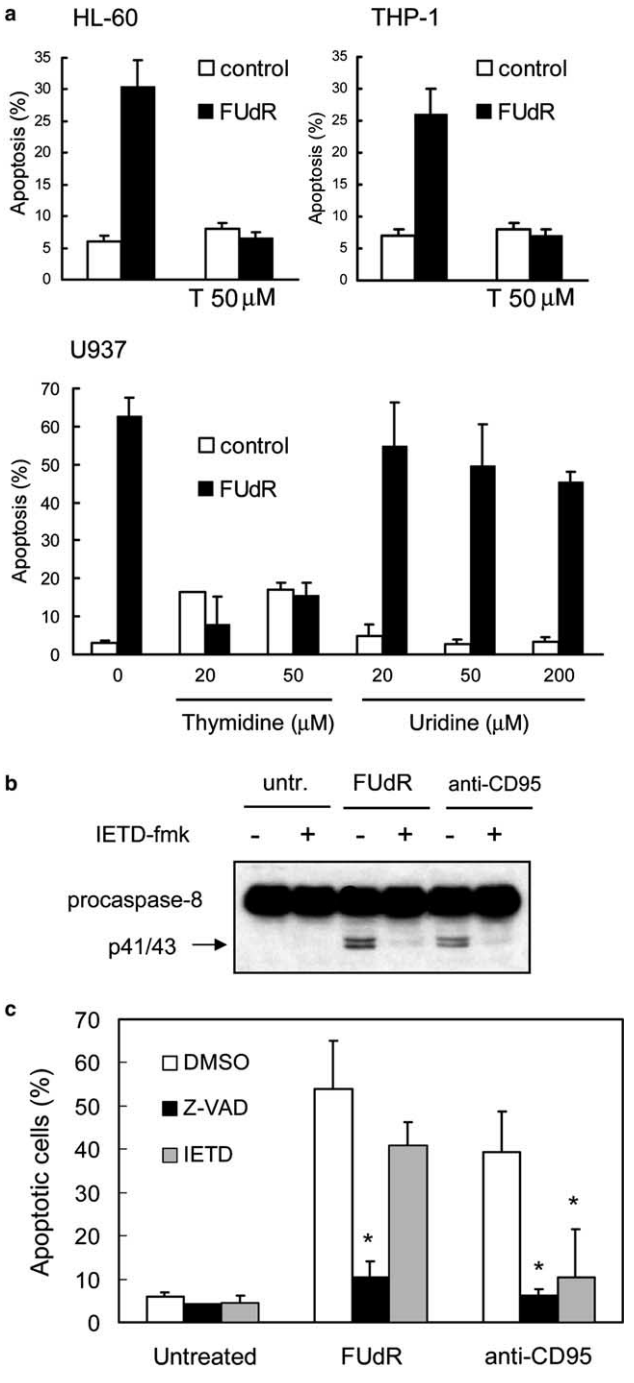


Fig. 1. FUdR induces apoptosis in p53-negative cells. (a) HL-60, THP-1 and U937 cells were incubated at a density of $5 \times 10^5$/ml and treated with FUdR (0.4 μM) for 15h in the presence of thymidine (T) or uridine at the concentrations shown. SubG1 population was measured. The average and standard deviation of at least three independent experiments are shown. In (b), processing of procaspase-8 is shown. Intact procaspase-8 and the intermediate cleavage products of 41/43 kDa were detected. Cells were pre-incubated for 1 h in the presence of IETD-fmk (100 μM) and subsequently treated with FUdR or 50 ng/ml anti-CD95 CH-11 mAb (Upstate Biotechnology) for 15 h before determining the processing of procaspase-8. Unt, untreated. Result shown is representative of three independent experiments. (c) Cells were pre-incubated for 1 h in the presence of either DMSO (0.2%), Z-VAD-fmk (100 μM) or IETD-fmk (100 μM) and further treated for 15 h with FUdR (0.4 μM) or anti-CD95 (50 ng/ml). Apoptosis was measured as percent of subG1 population. Results show average and S.E.M. of three independent experiments. Asterisk indicates significant inhibition.

CD95–CD95L is not a critical mediator of apoptosis induced by thymidylate-synthase inhibition. It has been reported, however, that certain genotoxic treatments may cause ligand-independent CD95 receptor activation and apoptosis [30]. Also, several groups have reported the activation of caspase-8 in drug-induced apoptosis [31–34]. In some of these cases, caspase-8 was cleaved through a re-amplification loop involving cytochrome *c* release from mitochondria and caspase-3 activation. To examine this issue, we first studied the requirements for caspase activation upon treatment of U937 cells either with FUdR or with CD95 agonistic antibody. Time course experiments of procaspase-8 processing demonstrated that this procaspase was cleaved in response to FUdR with similar kinetics to induction of apoptosis (not shown). We then used cell-permeable caspase inhibitors in order to test whether this caspase was required for FUdR-triggered death. As shown in Fig. 1(b), pre-incubation of U937 cells with the caspase-8 inhibitor IETD-fmk completely blocked the processing of caspase-8 by a subsequent treatment with FUdR or CD95 mAb. However, whereas induction of apoptosis by CD95 ligation was significantly inhibited by both IETD-fmk and the broad range caspase inhibitor Z-VAD-fmk, apoptosis triggered by dTTP depletion was markedly inhibited by Z-VAD-fmk but not by IETD-fmk (Fig. 1(c)). These results indicate that the processing of procaspase-8 induced by treatment with FUdR does not play an essential role in death of the cells.

### 3.2. *Caspase inhibitors prevent mitochondrial depolarization, but not cytochrome c release upon dTTP depletion in U937 cells*

Mitochondria play an important role in many models of apoptosis [35,36]. In anti-CD95 induced apoptosis of type II cells, activated caspase-8 cleaves Bid which translocates to mitochondria and induces release of cytochrome *c* and mitochondrial depolarization [37,38]. However, in genotoxic drug-induced apoptosis, the pathway to cytochrome *c* release has not been determined. Mitochondria are involved in apoptosis induced by thymidylate-synthase inhibition in different cell types, as Bcl-2 protects hematopoietic cells from FUdR-induced apoptosis [12] and FUdR triggers cytochrome *c* release in breast cancer cells [29]. We observed that overexpression of Bcl-2 protected U937 cells from FUdR-triggered apoptosis (not shown). We next determined whether FUdR triggered cytochrome *c* release and mitochondrial depolarization in U937 cells. As shown in Fig. 2(a), cytochrome *c* was released from mitochondria in cells treated with FUdR or anti-CD95. Both Z-VAD-fmk and IETD-fmk blocked cytochrome *c* release in anti-CD95 treated cells but not in FUdR-treated cells (Fig. 2(a)). These results suggest that while caspase activation is required for cytochrome *c* release in death receptor-induced death, this is not the case in cells treated with FUdR, in agreement with previous results obtained with other DNA-damaging drugs [34].

Cytochrome *c* may be released in some cases as a consequence of mitochondrial permeability transition [39]. In these cases, mitochondrial depolarization occurs before cytochrome *c* release and is not prevented by caspase inhibition. However, in other instances cytochrome *c* release appears to trigger caspase activation and mitochondrial depolarization [40]. In Fig. 2(b), we show that U937 cells treated either with FUdR or anti-CD95 underwent mitochondrial depolarization.

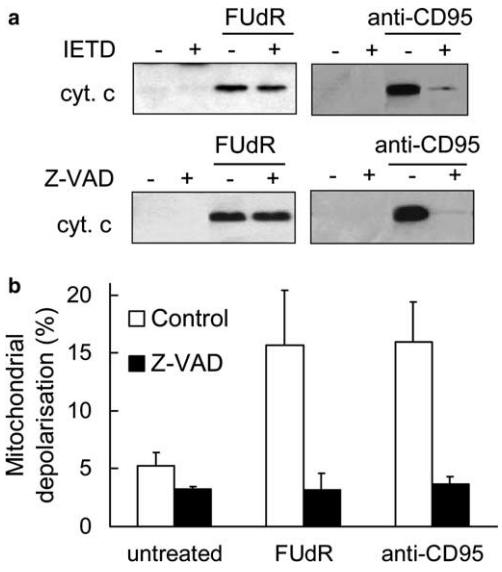


Fig. 2. Caspase inhibitors prevent mitochondrial depolarization but not cytochrome *c* release in FUdR-induced apoptosis. U937 cells were pre-incubated for 1 h with caspase inhibitors (100 μM) and treated subsequently for 15 h with FUdR (0.4 μM) or anti-CD95 (50 ng/ml). In (a), cytosolic fractions of treated cells were blotted for cytochrome *c*. Equal loading of protein was verified by incubation of the membrane with anti-tubulin (not shown). A representative experiment from at least three independent determinations is shown. In (b), cells were pre-incubated with Z-VAD-fmk and collected after treatments for measurement of mitochondrial depolarization. Depolarization was analyzed as described under Section 2. Percent of PI-negative cells with depolarized mitochondria is shown. Results show average and range of two independent experiments.

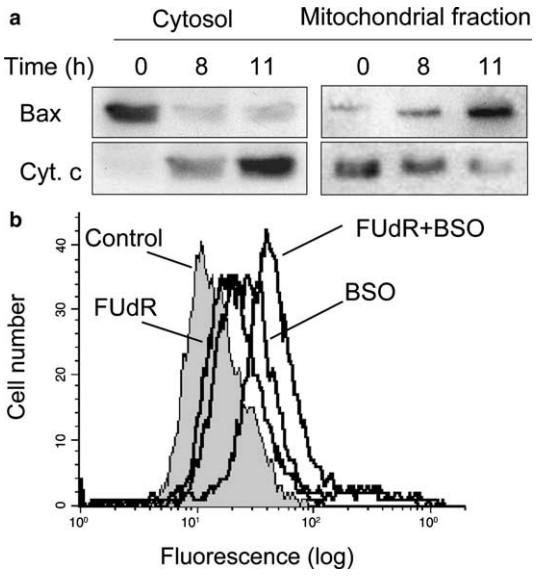


Fig. 3. Generation of ROS and translocation of Bax to the mitochondrial fraction after treatment with FUdR. In (a), cells were incubated with FUdR 0.4 μM for the indicated times, and collected and subjected to fractionation as described under Section 2. Immunodetection of cytochrome *c* and Bax in cytosolic and heavy-membrane fractions is shown. In (b), cells were treated with 0.4 μM of FUdR for 15 h, in the presence or absence of BSO 2 mM, and incubated for 30 min with $H_2$DCFDA as described under Section 2.

However, pre-treatment with Z-VAD-fmk completely abolished mitochondrial depolarization caused by a subsequent treatment with FUdR or anti-CD95. This indicates that in both apoptotic situations, mitochondrial depolarization occurs as a consequence of caspase activation, in agreement with other results [40,41]. Altogether, these results also demonstrate that unlike what is observed in CD95-activated apoptosis, release of cytochrome *c* from mitochondria upon FUdR treatment can be dissociated from the induction of mitochondrial depolarization. It has been reported that drug-induced, p53-mediated apoptosis proceeds through Bax translocation to mitochondria [42]. However, little is known about the mechanism of genotoxic drug-induced apoptosis in p53-negative cells. We show in Fig. 3(a) that treatment with FUdR caused Bax disappearance from cytosolic fractions and translocation to the heavy membrane fraction, concomitantly with cytochrome *c* release from mitochondria. Although we were not able to demonstrate a cause-effect relationship between these two events, both events occurred within the same time range, suggesting a possible role of Bax translocation to mitochondria in the release of cytochrome *c* from this organelle in a p53-independent manner.

### 3.3. Reactive oxygen species do not mediate FUdR-induced apoptosis

It has been proposed that drug-induced, p53-mediated apoptosis proceeds through alterations in redox metabolism [43]. Furthermore, it was recently described that in the cell line used in our studies, death induced by alkylating agents required production of ROS [44]. To our knowledge, there are no studies on the relationship of thymineless death and generation of ROS. We wished to test whether inhibition of thymidylate-synthase caused induction of ROS, and whether this production was involved in cell death. For this purpose, we analyzed the production of peroxides by measuring $H_2$DCFDA oxidation and we observed that FUdR caused the production of ROS (Fig. 3(b) and Table 1). To analyze whether production of ROS was involved in FUdR-induced death, we performed two types of experiments. First, we treated cells with FUdR in the presence of the antioxidants *N*-acetyl-L-cysteine (up to 10 mM) and pyrrolidine dithiocarbamate (up to 50 μM), and we observed no differences in apoptosis (not shown). Second, we depleted cells of reduced glutathione by adding L-buthionine-(*S*,*R*)-sulfoximine (BSO) to culture medium and we subsequently treated them with FUdR. BSO has been shown to enhance death of U937 cells treated with alkylating agents [44]. As shown in Table 1, although pretreatment with BSO enhanced the production of ROS by FUdR, the number of dead cells did not change, as measured by PI incorporation and subG1 analysis. These results indicate that apoptosis induced by inhibition of thymidylate-synthase does not require production of Reactive Oxygen Species.

### 3.4. Glucose deprivation reduces FUdR-induced but not CD95-triggered apoptosis

Apoptosis is an active process that may require ATP for its correct development. ATP seems to be necessary for drug-induced death in vivo, as an inhibitor of glucose uptake inhibits doxorubicin-triggered death [15], and in vitro, incubation of Jurkat cells in glucose-free medium in the presence of oligomycin inhibits apoptosis triggered by several chemotherapeutic drugs [14]. In order to test whether ATP was required for

FUdR-triggered apoptosis, we achieved ATP depletion by incubating cells in glucose-free medium, as we had previously observed that U937 cells depend mainly on glucose supply in order to maintain ATP levels [45]. By using this approach, we avoided the use of oligomycin, that may interfere with the mechanism of apoptosis by preventing cytosol acidification, cytochrome *c* release and Permeability Transition related events [46,47]. When cells were pre-incubated in the absence of glucose, apoptosis induced by FUdR was reduced (Fig. 4(a)). In contrast, anti-CD95-triggered apoptosis was enhanced by glucose deprivation, as we have previously shown [45]. It has been suggested that ATP depletion may affect drug-induced apoptosis by inhibiting the activation of Apaf-1 at the apoptosome, that requires ATP or dATP as well as cytochrome *c* for the activation of caspase-9 [14]. To determine the step in the apoptotic pathway that requires the presence of glucose in the medium, we analyzed several apoptotic parameters in cells treated either with anti-CD95 or FUdR under conditions of glucose starvation. Cleavage of caspase-3 and -8 correlated with the extent of apoptosis induced by these stimuli under conditions of glucose starvation (Fig. 4(b)), activation of these caspases was clearly reduced upon FUdR treatment in glucose-free medium; however, caspase activation in CD95 Ab-treated cells was not diminished by glucose depletion. Consistent with the results shown in Figs. 2(b) and 4(b), the extent of depolarization was reduced when cells were treated with FUdR under conditions of glucose starvation (Fig. 4(c)). Finally, we analyzed the release of cytochrome *c* induced either by FUdR or anti-CD95 Ab in glucose-free medium. As shown in Fig. 4(d), cytochrome *c* release was clearly inhibited in glucose-free medium when cells were treated with FUdR but not when treated with anti-CD95. These results indicate that glucose is required for FUdR-induced apoptosis at least at a mitochondrial or pre-mitochondrial step. Why ATP or glucose catabolism is required for drug-induced apoptosis but not for death caused by anti-CD95 is an issue that requires further attention, especially if we want to speculate with the therapeutical possibilities of a selective modulation of the different apoptotic signaling pathways. Some of these genotoxic drugs require progression through the cell cycle in order to cause DNA-damage and apoptosis. A possibility that has not been tested, to our knowledge, is that glucose removal may delay or inhibit cycling of cells, and this could affect drug- but not anti-CD95-induced apoptosis. As we have shown, one of the upstream common steps in apoptosis induced by most genotoxic drugs, cytochrome *c* release, is prevented in glucose-free medium. Then, the point of inhibition must affect the largely uncharacterized signals that link damage-sensing to mitochondrial events.

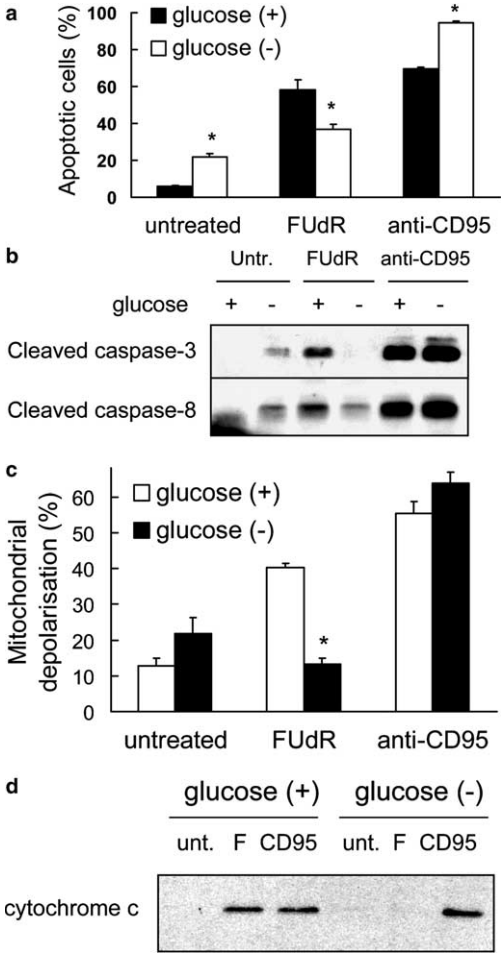


Fig. 4. Glucose withdrawal reduces FUdR-induced but not anti-CD95-triggered apoptosis. Cells were pre-incubated for 3 h in the absence of glucose with 1% FBS, as described in Section 2, and further treated for 13 h with FUdR (0.4 μM) or anti-CD95 (50 ng/ml). In (a), apoptosis was measured as percent of subG1 population. Results show average and S.E.M. of five independent experiments. (b) Analysis of caspase activation by western blotting. Active p17 subunit of caspase-3 and p18 fragment of caspase-8 are shown. Unt, untreated. Results shown are representatives of three independent experiments. (c) Percent of PI-negative cells with depolarized mitochondria is shown. Results show average and S.E.M. of three independent experiments. In (d), cytosolic fractions of treated cells were analyzed for the presence of cytochrome *c*. Equal loading of protein was verified by incubation with anti-tubulin (not shown). Unt, untreated cells; F, treated with FUdR; CD95, treated with anti-CD95. Results shown are representatives of three independent experiments. Asterisks indicate significant differences with respect to the glucose-replenished conditions.

In summary, our data may contribute to an understanding of the mechanism of "thymineless death", particularly in those aspects concerning the relationship between caspase activation, mitochondrial events and requirement of glucose/ATP. They may also help in the design of combination treatments to improve present therapeutic approaches to human neoplasias based on the use of drugs inhibiting dNTP metabolism.

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## References

[1] Bradley, M.O. and Sharkey, N.A. (1978) Nature 274, 607–608.
[2] Yoshioka, A., Tanaka, S., Hiraoka, O., Koyama, Y., Hirota, Y., Ayusawa, D., Seno, T., Garrett, C. and Wataya, Y. (1987) J. Biol. Chem. 262, 8235–8241.
[3] Reichard, P. (1988) Annu. Rev. Biochem. 57, 349–374.
[4] Miyashita, T. and Reed, J.C. (1992) Cancer Res. 52, 5407–5411.
[5] Zou, H., Li, Y., Liu, X. and Wang, X. (1999) J. Biol. Chem. 274, 11549–11556.
[6] Marzo, I., Brenner, C., Zamzami, N., Susin, S.A., Beutner, G., Brdiczka, D., Remy, R., Xie, Z.H., Reed, J.C. and Kroemer, G. (1998) J. Exp. Med. 187, 1261–1271.

[7] Oliver, F.J., Collins, M.K. and Lopez-Rivas, A. (1996) Biochem. J. 316, 421–425.
[8] Peters, G.J., van der Wilt, C.L., van Moorsel, C.J., Kroep, J.R., Bergman, A.M. and Ackland, S.P. (2000) Pharmacol. Ther. 87, 227–253.
[9] Ingraham, H.A., Dickey, L. and Goulian, M. (1986) Biochemistry 25, 3225–3230.
[10] Canman, C.E., Radany, E.H., Parsels, L.A., Davis, M.A., Lawrence, T.S. and Maybaum, J. (1994) Cancer Res. 54, 2296–2298.
[11] Genini, D., Adachi, S., Chao, Q., Rose, D.W., Carrera, C.J., Cottam, H.B., Carson, D.A. and Leoni, L.M. (2000) Blood 96, 3537–3543.
[12] Oliver, F.J., Marvel, J., Collins, M.K. and Lopez-Rivas, A. (1993) Biochem. Biophys. Res. Commun. 194, 126–132.
[13] Lotem, J. and Sachs, L. (1993) Cell Growth Differ. 4, 41–47.
[14] Ferrari, D., Stepczynska, A., Los, M., Wesselborg, S. and Schulze-Osthoff, K. (1998) J. Exp. Med. 188, 979–984.
[15] Thakkar, N.S. and Potten, C.S. (1993) Cancer Res. 53, 2057–2060.
[16] Kupfer, G., Bodley, A.L. and Liu, L.F. (1987) NCI Monogr. 4, 37–40.
[17] Varela, N., Munoz-Pinedo, C., Ruiz-Ruiz, C., Robledo, G., Pedroso, M. and Lopez-Rivas, A. (2001) J. Biol. Chem. 276, 17779–17787.
[18] Ruiz-Ruiz, C., Munoz-Pinedo, C. and Lopez-Rivas, A. (2000) Cancer Res. 60, 5673–5680.
[19] Palacios, C., Gutierrez del Arroyo, A., Silva, A. and Collins, M.K. (2000) Oncogene 19, 3556–3559.
[20] Elledge, R.M., Gray, R., Mansour, E., Yu, Y., Clark, G.M., Ravdin, P., Osborne, C.K., Gilchrist, K., Davidson, N.E. and Robert, N., et al. (1995) J. Natl. Cancer Inst. 87, 1254–1256.
[21] Pritchard, D.M., Watson, A.J., Potten, C.S., Jackman, A.L. and Hickman, J.A. (1997) Proc. Natl. Acad. Sci. USA 94, 1795–1799.
[22] Backus, H.H., Wouters, D., Ferreira, C.G., vanHouten, V.M., Brakenhoff, R.H., Pinedo, H.M. and Peters, G.J. (2003) Eur. J. Cancer 39, 1310–1317.
[23] Munoz-Pinedo, C., Oliver, F.J. and Lopez-Rivas, A. (2001) Biochem. J. 353, 101–108.
[24] Dou, Q.P. and Lui, V.W. (1995) Cancer Res. 55, 5222–5225.
[25] Bunz, F., Hwang, P.M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K.W. and Vogelstein, B. (1999) J. Clin. Invest. 104, 263–269.
[26] Oliver, F.J., Collins, M.K.L. and Lopez-Rivas, A. (1997) J. Biol. Chem. 272, 10624–10630.
[27] Houghton, J.A., Harwood, F.G. and Tillman, D.M. (1997) Proc. Natl. Acad. Sci. USA 94, 8144–8149.
[28] Ruiz-Ruiz, M.C. and Lopez-Rivas, A. (1999) Cell Death Differ. 6, 271–280.
[29] Kottke, T.J., Blajeski, A.L., Martins, L.M., Mesner Jr., P.W., Davidson, N.E., Earnshaw, W.C., Armstrong, D.K. and Kaufmann, S.H. (1999) J. Biol. Chem. 274, 15927–15936.
[30] Micheau, O., Solary, E., Hammann, A. and Dimanche-Boitrel, M.T. (1999) J. Biol. Chem. 274, 7987–7992.
[31] Engels, I.H., Stepczynska, A., Stroh, C., Lauber, K., Berg, C., Schwenzer, R., Wajant, H., Janicke, R.U., Porter, A.G., Belka, C., Gregor, M., Schulze-Osthoff, K. and Wesselborg, S. (2000) Oncogene 19, 4563–4573.
[32] Wesselborg, S., Engels, I.H., Rossmann, E., Los, M. and Schulze-Osthoff, K. (1999) Blood 93, 3053–3063.
[33] Wieder, T., Essmann, F., Prokop, A., Schmelz, K., Schulze-Osthoff, K., Beyaert, R., Dorken, B. and Daniel, P.T. (2001) Blood 97, 1378–1387.
[34] Sun, X.M., MacFarlane, M., Zhuang, J., Wolf, B.B., Green, D.R. and Cohen, G.M. (1999) J. Biol. Chem. 274, 5053–5060.
[35] Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P. and Wang, X. (1997) Science 275, 1129–1132.
[36] Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H. and Peter, M.E. (1998) EMBO J. 17, 1675–1687.
[37] Luo, X., Budihardjo, I., Zou, H., Slaughter, C. and Wang, X. (1998) Cell 94, 481–490.
[38] Li, H., Zhu, H., Xu, C.J. and Yuan, J. (1998) Cell 94, 491–501.
[39] Bradham, C.A., Qian, T., Streetz, K., Trautwein, C., Brenner, D.A. and Lemasters, J.J. (1998) Mol. Cell. Biol. 18, 6353–6364.
[40] Bossy-Wetzel, E., Newmeyer, D.D. and Green, D.R. (1998) EMBO J. 17, 37–49.
[41] Finucane, D.M., Waterhouse, N.J., Amarante-Mendes, G.P., Cotter, T.G. and Green, D.R. (1999) Exp. Cell Res. 251, 166–174.
[42] Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. and Korsmeyer, S.J. (2001) Science 292, 727–730.
[43] Polyak, K., Xia, Y., Zweier, J.L., Kinzler, K.W. and Vogelstein, B. (1997) Nature 389, 300–305.
[44] Troyano, A., Fernandez, C., Sancho, P., de Blas, E. and Aller, P. (2001) J. Biol. Chem. 276, 47107–47115.
[45] Munoz-Pinedo, C., Ruiz-Ruiz, C., Ruiz de Almodovar, C., Palacios, C. and Lopez-Rivas, A. (2003) J. Biol. Chem. 278, 12759–12768.
[46] Matsuyama, S., Llopis, J., Deveraux, Q.L., Tsien, R.Y. and Reed, J.C. (2000) Nat. Cell Biol. 2, 318–325.
[47] Shchepina, L.A., Pletjushkina, O.Y., Avetisyan, A.V., Bakeeva, L.E., Fetisova, E.K., Izyumov, D.S., Saprunova, V.B., Vyssokikh, M.Y., Chernyak, B.V. and Skulachev, V.P. (2002) Oncogene 21, 8149–8157.