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**Specific disintegration of complex II
succinate:ubiquinone oxidoreductase links pH changes to
oxidative stress for apoptosis induction**

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Running title:

Specific complex II inhibition signals apoptosis

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Abstract

The formation of reactive oxygen species (ROS) and the change of the intracellular pH are common phenomena during apoptosis. How they are interconnected, however, is poorly understood. Here we show that numerous anticancer drugs as well as cytokines such as FasL and TNF α provoke intracellular acidification and cause the formation of mitochondrial ROS. In parallel we found that the succinate:ubiquinone oxidoreductase (SQR) activity of the mitochondrial respiratory complex II is specifically impaired without affecting the second enzymatic activity of this complex as a succinate dehydrogenase (SDH). Only in this configuration is complex II an apoptosis mediator and generates superoxides for cell death. This is achieved by the pH_i decline that leads to the specific dissociation of the SDHA/SDHB subunits, which encompass the SDH activity, from the membrane-bound components of complex II that are required for the SQR activity.

Key words: apoptosis, mitochondria, respiratory chain, complex II, reactive oxygen species

Abbreviations: ROS, reactive oxygen species; SQR, succinate:ubiquinone oxidoreductase; SDH, succinate dehydrogenase; NHE1, Na⁺/H⁺ Exchanger 1; FasL, Fas ligand; TNF α , tumour necrosis factor; CoQ, coenzyme Q; TTFA, thenoyltrifluoroacetate; 3NP, 3-nitropropionic acid; SOD, superoxide dismutase; MnSOD, manganese SOD

Introduction

Intracellular acidification is, besides cell shrinkage, membrane blebbing, DNA fragmentation, and exposure of phosphatidylserine, a commonly described phenomenon during the apoptotic process. It constitutes an early event during apoptosis and precedes mitochondrial dysfunction.¹ A wide variety of apoptosis signals can cause this pH drop, typically in the range of 0.3-0.4 pH units, such as UV-irradiation, death receptor triggering, growth factor deprivation, and anticancer compounds but also genes such as p53 and Bax activation.²⁻¹² This can be accomplished by modulation of the activity of plasma membrane transporters such as the alkalinizing transporter Na⁺/H⁺ Exchanger 1 (NHE1), which seems to be inhibited during apoptosis.¹ While it has been suggested that the pH change can contribute to caspase activation,¹³ exactly how pH changes are linked to more downstream signals during apoptosis induction is only poorly understood.

Reactive oxygen species (ROS) are long known as general mediators of apoptosis. Mitochondria are regarded as the main, but not the sole source for ROS.¹⁴ While complex I and III have long been recognised as supplying ROS for apoptosis,¹⁵ in recent years complex II has also been established as an efficient producer of ROS, particularly superoxides, for apoptosis induction.^{16,17} Sensitive detection methods indicated that it can surpass even complex III for ROS formation.¹⁸ Structural studies likewise indicate that complex II can produce ROS.¹⁹ However, the exact mechanism by which complex II generates ROS in response to apoptosis signals remains unknown.

With only four subunits, complex II is the smallest protein complex of the mitochondrial respiratory chain and the only complex to be fully encoded by nuclear DNA.²⁰ The SDHC and SDHD proteins form the membrane anchoring components, which associate with two subunits facing the mitochondrial matrix: SDHB, an Fe-S protein, and SDHA, a FAD-containing protein²¹ (Supplementary Figure 1a). Complex II is also a component of the

Krebs cycle with its succinate dehydrogenase (SDH) activity oxidizing the metabolite succinate to fumarate. The electrons generated in this reaction are then channelled in a not completely understood pathway within complex II to ubiquinone, which is reduced to ubiquinol by the succinate:ubiquinone oxidoreductase (SQR) activity of complex II. Finally, the reduction equivalents are shuttled to complex III in the respiratory chain.²²

Here we demonstrate that various compounds and cytokines cause an intracellular pH acidification and ROS formation during apoptosis. We found that these prominent features of apoptosis are linked at complex II, which is specifically inhibited through the pH_i decrease that causes the dissociation of two of the complex II subunits. This, in turn, leads to ROS formation at the complex II level and subsequent apoptosis induction.

Results

Early intracellular acidification and ROS formation during apoptosis. We set up a suitable cellular system to study the early events of apoptosis signalling. Seven hours after addition of various anticancer agents HeLa cells showed no signs of apoptosis induction such as nuclear DNA degradation (Figure 1a) or phenotypic changes (not shown). However, after 24h, these drugs at the concentrations used caused apoptosis as evidenced by an increase of the sub-G1 cell population. The FasL and TNF α cytokines exerted a delayed effect on apoptosis with the 16h measurement showing only a marginal sub-G1 cell population but substantial apoptosis was recorded after 36h (Figure 1a). An additional assay for caspase-3 activity and poly (ADP-ribose) polymerase (PARP) cleavage confirmed apoptosis induction (Supplementary Figure 2). When we measured the intracellular pH (pH_i) 8h after treatment and prior to apoptosis manifestation, we observed a decrease in pH_i with all proapoptotic signals used (Figure 1b). EIPA, an inhibitor of the alkalinizing transporter Na⁺/H⁺ Exchanger 1 (NHE1) used as an acidification control, markedly potentiated apoptosis in co-treatments with all agents (Figure 1c). Conversely, when we transiently over-expressed NHE1, we prevented acidification following drug and cytokine treatment and strongly reduced apoptosis (Figure 1d). We were also able to show that the mitochondrial matrix acidified in parallel with the cytosolic pH_i drop (Figure 1e).

We then studied whether ROS could be causative for apoptosis triggered by the drugs and cytokines shown in Figure 1. Using the dihydroethidium (DHE) probe (Supplementary Figure 3a) and the mitochondria-specific probe MitoSOX (Figure 2a) we confirmed that all these agents could elicit the formation of ROS 8 or 16 hours after their application. Different efficiencies in ROS generation were noticed, probably because some compounds induce ROS formation through additional pathways, as is the case for

doxorubicin,²³ menadione²⁴ and etoposide.²⁵ Moreover, we showed that pre-treatment with PEG-catalase, a cell-permeable form of catalase,²⁶ could reduce their potential to induce apoptosis, underlining the fundamental role of ROS in the apoptotic cascade (Figure 2b). We also demonstrated that acidification impairment through NHE1 overexpression markedly reduced drug-induced mitochondrial ROS production (Supplementary Figure 3b). Finally, as our previous work showed a role of complex II in ROS production and apoptosis by various signals,²⁷ we used the SDH inhibitor malonate and observed a reduction of apoptosis induced by all compounds, suggesting that complex II-dependent ROS accumulation is at least partly responsible for apoptosis induction (Figure 2c).

Specific inhibition of complex II for apoptosis induction. Based on our previous work²⁷ and the results with the complex II inhibitor malonate (Figure 2c), we investigated the effects of the apoptosis inducers on complex II in more detail with two assays classically used for the enzymatic activities of complex II. The SDH assay traces the flow of electrons from the substrate succinate to its SDHA/SDHB subunits. The SQR assay covers the electron pathway from succinate through the whole complex II to the electron acceptor coenzyme Q (CoQ) (Supplementary Figure 1b,c). We found that 7h (anticancer drugs) and 16h (cytokines) after treatment, the SDH activity of complex II was not compromised, whereas its SQR activity was inhibited by approximately 20% (Figure 3a). Thus, we assumed that this early and selective inhibition of complex II SQR activity, and the associated SDH/SQR uncoupling, could be related to cell death induction. We hypothesised that complex II contributes to apoptosis induction only when the SQR activity is inhibited while the SDH activity is still fully functioning, creating an uncoupling phenomenon at the complex II level. This model, however, seemed to be in conflict with

the discovery in cancer cells with inactivating mutations in the complex II membrane anchoring subunits SDHC and SDHD²⁸ that appear to lead to apoptosis resistance and tumour formation rather than cell death induction. Consequently, we set up experiments to test the consequences on the SQR and SDH activity and on apoptosis induction upon specific disruption of the various complex II subunits by shRNA constructs. Figure 3b shows that the mRNAs of all targeted complex II components were reduced after 72h while the mRNA expression levels of their associated subunits remained unaffected. We also confirmed the downregulation of the respective proteins by Western-Blot (Figure 3c). Our expectation was that we detect apoptosis when the membrane-bound subunits are downregulated but not then the peripheral components of complex II were targeted. However, we did not observe any apoptosis induction following a 72h-transfection with these shRNA (Figure 3d). When we measured the enzymatic activities of complex II, we found that both, SDH and SQR activity were markedly and equally downregulated when SDHA and SDHB were targeted but also when SDHC and SDHD were inhibited (Figure 3e). This seems to be due to a major impairment of the whole complex assembly in mitochondria as revealed by Supplementary Figure 4. When we targeted SDHC, SDHD or SDHB, all the other subunits were likewise reduced in mitochondria, with the exception of SDHA. Hence, inactivation of complex II subunits as observed in cancer cells could impair the whole complex II and, because of this, the association of an active SDH activity with an inhibited SQR function is not possible, rendering complex II incapable of apoptosis induction and promoting tumorigenesis. To further prove that the observed effects depend on complex II, we made use of the SDHC *-/-* CCL16 B9 cells²⁹ and reconstituted wild type (*wt*) SDHC expression.³⁰ Five clones were picked and the expression of SDHC assessed. Clone 4 expressed this transgene at a level comparable to the parental *wt* B1 cells (Supplementary Figure 5a). We found that by expressing *wt*

SDHC (clone 4) we could restore both SDH and SQR activity to a similar level than in parental B1 cells (Supplementary Figure 5b). Importantly, complex II-reconstituted cells were also re-sensitized to apoptosis induction by anticancer drugs (Figure 3f). Thus, based on all these findings, we hypothesised that complex II contributes to apoptosis induction when functional complex II aggregates become inactive for the SQR activity while the SDH function is still fully effective. While this cannot happen in tumour cells with complex II inactivating mutations, in *wt* cells it creates uncoupling at the complex II level leading to ROS production and subsequent apoptosis.

In order to assess the cellular consequences of a selective and partial SQR inhibition as observed in Figure 3a, we turned to inhibitors of complex II, which facilitate a specific, concentration-dependent attenuation of complex II activities: 3-nitropropionic acid (3NP), a succinate analogue, binds to the active site of SDHA and inhibits complex II irreversibly.²¹ Thenoyltrifluoroacetate (TTFA) inhibits a later step of the electron flow as it binds to the ubiquinone docking sites and abrogates the transfer of electrons to this molecule.²¹ Apoptosis assays revealed that 3NP did not elicit an apoptotic effect at the concentrations tested, while TTFA caused a time- and dose-dependent increase of apoptosis (Figure 4a). In line with the known specificity of these compounds, we have observed an inhibition of both, SQR and SDH activity, with 3NP, while TTFA reduced only the SQR activity, prior to apoptosis (Figure 4b, upper panel). Of note, TTFA at a concentration of 0.5 mM, which caused apoptosis (Figure 4a), diminished the SQR activity by 20%, a reduction also observed with the various pro-apoptotic compounds (Figure 3a). An *in vitro* dose-escalation experiment on isolated mitochondria confirmed the concentration-dependent SQR inhibition by TTFA (Figure 4b, lower panel).

Apoptosis signals are mediated through complex II-dependent ROS production.

Based on the above experiments, which highlight the importance of an inhibited SQR activity without SDH impairment for apoptosis induction, we speculated that if electrons provided by succinate oxidation could not efficiently be transferred to ubiquinone, they would leak out from complex II, generate ROS and eventually trigger apoptosis. We asked whether this hypothesis was supported by the effects of the specific complex II inhibitors. Using DHE we found that after an 8h-treatment and prior to apoptosis, ROS were rapidly generated by TTFA in a dose-dependent manner. 3NP, on the other hand, did not show ROS production at all doses used at this early time point (Figure 5a). With MitoSOX, we also demonstrated that TTFA-induced ROS had a mitochondrial origin (Supplementary Figure 3c). Antioxidant enzymes such as PEG-catalase and superoxide dismutase (SOD) and ROS quenchers such as Trolox could, when added to the medium of TTFA-treated cells, reduced ROS and likewise apoptosis (Figure 5b,c). In addition, the transfection of plasmids coding for catalase and for mitochondrial manganese SOD (MnSOD) also potently diminished TTFA-induced apoptosis (Figure 5d). These results suggested that complex II, if specifically inhibited, can function as a source for ROS and apoptosis induction. ROS are known to also cause the passive cell death of necrosis.³¹ Hence, to examine whether the observed cell death was apoptosis, we analysed specific features of apoptosis, such as caspase-3 activity and PARP cleavage, and found that both of these apoptotic features increased upon TTFA treatment (Supplementary Figure 2).

We also performed PI and annexinV (AV) double-staining and found that necrotic PI+/AV- cells only marginally accumulated upon TTFA treatment (Supplementary Figure 6a) in contrast to PI-/AV+ apoptotic cells. PI+/AV+ double-stained necrotic or late-apoptotic cells significantly increased in response to TTFA. Nevertheless, the cell

populations positive in SubG1 (apoptotic) or 7-AAD (necrotic and late-apoptotic) assays could potentially be reduced by zVAD-fmk, a pan-caspase inhibitor (Supplementary Figure 6b,c). We hypothesised that, if the TTFA proapoptotic effects are due to specific inhibition of complex II at the SQR level, upstream inhibitors of complex II such as those of the Krebs cycle (fluoroacetate, fluorocitrate) or of the SDH activity (3NP, malonate), would also reduce ROS formation and apoptosis induction by TTFA. Figure 5e reveals that all the upstream inhibitors of complex II reduced both ROS and apoptosis in TTFA-treated cells. Hence, these results, summarized in Supplementary Figure 7, suggested that specific inhibition of complex II contributed to pro-apoptotic ROS formation through electron leakage between the SDH activity, which is linked to the Krebs cycle, and the SQR activity, which is directly connected with the respiratory chain. Also, in contrast to pcDNA3-transfected cells, SDHC-reconstituted B9 cells were again able to produce ROS and undergo apoptosis when treated with TTFA (Figure 5f) confirming the specificity of our observed effects for complex II.

pH change leads to specific disintegration of complex II. We tested both complex II activities in mitochondria kept in buffers with reduced pH *in vitro*, and observed a reduction in the SQR activity while the SDH activity remained unchanged (Figure 6a, left panel). Cytosolic acidification imposed on cells by EIPA also triggered a reduction of the SQR activity without affecting the SDH activity (Figure 6a, right panel). These experiments demonstrated that pH_i decrease was able to specifically impact on the SQR activity. To explain at a molecular level the observed different enzymatic activities of complex II during apoptosis, we investigated the integrity of this protein aggregate. We separated mitochondrial proteins in a blue native gel after incubating these organelles in a phosphate buffer with normal or reduced pH. A Western blot for SDHA revealed an

additional band indicating the dissociation of the 100kDa SDHA/B subcomplex, which is exposed to the mitochondrial matrix, from the integral membrane protein SDHC and SDHD (Figure 6b, left panel). This partial dissociation of complex II is correlated with the partial loss of enzymatic activity of SQR. The emergence of the SDHA/B subcomplex was likewise apparent when intact HeLa cells were treated for 7h with arsenic trioxide or doxorubicin and the proteins of whole cell lysates separated on a blue native gel (Figure 6b, right panel). The same dissociation pattern was also observed in human 293T cells (Supplementary Figure 8). Moreover, inhibition of acidification by NHE1 overexpression in HeLa cells significantly reduced the partial dissociation of complex II after drug-treatment (Figure 6c).

Discussion

pH changes cause specific inhibition of complex II

Mitochondria contain a number of master regulators for apoptosis. We have proposed that complex II of the respiratory chain constitutes such a mitochondrial factor for apoptosis control. In a previous study, we showed that defects in complex II rendered cells resistant to apoptosis induction by a wide array of proapoptotic signals. This ranged from anticancer drugs such as cisplatin and etoposide to biologicals such as $\text{TNF}\alpha$.²⁷ Additional, independent studies also implicate complex II as an important sensor that is used by various signals for apoptosis induction. Downregulation of SDHD by RNAi, for example, was shown to protect neuronal cells against apoptosis by NGF withdrawal.³² Moreover, Ishii et al. found that inhibiting complex II by transfecting a SDHC mutant for the ubiquinone-binding site induced apoptosis.¹⁷ In this study we addressed why complex II constitutes a major target in pro-apoptotic signalling and the mechanism of this process. Our results show that various anticancer compounds and pro-apoptotic cytokines are able to inhibit complex II specifically at the SQR level, which is responsible for ubiquinone reduction and links complex II with the respiratory chain. At the same time, the upstream SDH activity, which is associated with succinate oxidation and hence with the Krebs cycle, is not impaired. We provide evidence that this is accomplished through intracellular acidification, concomitant with mitochondrial pH decrease. This pHi drop appeared to be a synchronic event in the whole HeLa cell population, as shown by the flow cytometry density blots depicted in Supplementary Figure 9. As intracellular pH decline is a general phenomenon during apoptosis,¹ it could explain the ubiquitous involvement of complex II in apoptosis induction. Accordingly, pH changes were previously found in connection with some of the compounds tested here such as FasL,³³ $\text{TNF}\alpha$,³⁴ etoposide,³⁵ cisplatin,³⁶ or arsenic.³⁷ Different upstream pathways were identified

such as NHE inhibition, chloride channel inhibition, lysosomal proton leakage or cystic fibrosis transmembrane conductance regulator (CFTR) involvement. However, very few studies analysed the role of mitochondrial pH during chemically induced apoptosis. While one work described an early mitochondrial matrix alkalinization,¹³ it appears that this transient phase is followed by a significant matrix acidification leading to apoptosis.³⁸ In our study, pH_i acidification, associated with pH_M drop, appears to be essential for specific SQR inhibition and subsequent apoptosis, since pH_i modification by EIPA (acidifying compound) or NHE1 overexpression (acidification inhibition) can either potentiate or block cell death, respectively. Moreover, assays both *in vitro* (Figure 6a) and in intact cells (Figures 3a with 1b,e) for complex II activities underline the specific sensitivity of the SQR function to acidification. These data suggest that complex II is an important sensor for pro-apoptotic signals through its pH sensitivity (schematized in Figure 7).

Our experiments with isolated mitochondria in buffers with different pH or recovered from cells treated with anticancer compounds indicated that complex II is unstable and can specifically dissociate. Blue native gel electrophoresis of complex II indicated that SDHA and SDHB, the two subunits exposed to the matrix and attached to the membrane anchors of complex II can be released from this complex (Figure 6b). This is in agreement with earlier studies that showed that their association can be broken by chaotropic ions and treatments such as freeze-thawing.^{39,40} Since the SDH activity of complex II is associated with SDHA and SDHB this dissociation retains this enzymatic activity but abrogates the downstream SQR function of complex II, leading to the uncoupling between SDH and SQR activity (Figure 7).

ROS generation by respiratory chain complex II

Prior to apoptosis induction we observe a partial reduction of the total cellular complex II SQR activity (Figure 3a). We believe that this is due to some of the complex II aggregates in the affected cells shutting down the SQR activity while maintaining the SDH activity (Fig 7b). As a result of this specific complex II inhibition, the electron flux from the citrate cycle through complex II is altered in three ways (i) electrons cannot efficiently be transferred to the ubiquinone-binding sites, (ii) the electron flow is disrupted within the complex, and (iii) the electrons react with molecular oxygen to produce superoxide radicals which eventually trigger apoptosis (Figure 7). Of note, the DHE and MitoSOX probes used in this study have been described as relatively specific for superoxide detection, even though they may give an unspecific response for superoxides,⁴¹ notably at the 488nm excitation wavelength used for FACS application. However, we showed that superoxide scavengers such as SOD pretreatment or MnSOD overexpression can potentially reduce (i) the DHE and MitoSOX signals induced upon complex II inhibition and (ii) the subsequent apoptosis features (Figure 5b-d and Supplementary Figure 3d), confirming that the complex II blockade generates superoxide anions. A block in various complexes of the respiratory chain has been shown to induce the formation of ROS and subsequent apoptosis,¹⁴ particularly at the complex I and III level.¹⁵ Interestingly, the cleavage of complex I subunits NDUFS1 or NDUFS3 by caspases⁴² or granzyme A,⁴³ respectively, can lead to superoxides and apoptosis. Long considered a physiologically negligible site for ROS production, complex II has been recently described by us²⁷ and others to mediate intracellular signalling through superoxide generation in a number of scenarios such as (i) mutations of the CoQ-binding site of SDHC in *C. Elegans*,⁴⁴ *E. Coli*,⁴⁵ *S. Cerevisiae*⁴⁶ and mammals,¹⁷ (ii) mutation or destabilization of SDHB,⁴⁷ and (iii) chemical inhibition of the succinate or CoQ-binding site.^{16,48} Different sites for superoxide

generation within complex II have been proposed depending on the cause of complex II inhibition, notably at the SDHC/D CoQ-binding site⁴⁶ or between the SDHA-embedded FAD group and a downstream obstruction.⁴⁷ Nevertheless, the precise molecular mechanisms of such a blockade were rarely explored. Some recent studies have evoked either a stabilization of the ubisemiquinone radical⁴⁹ or a modification of the glutathionylation and the tyrosine nitration status of SDHA.^{50,51} Our data demonstrate that another major cellular factor, pH_i homeostasis, can impact on complex II functions in living cells and facilitate ROS production.

Role of complex II in tumorigenesis

Mutations in SDHB, C and D subunits that cripple complex II have been found in various cancers, particularly paragangliomas and pheochromocytomas,²⁸ suggesting that this confers a selective growth advantage to cells and led to define its subunits as tumour suppressor proteins.²⁰ During the last ten years, research exploring the biochemical causes of complex II inhibition and tumorigenesis found different metabolic pathways to be involved. Firstly, in complex II-impaired cells, the SDH substrate succinate accumulates in mitochondria, is transported into the cytosol, and inhibits HIF1 α prolyl hydroxylase. This leads to HIF1 α stabilization and activation⁵² and finally to a pseudohypoxia tumor phenotype⁵³ and apoptosis inhibition.³² A second pathway involves the generation of ROS in association with mutated or destabilized complex II,^{17,47,54} leading to mtDNA damage⁵⁵ and likewise HIF1 α activation.⁴⁷ The results of the present study allow us to propose a third pathway that highlights the role of complex II as a major apoptosis regulator: Numerous pro-apoptotic signals target complex II and lead to (i) a specific inhibition of SQR activity without SDH impairment, (ii) uncoupling of these two enzymatic activities, (iii) superoxide production, and finally (iv) cell death induction. Our

shRNA experiments (Figure 3b-e and Supplementary Figure 4) and data from SDHC-negative B9 cells (Figures 3f and 5f) indicate that specific downregulation of complex II subunits inhibits the aggregation of the whole complex II in mitochondria. This, on its own does not elicit apoptosis (HeLa cells, Figure 3d) and even leads to a resistance to anticancer drug treatment (B9 cells, Figure 3f). These findings are also supported by other independent studies showing that, in cancer patients with mutations in complex II subunits, the remaining components of this complex are either not present⁵⁶ or display a profoundly reduced complex II activity.⁵⁷ Hence, in tumours harbouring such altered genes, the combination of a partial SQR inhibition and a fully active SDH is not possible, since both activities are downregulated, therefore impairing the apoptotic process. We believe that this is the reason why these cells can survive the onset of such mutations and eventually form tumour cells. This model does not exclude that parallel processes, such as a hypoxia-like pathway through the stabilisation of the HIF1 α transcription factor,⁵² also contribute to tumorigenesis. Nevertheless, our findings can contribute to explain why components of complex II function as tumour suppressor genes.

In conclusion, our study brings together two common features of apoptosis in response to pro-apoptotic signals: pHi change and the formation of ROS. We found that they are interconnected by the specific inhibition of complex II of the respiratory chain, which is accomplished by the release of the SDHA/SDHB subunits from the whole complex.

Materials and Methods

Cell culture and treatments. Human HeLa cells and the SDHC-deficient B9 cells, derivatives from the Chinese hamster CCL16 parental cell line (B1 cells)²⁹ were cultivated as described.²⁷ Concentrations used for treatments are shown in Supplementary Table 1.

Chemicals. All the reagents are described in Supplementary Experimental Procedures.

ShRNA constructs. ShRNA against human SDHA/B/C/D were designed (Supplementary Table 2) and checked for specificity with the iRNAi software (www.mekentosj.com/irnai/). Specific primers for SDHA/B/C/D as well as scrambled primers (of the SDHB sequence) as a negative control were synthesised and inserted into pSuper vector (Oligoengine).

Transfections and plasmids. Cells were transfected with plasmid DNA either by Lipofectamine 2000 or Effectene in B9 (30% efficiency after 48h) or HeLa cells (60-70% after 24h), respectively. For stable transfections, B9 cells were selected 3 weeks with 800µg/mL G418. Previously described expression vectors for mitochondrial MnSOD, catalase, luciferase, NHE1 and *wt* SDHC were used.^{27,58,59}

Apoptosis and necrosis quantification. Apoptotic cells were quantified using flow cytometry by determining the percentage of cells with subG1-DNA content. This subG1 population was analysed after cell permeabilization and subsequent propidium iodide staining, as previously described.^{58,60} For some experiments, apoptosis and necrosis were quantified at the same time on non-permeabilized cells by flow cytometry with an Alexa Fluor 488 conjugated annexin V (AV) and propidium iodide (PI) kit, according to the manufacturer's protocol (Invitrogen). Cells with compromised membrane integrity were also specifically quantified by flow cytometry using 7-aminoactinomycin D (7-AAD), according to the supplier's protocol (Invitrogen).

ROS detection. The ROS-sensitive probe dihydroethidium (DHE) and its mitochondria-targeted derivative MitoSOX were used, according to previously published protocols.^{47,61} Briefly, cells were treated with the indicated conditions, harvested and washed in Phosphate Buffered Saline (PBS) before the addition of 150µL of a PBS solution containing DHE (5µM) or MitoSOX (5µM). Cells were incubated at 37°C for 25 or 10min, respectively, and then washed and resuspended in PBS. Fluorescence was measured by flow cytometry using a FACScalibur (FL2-H channel), and a minimum of 4,000 cells were counted for each sample.

Total RNA isolation and reverse transcription-PCR (RT-PCR) assay. Total RNAs were extracted with TRIzol and subjected to RT-PCR.⁶² The primers used are described in Supplementary Table 3.

Mitochondria isolation. Mitochondria were isolated with established protocols,⁶³ resuspended in lysis buffer and kept at -80°C.

Complex II activity assays. Complex II activities were measured by spectrophotometric tests on 10µg of mitochondria, using the SDH (PMS-mediated DCPIP reduction) and the SQR assay (CoQ-dependent reduction of DCPIP).^{27,30} For measurements at pH 6.7 and 7.3, we used the normal phosphate buffer with different ratios K_2HPO_4/KH_2PO_4 to obtain the acidic or neutral pH (Gomorri Buffers tables).

Western blotting. For each sample, mitochondria (30µg) were separated in a 14% SDS-PAGE and electroblotted onto nitrocellulose membranes (BioRad), which were processed as described.⁶² Goat polyclonal Abs against SDHB and SDHD were purchased from Santa Cruz, mouse mAbs against SDHA and COX IV from Abcam and mouse mAbs against Cyt C and SDHC from BD Pharmingen and Abnova, respectively.

Measurements of intracellular pH (pH_i). The pH_i of HeLa cells was monitored using the pH-sensitive fluorescent probe carboxy-SNARF-1. After a 7h-treatment, cells were

collected and loaded with SNARF (5 μ M) for 30 min at 37°C, in a cell suspension buffer (CSB). The mean fluorescence intensity (MFI) of 10,000 cells was determined by flow cytometry ($\lambda_{\text{ex.}}$ = 488 nm, $\lambda_{\text{em.}}$ = FL2-H 580nm and FL3-H 640nm). The emission ratio 640/580 was converted into pH value by using the calibration curve obtained *in situ* on control cells exposed to calibration buffers containing 10 μ M nigericin.⁵⁹

Mitochondrial matrix pH (pH_M). The pH_M was monitored using two mitochondrial targeted plasmids (Aequotech): mt-EYFP (pH-sensitive enhanced yellow fluorescent protein) and mt-dsRed (pH-insensitive red fluorescent protein) used as a transfection rate control.⁶⁴ HeLa cells were co-transfected overnight with these two plasmids. After 24h, cells were treated for 7h with the different proapoptotic agents. Cells were then collected, re-suspended in CSB and analysed by flow cytometry. MFI was determined on 10,000 cells ($\lambda_{\text{ex.}}$ = 488nm, $\lambda_{\text{em.}}$ EYFP = FL1-H 530nm and $\lambda_{\text{em.}}$ DsRed = FH3-H 640nm). A standard curve was generated *in situ* on control cells exposed to calibration buffers containing ionophores nigericin and monensin, allowing the conversion of the MFI ratio 530/640 into pH value.⁶⁵ CCCP, a mitochondrial protonophore, was used as control for pH_M acidification.⁶⁴

Blue native gel PAGE. For *in vitro* pH experiments, fresh mitochondria isolated using a commercial isolation kit (Pierce) were incubated in the indicated phosphate buffer (see *Complex II activity assays*). Mitochondrial complex II was gently solubilized in a 50mM Bis-Tris buffer containing 1.25% n-dodecyl-D-maltoside and 750mM 6-aminocaproic acid on ice for 30min and the supernatant collected after centrifugation (20,000g, 30min). For experiments on transfected and/or treated cells, HeLa cells were lysed using 5% n-dodecyl-D-maltoside in PBS. Samples were incubated on ice for 30min and centrifuged (17,000g, 30min). For both experiments, Native Sample buffer (Bio-Rad), supplemented with 1.25% (final concentration) Coomassie Brilliant Blue G solution (5% Coomassie

Brilliant Blue G, 750mM 6-Amicocaproic acid and 50mM Bis-Tris), was added to the supernatants. Proteins were loaded on a Ready-Gel 4-15% (Bio-Rad) for Blue Native Page and Western Blot was performed according to established protocols. Blots were probed with a SDHA antibody (Invitrogen).

Statistical analysis. The results are presented as means \pm SD of at least three independent experiments. Significant differences ($p < 0.05$) were evaluated with the Student *t*-test. Western-Blots and PCR results are representative of at least three different experiments.

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Conflict of interest.

The authors declare no conflict of interest.

Supplementary data

Supplementary information is available at the Cell Death and Differentiation's website.

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Titles and legends to figures

Figure 1 pH change during apoptosis. **(a)** Apoptosis induction by anticancer drugs and cytokines. HeLa cells were treated with the indicated drugs for 7h and 24h or exposed to FasL or TNF α for 16h and 36h (see *Materials and Methods*). After treatment, apoptotic cells were quantified by flow cytometry using PI staining of the Sub-G1 population. *, $p < 0.05$ compared to the related control. **(b)** The proapoptotic agents lead to cytosolic acidification. HeLa cells were treated for 7h with the indicated drugs or cytokines. EIPA (20 μ M), an inhibitor of NHE1, was used as a positive control for cytosolic acidification. Cells were collected and incubated with the pH-sensitive fluorescent probe carboxy-SNARF-1. pH-related fluorescence ratios were analysed by flow cytometry and converted into pH values. Results are normalised to pH_i in untreated cells and expressed as Δ pH_i values. *, $p < 0.05$ compared to pH_i in untreated cells. **(c)** Inhibition of the NHE1 transporter by EIPA potentiates the apoptosis induced by the proapoptotic agents. HeLa cells were treated for 26h with the anticancer drugs or cytokines in the presence or absence of 20 μ M EIPA. Apoptosis was measured as in (a). #, $p < 0.05$. **(d)** Overexpression of NHE1 reduces cell death induced by the various proapoptotic signals and inhibits cytosolic acidification. HeLa cells were transfected either with pcDNA3 or with a NHE1 expression plasmid. After 48h, when the NHE1 mRNA level was upregulated (see inset), the cells were treated with the anticancer drugs or cytokines. Changes in pH_i (left panel) were quantified by flow cytometry after a 7h-treatment, as in (b). Apoptosis (right panel) was measured after 26h as in (a). Results are shown after subtraction of the pcDNA3-associated apoptosis background. #, $p < 0.05$. **(e)** The different proapoptotic agents responsible for complex II inhibition lead to mitochondrial matrix acidification. HeLa cells were co-transfected for 24h with an mt-EYFP plasmid, sensitive to pH_M changes, and with a mt-dsRed plasmid (insensitive). Cells were treated for 7h with the different anticancer drugs or cytokines. CCCP (10 μ M for 20min), a

mitochondrial protonophore, was used as a positive control for mitochondrial matrix acidification. pH_M -related fluorescence ratios were analysed by flow cytometry (see *Materials and Methods*) and converted into pH_M values. *, $p < 0.05$ compared to control.

Figure 2 Involvement of ROS in apoptosis induced by various apoptotic signals. (a) Effects of the different anticancer drugs and cytokines on mitochondrial ROS production. HeLa cells were treated with the indicated drugs (8h) or cytokines (16h), at the concentrations shown in Supplementary Table 1. ROS were quantified by flow cytometry through MitoSOX-related fluorescence. (b, c) Effects of the ROS scavenger PEG-catalase (b) and the SDH inhibitor malonate (c) on apoptosis induced by the different agents. HeLa cells were treated with the compounds for the indicated times and apoptosis was measured by flow cytometry using PI staining of the Sub-G1 population. *, $p < 0.05$ compared to the related control; #, $p < 0.05$.

Figure 3 Effect of various anticancer drugs and cytokines and the shRNA-mediated downregulation on the enzymatic activities of complex II. (a) Effects of the various proapoptotic signals on the SQR and SDH activities of complex II. HeLa cells were treated with the indicated drugs (7h) or cytokines (16h). Mitochondria were isolated and SQR and SDH activities were assessed by the appropriate assay. *, $p < 0.05$ compared to the related control. (b, c) Downregulation of SDHA/B/C/D mRNA (b) and proteins (c) by shRNA. HeLa cells were transfected with an empty pSuper vector, a scrambled pSuper-shRNA or shRNA constructs targeting SDHA/B/C/D. After 72h, mRNA levels (b) of the various complex II subunits were quantified by RT-PCR. Proteins levels (c) were analysed by Western Blot in mitochondrial fractions. Equal gel loading and transfer efficiency were checked with anti-Cox IV or Cyt C antibodies. (d) ShRNA-mediated downregulation of

SDHA/B/C/D does not induce apoptosis. HeLa cells were transfected either with a luciferase vector, a scrambled shRNA or a specific SDHA/B/C/D shRNA. After 72h, apoptosis was measured by flow cytometry using PI staining. A *wt* SDHC expression vector was transfected as a positive control for apoptosis induction after 24h. The same amount of a GFP plasmid was introduced in parallel to normalize the cell death induction to the transfection efficiency. Results are shown after subtraction of the luciferase-associated apoptosis background. #, $p < 0.05$. (e) Potent inhibition of SQR and SDH activity by shRNA-mediated downregulation of SDHA/B/C/D. SQR and SDH activities of complex II were measured in mitochondrial fractions isolated 72h after transfection. Shown are the activities relative to the scrambled shRNA-transfected cells. *, $p < 0.05$ compared to the related scrambled shRNA-activity. (f) Reconstitution of SDHC expression in B9 cells sensitises cells for apoptosis. PcDNA3 clone 2 (control cells stably transfected with an empty pcDNA3 vector) and *wt* SDHC clone 4 (stably reconstituted cells) were treated with the indicated anticancer drugs for 48h, at the concentrations shown in the Supplementary table 1. Apoptosis was quantified as in (d). #, $p < 0.05$.

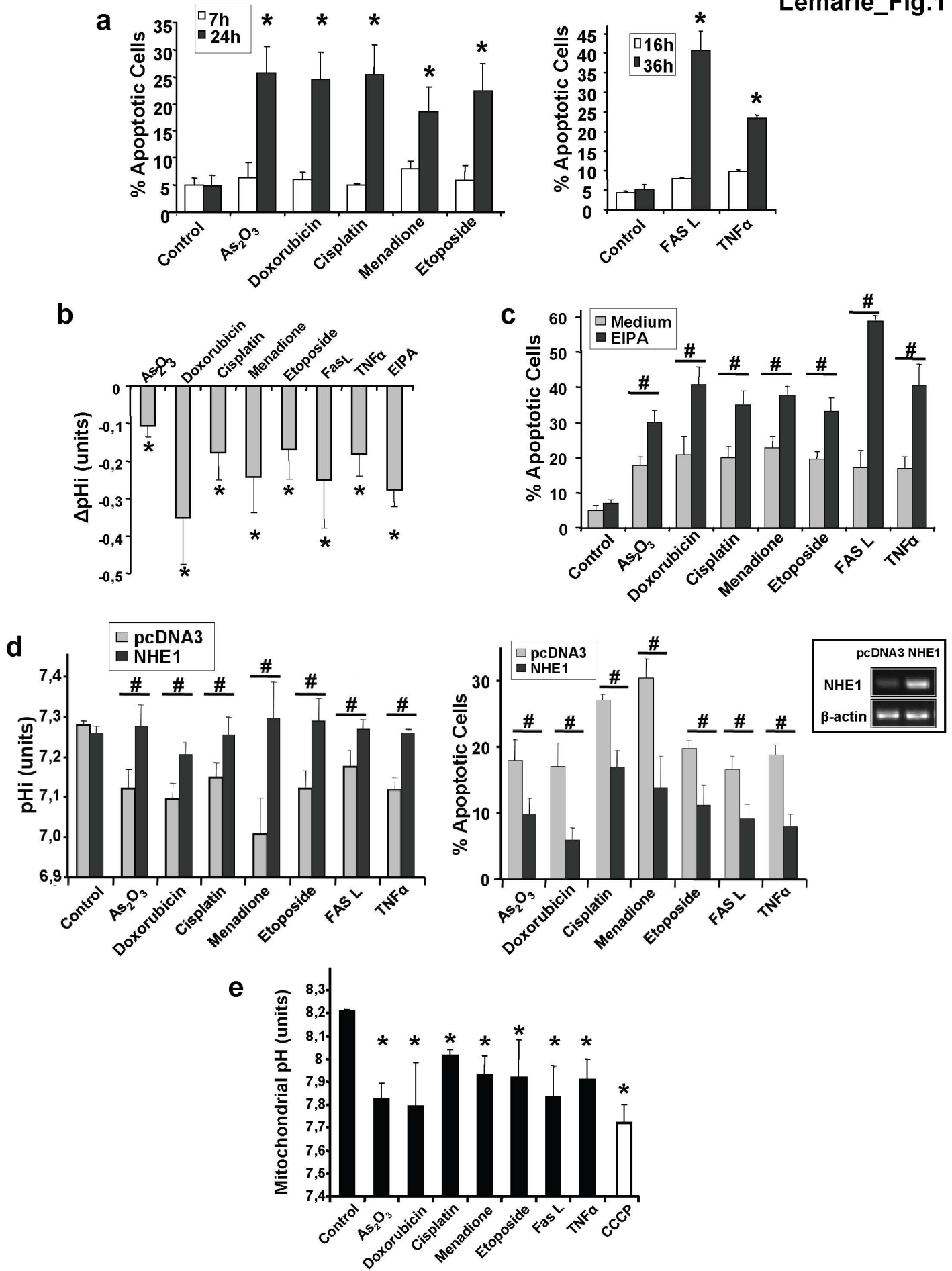
Figure 4 Effect of TTFA and 3NP, two complex II inhibitors with different target specificities. (a) Time- and dose-dependent proapoptotic effects by TTFA or 3NP. HeLa cells were treated with the indicated concentrations of TTFA or 3NP. After incubation, apoptotic cells were quantified by flow cytometry using PI staining of the Sub-G1 population. (b) Effects of TTFA and 3NP on SQR and SDH activities. (*top panel*) HeLa cells were treated for 8h with TTFA (0.5mM) or 3NP (1mM). Mitochondria were isolated and SQR and SDH activities were assessed by specific assays. (*bottom panel*) Isolated mitochondria were treated by the indicated concentrations of TTFA immediately prior to the appropriate enzymatic assay. *, #, $p < 0.05$ compared to the related control; #, $p < 0.05$.

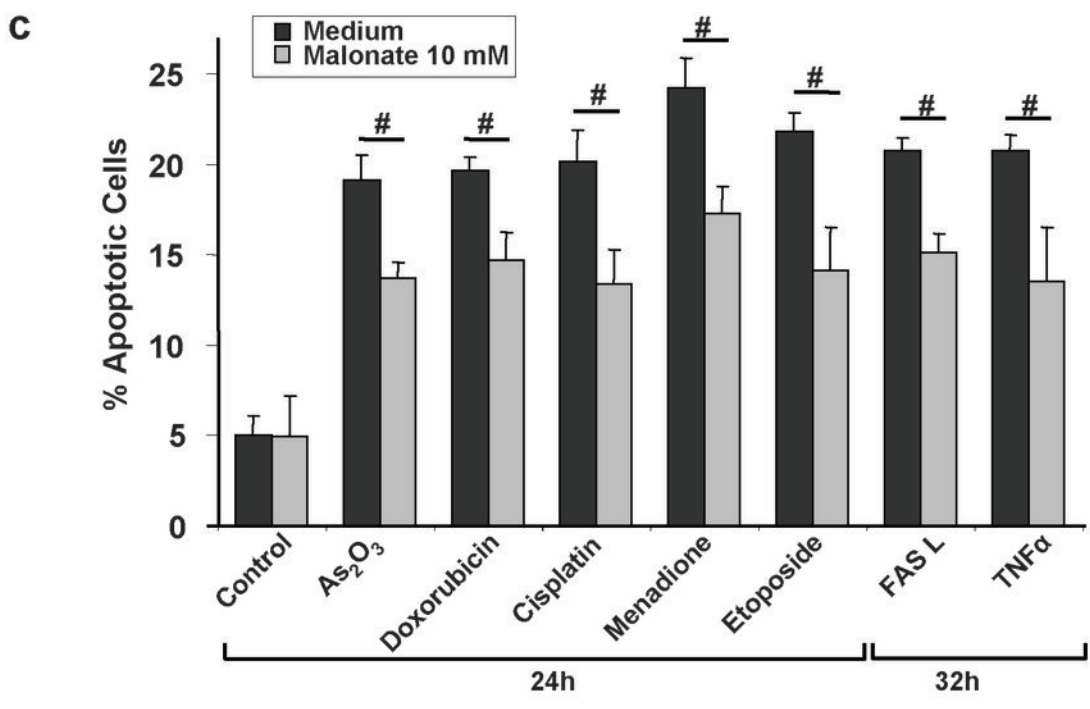
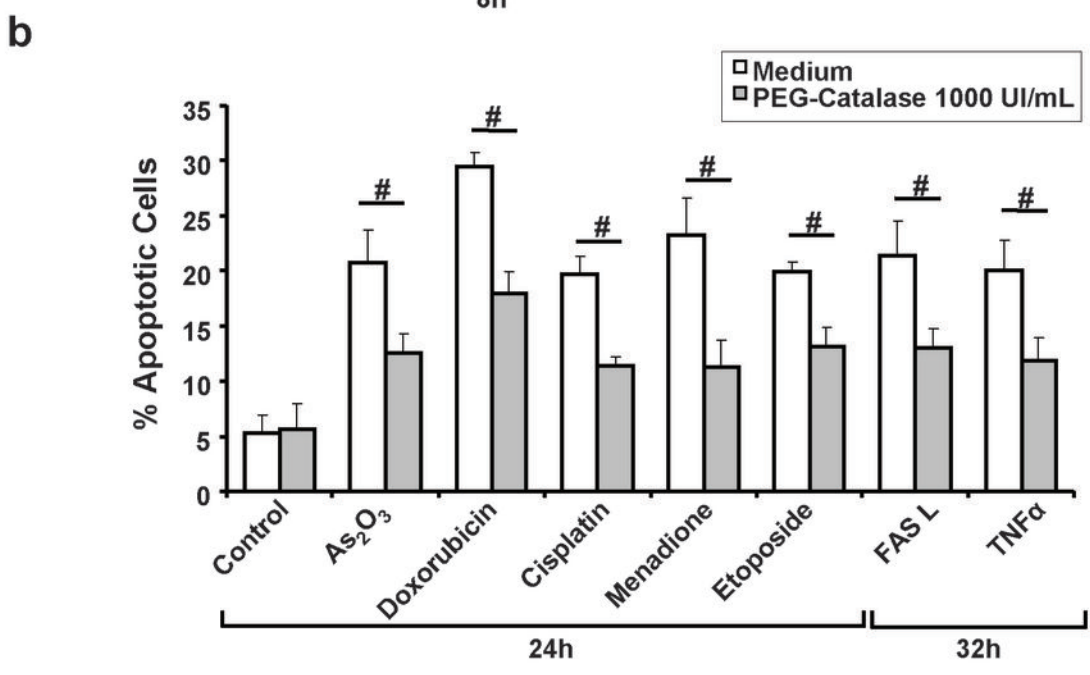
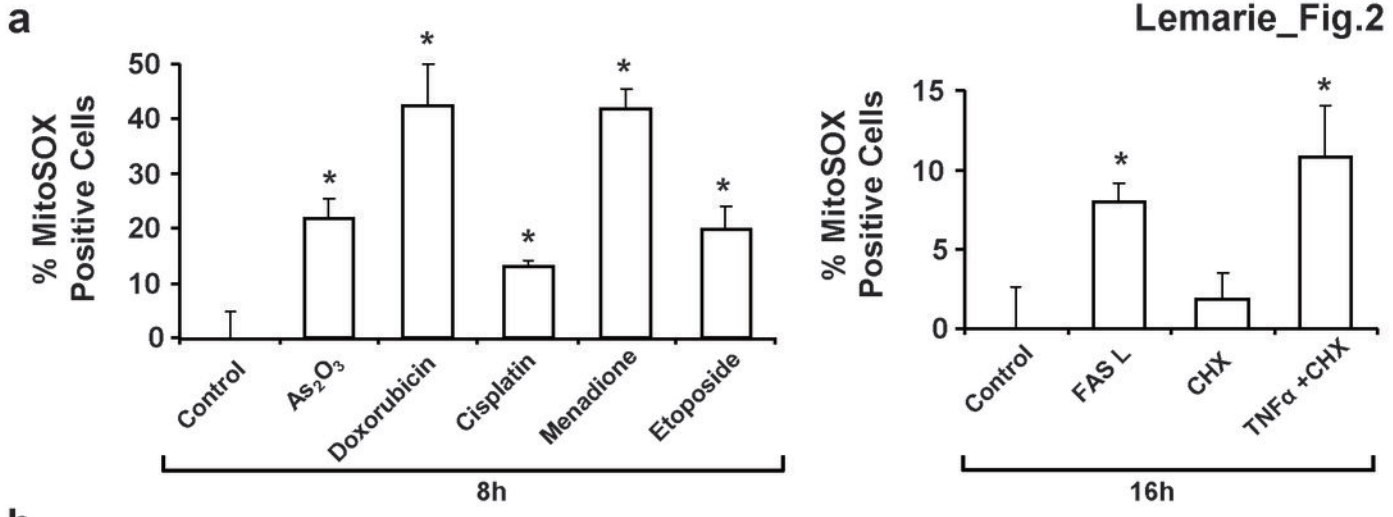
Figure 5 Role of ROS in apoptosis induced by specific complex II inhibition. **(a)** Dose-dependent ROS production induced by complex II inhibitors. HeLa cells were treated with the indicated concentrations of TTFA or 3NP for 8h. The superoxide-sensitive dye DHE was incubated with the cells and fluorescence was measured by flow cytometry. *, $p < 0.05$ compared to the related control. **(b, c)** ROS scavengers reduce superoxide generation (b) and apoptosis (c) in response to specific complex II inhibition. HeLa cells were pre-treated with superoxide dismutase (SOD, 4000 UI/mL), Trolox (100 μ M) or PEG-catalase (1000 UI/mL), and exposed to 1mM TTFA for 20h. ROS were determined as in (a) and apoptosis was measured by flow cytometry using PI staining of the Sub-G1 population. #, $p < 0.05$. **(d)** Expression plasmids for MnSOD and catalase can reduce apoptosis by TTFA. Vectors containing luciferase, MnSOD or catalase were transfected into HeLa cells. In parallel the same amount of a GFP plasmid was transfected to normalize the cell death inhibition to the transfection efficiency. After 24h, cells were treated by 1mM TTFA for 8h. Apoptosis was measured as in (c). #, $p < 0.05$. Luciferase expression had no effect on apoptosis (Supplementary figure 10). **(e)** Effects of different SDH inhibitors (3NP, malonate), Krebs cycle inhibitors (fluorocitrate, fluoroacetate) or a Krebs cycle intermediate (succinate DBE) on apoptosis and superoxide production induced by TTFA. HeLa cells were treated with 1mM TTFA and the indicated compounds. As a control the inhibitors were used in their own. Superoxides and apoptosis were quantified as in (a) and (c), respectively. §, $p < 0.05$ for PI values; \$, $p < 0.05$ for DHE values. **(f)** Effects of TTFA on the SDHC -/- clone 2 and on the *wt* SDHC reconstituted clone 4. These B9 clones were treated with 1mM TTFA for 48h. Superoxides and apoptotic cells were measured as in (a) and (c), respectively. §, $p < 0.05$ for PI values; \$, $p < 0.05$ for DHE values.

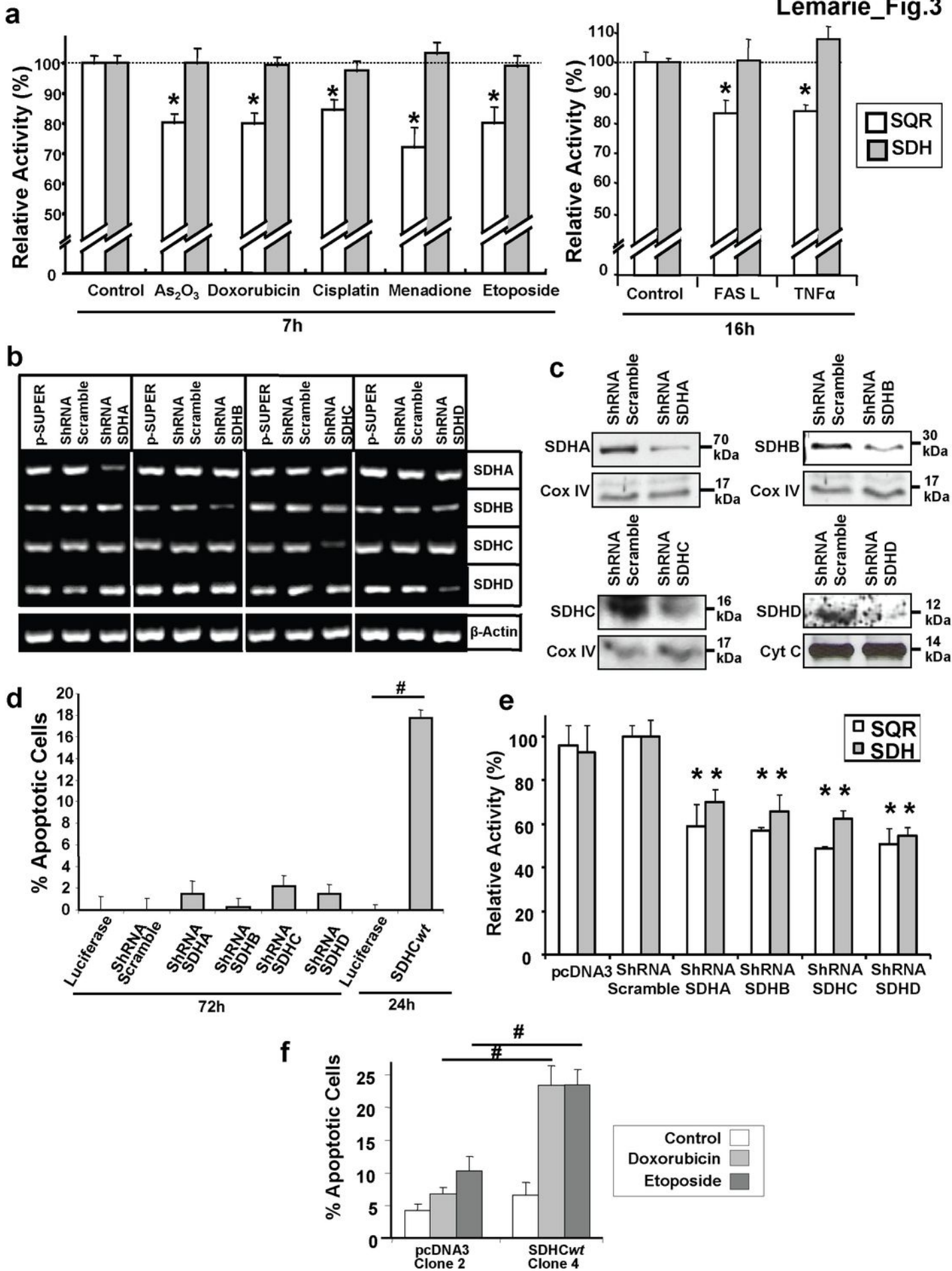
Figure 6 Specific complex II disintegration by pH change. (a) Effects of pH_i acidification and NHE1 inhibition on complex II enzymatic activities. (*left panel*) Isolated mitochondria were incubated in a phosphate buffer at normal pH 7.3 or acidic pH 6.7 and the appropriate assay was performed. (*right panel*) HeLa cells were treated for 7h with EIPA (20μM), mitochondria were isolated, and SQR and SDH activities were assessed by specific enzymatic assays. #, $p < 0.05$. (b) Integrity of complex II as revealed by immunoblots of blue native PAGE. (*left panel*) Isolated mitochondria from HeLa cells were incubated in phosphate buffers with the indicated pH for 30min and complex II solubilization was performed as described in *Materials and Methods*. (*right panel*) HeLa cells were incubated for 7h with 10μM As₂O₃ or 1.4μM doxorubicin. Whole cell lysates were prepared and their proteins solubilized as described in *Materials and Methods*. Equal amounts of protein were loaded onto a native gel, blotted onto a membrane, which was probed for SDHA. (c) Overexpression of NHE1 reduces drug-induced complex II dissociation. HeLa cells were transfected either with pcDNA3 or with a NHE1 expression plasmid. After 24h, the cells were treated with 1.4μM doxorubicin (*left panel*) or 10μM As₂O₃ (*right panel*) for 7h. Proteins from whole cell lysates were processed as in (b) and loaded onto a native gel. For (b) and (c), the percentage of SDHA/B subcomplex dissociation was determined using densitometric analyses of at least 4 independent experiments and shown as graphs under the related blots. *, $p < 0.01$ compared to the related control; #, $p < 0.01$.

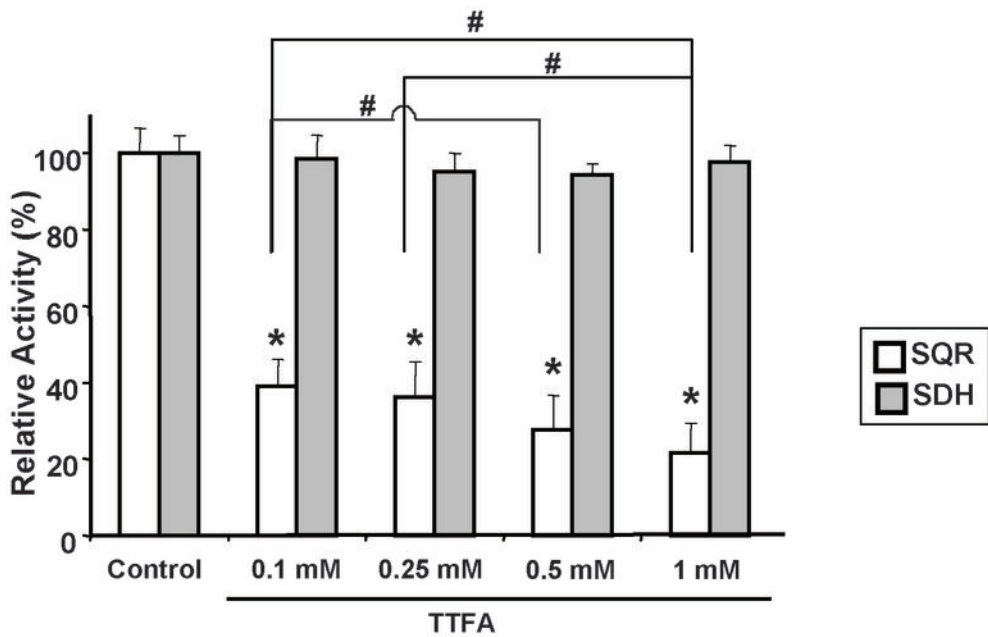
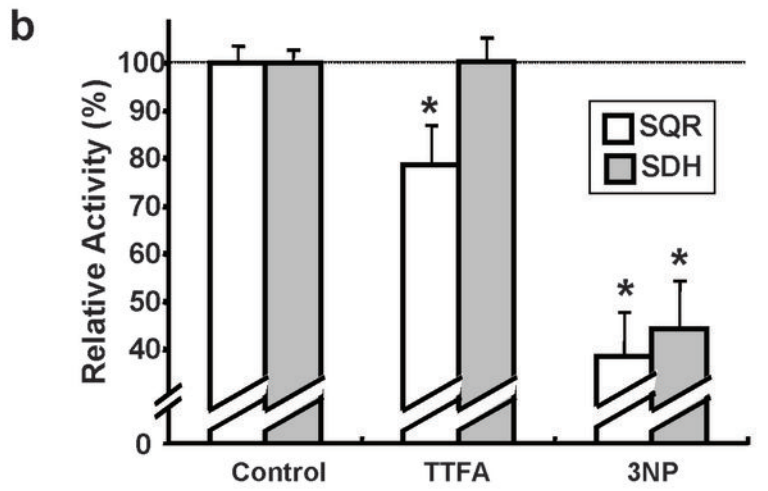
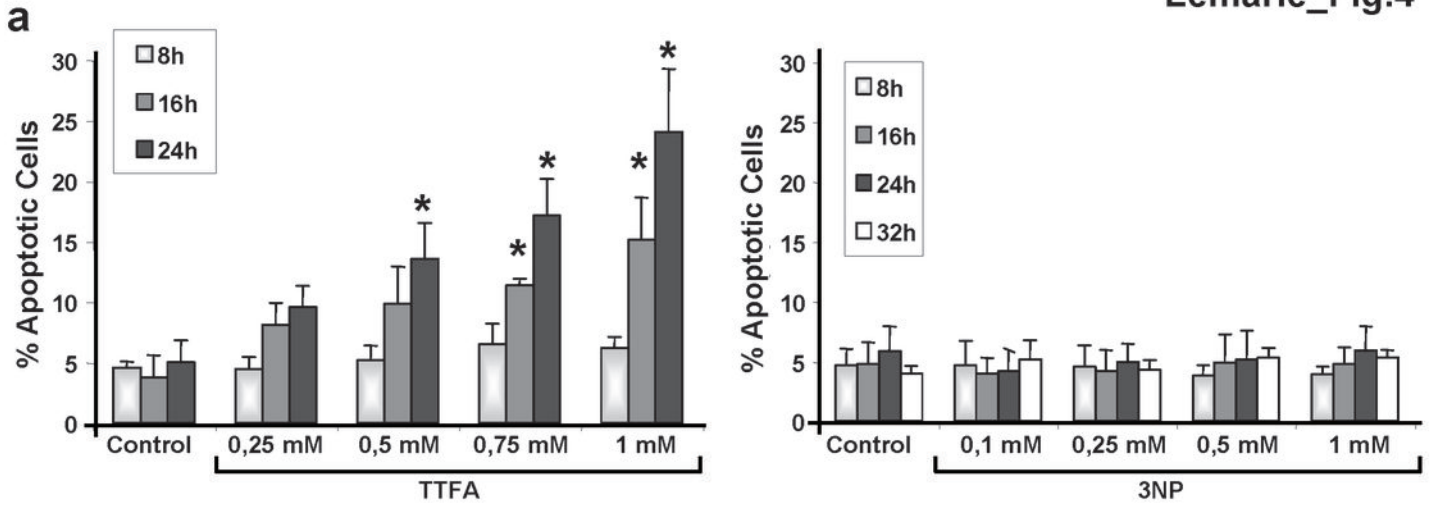
Figure 7 Model for the role of specific complex II inhibition for apoptosis induction by various proapoptotic compounds. (a) In healthy cells complex II serves to funnel electrons derived from the Krebs cycle to the respiratory chain. SDHA-mediated oxidation of succinate to fumarate by the succinate dehydrogenase activity (SDH), as part of the Krebs

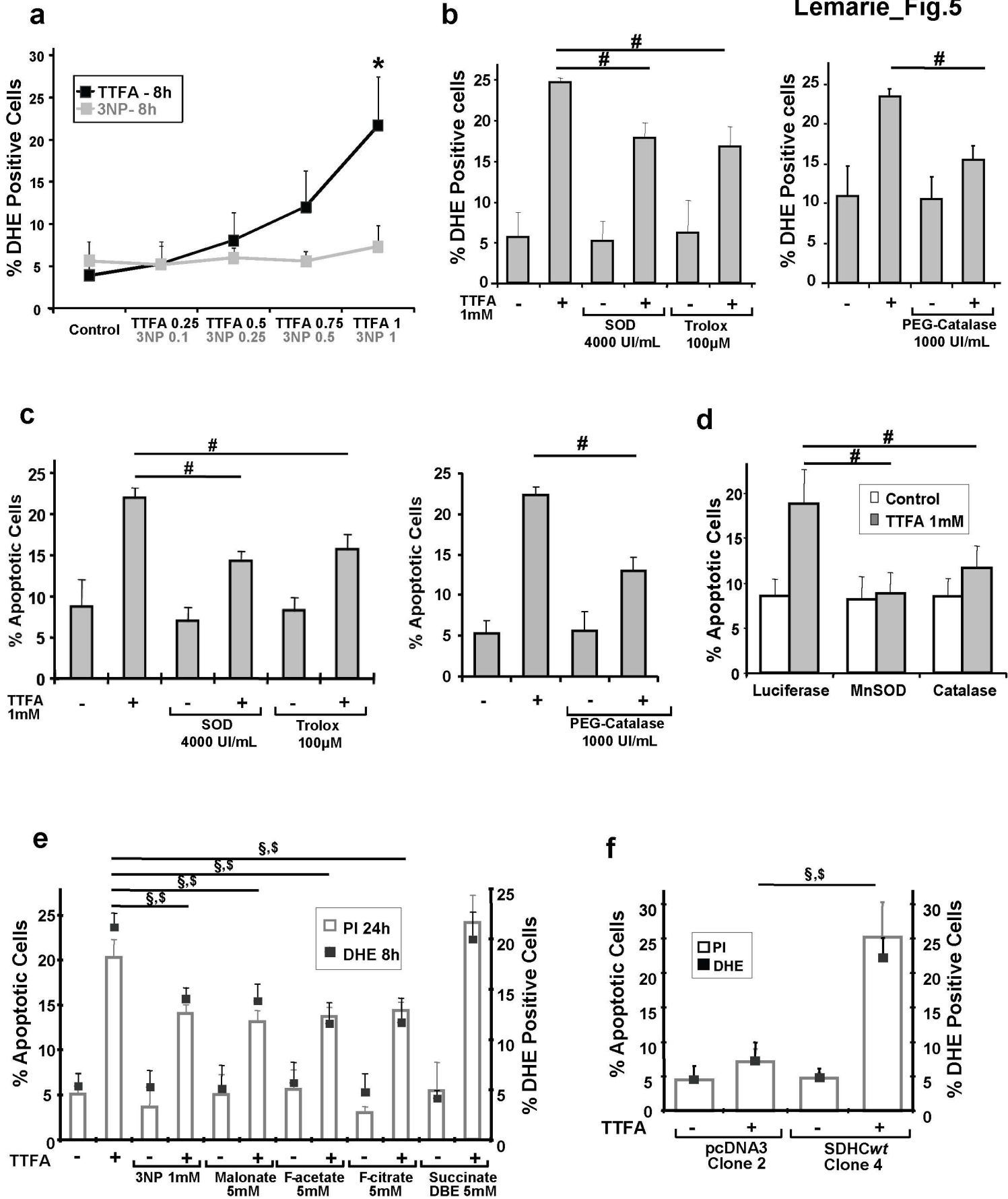
cycle, provides electrons to complex II. They are transferred to the iron-sulfur centers of the SDHB subunit and finally to the CoQ reduction site at the interface between SDHC and SDHD, the two transmembrane subunits of complex II. The succinate CoQ oxidoreductase (SQR) reaction comprises in addition the transfer of the electrons to CoQ. **(b)** Proapoptotic compounds such as various anticancer drugs, FasL or TNF α induce intracellular (pHi) and mitochondrial (pH_M) acidification. These pH changes lead to the dissociation of the SDHA/B subunits from complex II and finally to the partial inhibition of the SQR activity without any impairment of the SDH reaction. This specific inhibition leads to complex II uncoupling, superoxide production and apoptosis.



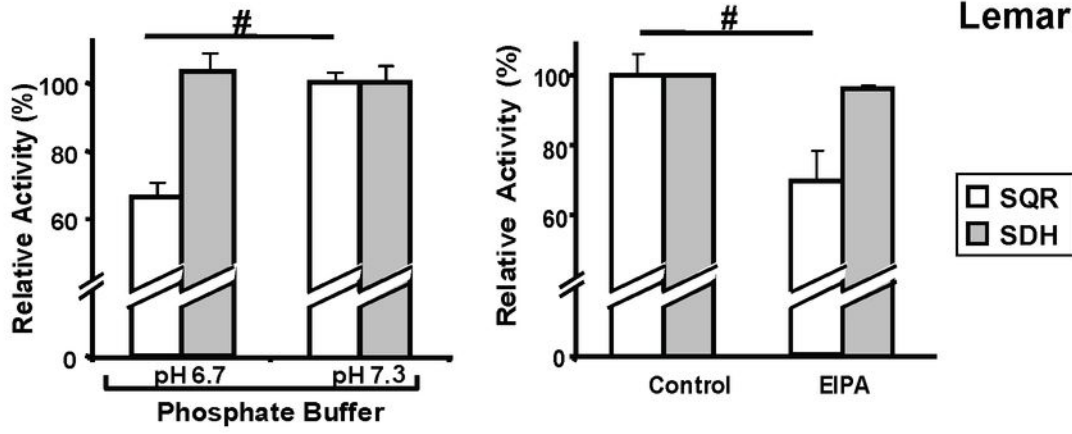




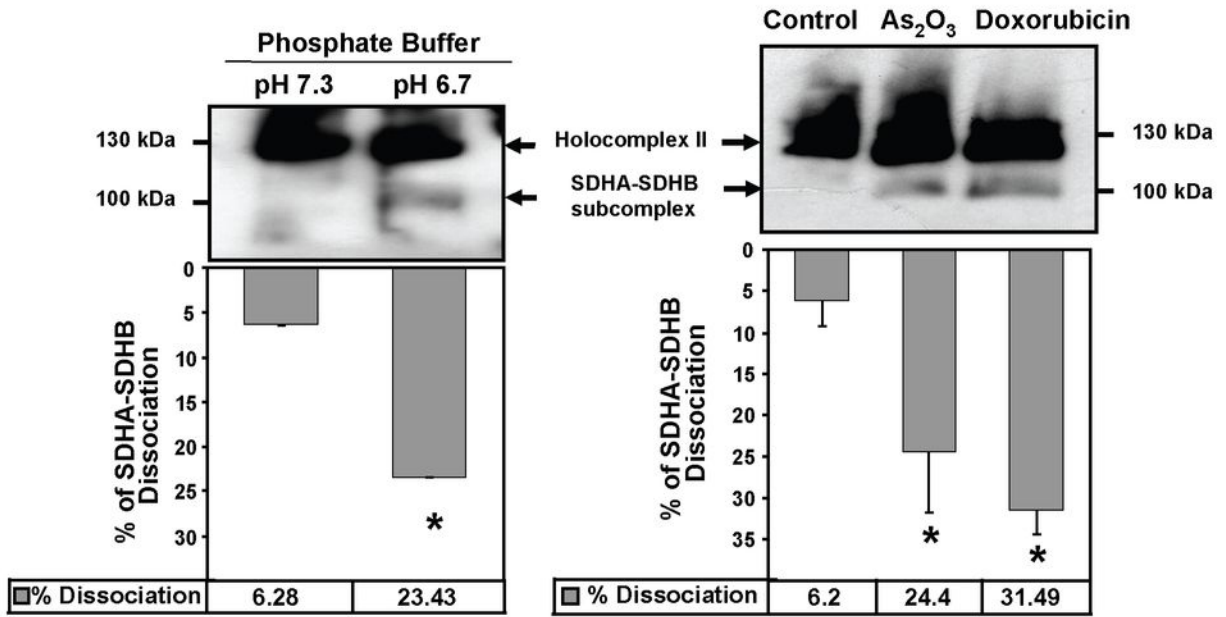




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