Hydrogen peroxide produced by *Aplysia* ink toxin kills tumor cells independent of apoptosis via peroxiredoxin I sensitive pathways

D Butzke^{1,2}, N Machuy², B Thiede², R Hurwitz³, S Goedert² and T Rudel^{1,2}*

¹ Institut fuer Oekotoxikologie und Biochemie der Freien Universität Berlin, Ehrenbergstr. 26-28, Berlin, Germany

² Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, Berlin, Germany

³ Max-Planck-Institut für Infektionsbiologie, Serviceabteilung Proteinreinigung, Berlin, Germany

* Corresponding author: T Rudel, Department of Molecular Biology, Max-Planck-Institute for Infection Biology, Schumannstr. 21/22, D-10117 Berlin, Germany. Tel.: +49 30 28460 415; Fax: +49 30 28460 401; E-mail: rudel@mpiib-berlin.mpg.de

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Abstract

Marine snails of the genus *Aplysia* possess numerous bioactive substances. We have purified a 60 kDa protein, APIT (*Aplysia punctata* ink toxin), from the defensive ink of *A. punctata* that triggers cell death with profound tumor specificity. Tumor cell death induced by APIT is independent of apoptosis but is characterized by the rapid loss of metabolic activity, membrane permeabilization, and shrinkage of nuclei. Proteome analysis of APIT-treated tumor cells indicated a modification of peroxiredoxin I, a cytoplasmic peroxidase involved in the detoxification of peroxides. Interestingly, knockdown of peroxiredoxin I expression by RNA interference sensitized cells for APIT-induced cell death. APIT induced the death of tumor cells via the enzymatic production of H₂O₂ and catalase completely blocked APITs' activity. Our data suggest that H₂O₂ induced stress and the modulation of peroxiredoxins might be a promising approach for tumor therapy.

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Abbreviations: APIT, *Aplysia punctata* ink toxin; chx, cycloheximide; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; Prx I, peroxiredoxin I; RNAi, RNA interference; ROS, reactive oxygen species.

Introduction

Marine sea hares produce purple ink that is discharged in defense against predators.¹ A broad range of small pharma-

cologically active compounds, some of which originate from ingested Cyanobacteria, have been isolated from different sea hare genera including *Aplysia* and *Dolabella*.² In addition, several *Aplysia* and *Dolabella* species produce glycoproteins with antitumor and bactericidal activity including the Aplysia-nins, Dolabellanins, and Cyplasins.^{3,4} The molecular mechanism of the antitumor activity of these factors has so far not been elucidated.

At least three pathways of cell death with distinct phenotypes are known to date; apoptosis, necrosis, and oxidative damage.⁵ Apoptosis, a genetically fixed physiological form of cell death, is accompanied by shrinkage, membrane blebbing, nuclear fragmentation, and final disintegration of the dying cell into so-called apoptotic bodies.⁶ In contrast, necrosis is a pathological process characterized by membrane disruption and cell swelling. The phenotype of cell death induced by reactive oxygen species (ROS) is less well defined; for example, induction of apoptosis and necrosis,⁷ and other forms of cell death,⁸ have been described.

Tumor cells show an increased ROS production compared to normal cells^{9,10} and ROS are thought to play multiple roles in tumor initiation, progression, and maintenance.¹¹ ROS-induced DNA damage may promote tumorigenesis. Moreover, cellular stress response kinases are activated by ROS like extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNK), and p38. Interestingly, tumor cells sensitive to anticancer therapy show higher levels of ROS and stress responses than drug-resistant tumor cells.¹² Eucaryotic cells have developed different enzymatic systems to neutralize potential harmful ROS such as superoxide dismutase, catalase, glutathione peroxidases, and thioredoxin peroxidases. Overproduction of thioredoxin peroxidases, also known as peroxiredoxins,^{13,14} is found in many types of cancers.^{15,16}

Bcl-2 family proteins are involved in the regulation of apoptosis. They are of major medical importance since overexpression of inhibitory members or mutational inactivation of activating members has been implicated in tumor cell growth and tumor therapy resistance.¹⁷ However, besides playing an important role in the regulation of apoptosis, they have also been found to regulate apoptosis-independent stress-related forms of cell death.¹⁸

We have identified and purified a new protein from the ink of *Aplysia punctata* that causes death of tumor cells but not of peripheral blood mononuclear cells (PBMC) and human umbilical vein endothelial cells (HUVEC). The characteristics of cell death induced by the *Aplysia punctata* ink toxin (APIT) reflects neither an apoptotic nor a clear necrotic form of cell death but is rather typical of oxidative damage. The mechanism of the cytolytic activity of APIT is the continuous enzymatic production of H₂O₂. Furthermore, knockdown of peroxiredoxin I (Prx I) expression by RNA interference (RNAi) demonstrated the specific involvement of Prx I in signaling pathways induced by APIT.

Results

Purification of a new compound with tumor lytic activity from *A. punctata*

The ink of *A. punctata* contains an unknown activity that efficiently kills tumor cells (Figure 1a). Tumor cells exposed to ink exhibited a characteristic phenotype; loss of contact to neighboring cells, appearance of vacuoles in the cytoplasm, and nuclei shrinkage (Figure 1a). In order to purify the tumor lytic factor, ink from *A. punctata* was subjected to ion exchange chromatography (Figure 1b) and subsequent gel filtration chromatography (Figure 1c). Each fraction was tested for toxic activity by measuring the metabolic activity of treated Jurkat cells (Figure 1b,c). Activity correlated with the presence of a protein of approximately 60 kDa (Figure 1d), whereas inactive fractions did not contain a 60 kDa band (not shown). Since Jurkat cells exhibited the same phenotype when either treated with the purified APIT or the crude ink (Figure 1a), we concluded that APIT is the main factor exhibiting cytolytic activity in the ink. The gene of APIT was cloned and sequenced (Genbank accession numbers AY442281, AY442282, AY442283). APIT exhibited significant homology to L-amino-acid oxidases, Aplysianins, and Cyplains (manuscript in preparation).

APIT-induced cell death is independent of apoptosis

To further characterize the mode of cell death induced by APIT, metabolic activity, membrane permeabilization, as well as apoptotic features of treated cells were investigated. Metabolic activity of tumor cells was blocked as early as 30 min after exposure to APIT (Figure 2a). Tumor cells took up propidium iodide (PI) 2 h after APIT treatment indicating plasma membrane permeabilization and cell death (Figure 2b). Treated cells exhibited neither sole morphological signs of apoptotic or necrotic cell death (Figure 1a, 2c), as no blebbing or swollen cells were detected when cells were treated with a lethal dose of APIT. Corresponding to the absence of apoptosis, caspases were not activated in treated cells as determined by 2-DE analysis of previously identified caspase substrates like Rho GDI-2 or hnRNPs (not shown).¹⁹ Consistent with the absence of active caspases, the broad-range caspase inhibitor zVAD-fmk failed to prevent cell death induced by ink or by purified APIT, but efficiently blocked apoptosis induced by doxorubicin or low H₂O₂ concentrations (Figure 2c). As a further hallmark of apoptosis, the fragmentation of DNA was investigated in treated cells. No processing of DNA into oligonucleosomal fragments occurred in treated cells as monitored by gel electrophoresis (Figure 2d). However, treated cells were positive in TUNEL analyses, indicating that APIT induced DNA-strand breaks, probably single-strand breaks, with high efficiency (Figure 2e). Collectively, these data demonstrate that APIT-induced cell death occurs independent of apoptosis.

Tumor specificity of APIT

To analyze the specificity of APIT for tumor cells, PBMC from healthy donors and Jurkat cells were incubated with increas-

ing amounts of purified APIT and analyzed for PI uptake (Figure 3a). Even at the highest APIT concentrations used in this experiments, only 28% of the peripheral blood cells took up PI, indicative of membrane disintegration, compared to 80% of the tumor cells. Determination of the release of cytoplasmic lactate dehydrogenase (LDH) as a further marker of membrane permeabilization strongly confirmed these data (not shown). Beside PBMC, also HUVEC were highly resistant against APIT-induced cell death as shown in Figure 3b. Even at a APIT concentration of 40 ng/ml, LDH release of HUVEC cells remained below 10%, in contrast to LDH release of about 70% of Jurkat cells. Since several tumor cell lines showed a similar sensitivity as the Jurkat cells (Table 1) but normal PBMC and HUVEC were resistant to APIT, our data suggest that the toxic effect induced by APIT is tumor specific.

Antiapoptotic Bcl-2 family proteins are involved in the regulation of apoptotic cell death induced by a wide variety of stimuli.²⁰ Since many anticancer drugs kill tumor cells by inducing Bcl-2-dependent apoptotic cell death, overexpression of Bcl-2 family proteins has been recognized as an important aspect of tumor therapy resistance.¹⁷ In order to test the influence of antiapoptotic Bcl-2 proteins on APIT-induced cell death, tumor cell lines overexpressing Bcl-2 or Bcl-X_L and the respective control cell lines were analyzed. In contrast to control cells, doxorubicin-induced reduction of metabolic activity was inhibited in the Bcl-2- or Bcl-X_L-overexpressing cells demonstrating the functional expression of Bcl-2 and Bcl-X_L (Table 1). Interestingly, tumor cells overexpressing Bcl-2 or Bcl-X_L exhibited the same sensitivity for APIT as the corresponding control lines (Table 1). These results suggested that APIT-induced cell death does not interfere with Bcl-2- or Bcl-X_L-sensitive apoptosis pathways.

APIT kills tumor cells by H₂O₂ production

In order to investigate the mechanism by which APIT induces cell death, proteome analyses of treated and untreated tumor cells were performed. Interestingly, the most prominent change observed was the shift of a 22 kDa protein spot from pI 8.3 to 7.7 (Figure 4a). These protein spots were identified as Prx I by peptide mass fingerprinting.

Prx I is located in the cytoplasm and is involved in the detoxification of H₂O₂ and other peroxides.^{21–24} Cells were treated with APIT and the H₂O₂-degrading enzyme catalase to investigate whether H₂O₂ is involved in APIT-induced toxicity. Catalase completely prevented cytolysis (Figure 4b) and the rapid loss of metabolic activity (Figure 4c). As expected, catalase was ineffective in blocking CD95(Fas/Apo-1)-induced cell death in the same assay (Figure 4c). In the presence of catalase, APIT failed to induce morphological changes of tumor cells as judged by microscopy (Figure 4d). Catalase treatment also completely prevented the permeabilization of PBMC, suggesting that the minor toxic effect on normal cells also depends on the enzymatic activity of APIT (data not shown). The highly efficient inhibition of catalase in particular suggested that no other substance than H₂O₂ elicits the toxic effect observed in APIT-treated samples. Moreover, catalase also inhibited tumor cell lysis induced by ink (Figure 4b,d), and high concentrations of H₂O₂ induced the

phenotype typical for APIT-treated cells (Figure 4d), indicating that the cytotoxic effect of ink is caused by H₂O₂ production. Furthermore, proteome analyses revealed the same changes in the protein pattern in H₂O₂-treated and APIT-treated cells (data not shown).

The data obtained so far did not allow us to determine if APIT induces peroxide production in treated cells or if it contains an enzymatic activity that produces peroxide. To distinguish between these possibilities, H₂O₂ was measured using an enzymatic assay based on the conversion of ABTS 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) by horseradish peroxidase in samples of APIT-treated cells, and also in medium containing APIT in the absence of cells. As shown in Figure 4e, H₂O₂ was produced by APIT in cell culture medium even in the absence of cells. Under these conditions, H₂O₂ was produced at 300 μ M after 90 min (Figure 4e), which would be sufficient to induce cell death (data not shown). H₂O₂ concentrations measured in the presence of cells were generally lower due to H₂O₂ consumption by the cells. These data clearly demonstrated that the cytotoxic activity is dependent on the H₂O₂-producing enzymatic activity of APIT.

Knockdown of Prx I sensitized cells for APIT-induced cell death

Prx I exhibited the most significant modification observed in 2-DE protein patterns of APIT-treated cells in comparison to untreated Jurkat cells. Since Prx I is involved in ROS detoxification,^{21–24} we asked whether Prx I was involved in a signaling pathway induced by APIT. If Prx I was involved in the detoxification of H₂O₂ produced by APIT, we expected to observe a sensitization in cells in which Prx I expression is decreased. To measure sensitization, conditions were chosen under which the reduction of metabolic activity of treated cells was 50% or less of the untreated cells. siRNAs were transfected into HeLa cells and after 72 h cells were treated with APIT for 6 h and the metabolic activity was determined. In parallel, cells were harvested for quantitative analysis of the respective mRNAs by realtime PCR (Figure 5a). The mRNA of Prx I was reduced by more than 90% compared to the mRNA level of GAPDH. Interestingly, this reduction of Prx I expression significantly sensitized the cells for killing by APIT, whereas control siRNA directed against Luciferase and Lamin A/C had no effect (Figure 5b). Thus, our data show that Prx I is involved in a protective pathway against APIT toxicity in tumor cells.

Discussion

The overall aim in tumor therapy is the selective eradication of transformed cells without harming healthy cells. Several

glycoproteins isolated from sea hares have attracted attention because of their reported antitumor activity.³ The underlying mechanism for such activity has, however, not been elucidated. Here we demonstrate that APIT, a protein of the ink of *A. punctata*, lyse tumor cells by the production of H₂O₂. This basic mechanism of APITs tumorizidal activity was confirmed by our biochemical and molecular analyses which revealed that APIT is an L-amino-acid oxidase producing H₂O₂ via oxidation of amino acids (manuscript in preparation). Since the amino-acid sequence of APIT is homologous to Aplysianins and Cyplasins, we suggest that these toxins are H₂O₂-producing amino-acid oxidases. Moreover, we present evidence that Prx I is involved in the detoxification of H₂O₂ in tumor cells.

The major modification identified in 2-DE patterns of cells treated with APIT was a significant shift in the pI of Prx I. Prx I belongs to a class of peroxidases involved in the detoxification of H₂O₂ and other peroxides.²⁴ Therefore, we concluded that the generation of peroxides is the mechanism by which APIT induced the lysis of tumor cells. Thus, the modification of Prx I could be used as a marker for APITs antitumor activity. Since the same modification of Prx I was detected in cells treated with H₂O₂, antitumor activities of APIT other than production of H₂O₂ are very unlikely. This conclusion was confirmed by the inhibition of APIT-induced tumor cell death by catalase, an H₂O₂ hydrolyzing enzyme. Catalase also prevented tumor cell death induced by crude ink, further demonstrating that no other activity than the production of H₂O₂ is responsible for the ink-mediated killing of tumor cells.

Tumor cells treated with APIT displayed an unusual morphology which was neither typical for apoptosis or for necrosis. Shrunken nuclei and lack of cell swelling are typical characteristics of apoptosis, and early membrane permeabilization of necrosis. Interestingly, APIT induced no oligosomal DNA fragmentation but probably single-strand DNA breaks detected by TUNEL assay. High H₂O₂ concentrations are able to induce single-strand breaks by activating endonuclease.²⁵ The phenotype induced by APIT could be reproduced by treatment of the cells with H₂O₂ at concentrations above 200 μ M (Figure 4d). Interestingly, H₂O₂ concentrations <100 μ M induced typical apoptosis in Jurkat cells (Figure 2c). These observations are confirmed by recent publications showing that H₂O₂ may either induce apoptosis or a further noncharacterized form of cell death depending on the amount of H₂O₂.²⁶ The dose dependency of the phenotype induced by H₂O₂ treatment is also in agreement with the notion that many toxic insults may either induce apoptosis or necrosis, dependent on the magnitude rather than on the nature of the insult.⁷ Interestingly, a phenotype very similar to that induced by APIT has been described as a result of treating cells with the electron transport chain inhibitor antimycin A.⁸ Cells die by apoptosis at low doses of

Figure 1 (a) Phenotype of APIT-induced cell death. Jurkat T cells (upper panel) or HeLa cells (lower panel) were cultured for 7 h in medium, ink or APIT as indicated and bright field and phase contrast images were recorded, respectively. (b) Anion exchange chromatography of ink and activity assay of single fractions. Proteins were eluted by a linear gradient from 0 to 800 mM NaCl. Absorption was measured at 280 nm (AU: absorption unit). Horizontal bar indicates active fractions, which were determined as shown in the adjacent diagram: Jurkat cells were treated with diluted fractions (1/2,700) and their metabolic activity was recorded as percent of medium control. (c) Active fractions of (b) were subjected to gel filtration chromatography and active fractions were determined as described under (b). (d) Silver-stained SDS-PAGE of the active fractions obtained by gel chromatography. Both protein bands at 60 kDa consist of the same amino-acid sequence as indicated by identical peptide mass fingerprints

Figure 3 (a) Sensitivity of tumor cells and PBMC to APIT-induced cell death. Jurkat cells and PBMC from healthy donors were cultured for 14 h in the presence of increasing amounts of APIT. Cell death was measured as PI uptake. (b) HUVEC cells are resistant to APIT-induced cell death. HUVEC (black bars) and Jurkat cells (white bars) were incubated overnight with increasing amounts of APIT. Subsequently, LDH release in the culture supernatant was measured photometrically. The results of two independent experiments $\pm$ the S.D. are shown

antimycin A, while at higher doses features of apoptotic and necrotic cell death can be detected.⁸ Interestingly, antimycin A induces the rise of intracellular H₂O₂.²⁷ Both APIT and antimycin A, although by different mechanisms, might thus

Table 1 APIT kills apoptosis-resistant tumor cell lines

Tumor cell line	IC ₅₀ (APIT, ng/ml)	Metabolic activity after doxorubicin treatment (%)
Jurkat neo	3.2 $\pm$ 0.7	32.5 $\pm$ 5.5
Jurkat Bcl-2	2.7 $\pm$ 0.6	80.0 $\pm$ 4.0
CEM neo	5.6 $\pm$ 0.7	27.0 $\pm$ 7.0
CEM Bcl-X _L	4.1 $\pm$ 0.1	75.0 $\pm$ 1.0
K562	4.3 $\pm$ 0.2	80.5 $\pm$ 8.5

The IC₅₀ values reflect the APIT concentration at which the metabolic activity of the tumor cells is decreased to 50% compared to the medium control. Apoptosis resistance of the Bcl-2 and Bcl-X_L transfected and of the naturally resistant K562 cell lines was confirmed via increased metabolic activity after doxorubicin treatment (10 μ M) in comparison to mock-transfected cell lines. The results of three independent experiments $\pm$ the S.D. are shown.

lead to the accumulation of the same effector molecule, H₂O₂, which at a certain level induces this special form of cell death.

H₂O₂ and the resulting free radicals are involved in a plethora of physiological and pathological signaling pathways.²⁸ The mechanism by which H₂O₂ itself interacts with protein function depends on its oxidative potential for thiol proteins. Such a group of target proteins influenced by H₂O₂ might be the caspases, a family of cysteine proteases that play an important role in apoptotic signaling.²⁹ Interestingly, H₂O₂ has been shown to have a dual function on caspases; addition of H₂O₂ to cells at low concentrations (50 μ M) activates caspases and induces apoptosis. At higher concentrations (>200 μ M), H₂O₂ inhibits the activation of caspases and rather induces necrosis.³⁰ H₂O₂ also inhibits the activation of recombinant caspases suggesting that the caspase inhibitory effect is dependent on direct interference with caspase activity.³¹ Therefore, it might well be that APIT produces levels of H₂O₂ that damage the cell but prevent apoptosis by blocking caspase function.

Knockdown of Prx I by RNAi rendered the cells hypersensitive for APIT, suggesting that Prx I is part of an H₂O₂ detoxifying pathway. Peroxiredoxins comprise a class of highly conserved oxidases.³² In mammals, six different isoforms are known.^{13,14} Peroxiredoxins catalyze the reduction of peroxides by using reducing equivalents that are provided by thioredoxin or glutathione. During catalysis, Prx I is inactivated by oxidation of the active site cysteine to cysteine sulfinic acid,³³ a modification which is reversible upon removal of H₂O₂.³⁴ The modification of Prx I that we observed in 2-DE gel analysis of APIT-treated cells resembles that described for the oxidized and inactivated Prx I,³³ indicating that APIT inactivates this detoxification system. Previously, overexpression of both Prx I and Prx II has been shown to render cells resistant to H₂O₂-induced

Figure 2 (a) Cell death induced by APIT measured by the loss of metabolic activity. APIT and the tetrazolium-salt WST-1 were added simultaneously to Jurkat cells and the turnover of WST-1 was measured photometrically. White circles: medium control; black circles: APIT-treated samples; mean absorbance of eight replicates $\pm$ S.D. (b) Jurkat cells were treated with ink and PI uptake was measured. (c) Influence of the caspase inhibitor zVAD-fmk on APIT-induced cell death or apoptosis. Jurkat cells were either treated for 6 h with ink or APIT or with the apoptosis inducers doxorubicin or H₂O₂ at 62.5 μ M (H₂O₂ low), as indicated in the absence (upper panel) or presence of zVAD-fmk (lower panel). Arrowheads point to apoptotic cells. Note that apoptosis but not the APIT-induced cell death is blocked by zVAD-fmk. (d) Lack of apoptotic DNA fragmentation in ink-treated cells. Jurkat cells were incubated in medium (control) or treated with cycloheximide (chx) or ink (ink) for 2, 4, and 6 h. Isolated DNA was visualized on a 1.6% agarose gel by ethidium bromide staining. (e) DNA-strand breaks in APIT- and apoptosis-induced cell death. Jurkat cells were either left untreated (upper panel), treated with APIT (middle panel) or cycloheximide (chx, lower panel); m = marker. Apoptotic and APIT-treated cells were both TUNEL positive, but only the nuclei of apoptotic cells fragmented into apoptotic bodies

Figure 4 (a) Proteome analysis of Jurkat cells cultured for 8 h in the presence (ink) or absence (medium) of ink. The pI of Prx I in control (8.3) and ink-treated cells (7.7), as well as the mass of 22.1 kDa is indicated. (b) Catalase inhibits ink-induced cell death. Jurkat T cells were incubated for 8 h with ink in the presence (black bars) or absence (white bars) of catalase. Cytotoxicity was measured as PI uptake (mean of triplicates $\pm$ S.D.). (c) Catalase protects from APIT-induced loss of metabolic activity. Metabolic activity of Jurkat cells was measured after incubation with APIT or anti-CD95 for 3 h in the presence (black bars) or absence (white bars) of catalase (mean of five replicates $\pm$ S.D.). (d) Phenotype of ink and APIT-induced cell death is mediated by H₂O₂. Jurkat cells were cultured for 7 h in medium, ink, APIT, or H₂O₂ (500 μM; high) in the absence (upper panel) or presence of catalase (lower panel). Cell morphology was analyzed by bright field microscopy. (e) APIT generates H₂O₂ in cell culture medium. Jurkat cell suspension (filled) or medium alone (open) was incubated with APIT (circles) or 200 μM H₂O₂ (triangles). H₂O₂ in the supernatants was measured quantitatively at the indicated time points (means of three independent experiments $\pm$ S.D.).

apoptosis.^{24,35,36} Our finding of a specific involvement of Prx I in a pathway counteracting the toxic activity of H₂O₂ is, to our best knowledge, the first report on a specific function of Prx I demonstrated in a loss of function experiment.

Why do amino-acid oxidases of *Aplysia* exhibit tumor specific toxicity? From many studies, it is known that tumor cells have an increased rate of metabolism compared to normal cells. A result of this high metabolic rate is a high concentration of ROS that originate from oxidative phosphorylation reactions by the electron transport chain of the mitochondria. As a consequence, ROS detoxification reactions are increased in tumor cells, and interference with detoxification has a selective toxic effect on tumor cells but not on normal cells.¹¹ Likewise, increasing the concentration of ROS at a certain level might overcome the detoxification reactions and kill the tumor cell. The same level of extra ROS produced by exogenous APIT might not affect normal cells because of their higher tolerance for additional ROS.

In summary, we have shown that the tumor lytic activity of the ink from *A. punctata* is mediated by the ink toxin APIT via the enzymatic production of H₂O₂. APIT-induced tumor cell death is apoptosis independent and characterized by early membrane permeabilization. We identified the modification of Prx I as an important step in the APIT-mediated death pathway, which according to literature reflects an oxidation and inactivation of this detoxification system. The fact that the knockdown of Prx I expression by RNAi increased the sensitivity of tumor cells for the cytolytic activity of APIT underlines the impact of this event. In addition, APIT might lead to inactivation of caspases and other molecules necessary for the integrity and metabolism of the cells. The knowledge about the mode of action of this interesting class of antitumor enzymes may allow for the design of novel anticancer therapies.

Materials and Methods

Animals

A. punctata were gained from the Station Biologique de Roscoff, France. Crude ink was prepared by gentle squeezing the sea hares in sterile seawater. Insoluble particles were removed by ultracentrifugation (82 000 g, 30 min, 4°C) and supernatants were stored at -70°C.

Cells, media, and reagents

Jurkat, HeLa, and K562 cells were obtained from ATCC. HUVEC were prepared as described before.³⁷ Mock- and Bcl-2-transfected Jurkat cells (Jurkat neo, Jurkat Bcl-2) were a kind gift of Dr. K Tomaselli (Idun Pharmaceuticals, La Jolla, USA), mock- and Bcl-X_L-transfected CEM cells (CEM neo, CEM BCL-X_L) were kindly provided by Dr. M Peter (University of Chicago, USA). Cells were cultured in RPMI medium containing 10% FCS (Invitrogen), 100 U/ml penicillin, 100 µg/ml streptomycin. PBMCs were isolated from the heparin-treated blood of healthy donors by density centrifugation. In brief, blood was diluted 1:2 with PBS w/o Ca²⁺ and Mg²⁺, overlaid on Ficoll/Isopaque ($P=1.077$ g/ml, Seromed) and centrifuged at 600 × *g* and 20°C for 25 min. The mononuclear cells of the interphase were harvested and washed three times. In addition, the following reagents were used: horseradish peroxidase and catalase from Roche; ABTS 2,2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid),

Figure 5 (a) Quantification of the mRNAs of Lamin A/C and Prx I after transfection of specific siRNA (open bars) and control Luciferase siRNA (black bars) with quantitative realtime PCR. The relative mRNAs levels compared to the mRNA of GAPDH measured in the same RNA preparation are shown. (b) Sensitization of Jurkat cells by knockdown of Prx I. Specific siRNAs directed against the mRNA of Luciferase (Luc, transfection control), Lamin A/C (control knock down), and Prx I were transfected in HeLa cells and the metabolic activity of transfectants treated in the presence (black bars) or absence of APIT (open bars) was measured. Note that the knockdown of Prx I but not of the other genes sensitized cells for the cytotoxic activity of APIT

doxorubicin, and H₂O₂ from Sigma; anti-CD95 antibody clone CH11 from Immunotech, *N*-benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (zVAD-fmk) from Bachem.

Purification of APIT

Crude ink was filtered and concentrated using Ultrafree-15 Units (exclusion weight 30 kDa) followed by three washing steps with 20 mM Tris HCl (pH 8.2). The concentrate (20–60-fold) was applied to a Source Q15 anion exchange column (Ø 10 mm, length 40 mm) equilibrated with 20 mM Tris HCl, pH 8.2. Proteins were eluted using a linear gradient from 0 to 800 mM NaCl. The fractions containing cytotoxic activity were pooled, concentrated and loaded onto a Superose 12 HR 10/30 column

(Pharmacia). Proteins were eluted with 100 mM potassium phosphate buffer (pH 7.2) at a flow rate of 0.4 ml/min. The purity of the fractions was determined by SDS-PAGE and silver staining.

2-DE

Jurkat cells were treated with or without ink for 8 h and analyzed by 2-DE as described.³⁸ Prx I protein was identified by peptide mass fingerprinting analysis after trypsin digestion using the MS-Fit software (<http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm>) as previously described.³⁹

Vitality, toxicity, and apoptosis assays

Jurkat cells suspended at 50 000 cells in 100 μ l cell culture medium in 96-well plates were treated with ink or APIT. Cell vitality was determined as metabolic activity by the turnover of WST-1 (Roche), a substrate of the mitochondrial dehydrogenase, to red formazan. Absorbance of the cell suspension was measured photometrically at 450 nm (690 nm reference). Toxicity was determined by quantifying PI (1 μ g/ml in PBS) uptake by flow cytometry. Cytolytic activity was determined furthermore via the release of cytoplasmic LDH in the supernatant. Release of LDH is found only upon membrane permeabilization by APIT. After APIT treatment, half of the culture supernatants (50 μ l) were transferred in fresh wells and mixed with 50 μ l reagent of Cytotoxicity Detection Kit-LDH, according to the manufacturer's instruction (Roche 1644793). LDH release was measured photometrically at 490 nm (690 nm reference) and calculated as the ratio of LDH activity of APIT-treated cells relative to the LDH activity of Triton X-100 lysed cells. Fragmented DNA of apoptotic cells was analyzed according to Herrmann et al.⁴⁰ For detection of DNA-strand breaks, cells were treated for 4 h with APIT, cycloheximide (chx), or medium alone. Subsequently, the 'DeadEnd™ Fluorimetric TUNEL-Assay' was used according to the manufacturer's instruction (Promega, Technical bulletin No. 235). Catalase was prepared according to Dahlgren and Karlsson⁴¹ and used at a concentration of 2000 U/ml. Generally, ink was diluted 1/200 and APIT was used at a concentration of 10 or 40 ng/ml for the treatment of Jurkat or HeLa cells, respectively. The working concentrations of chx was 10 μ M, of zVAD-fmk 50 μ M, and of doxorubicin 10 μ M.

Enzymatic assays

The production of H₂O₂ by APIT was determined via its turnover by horseradish peroxidase. Native ink or APIT were incubated with L-lysine (1 mM) in 100 μ l of 100 mM potassium phosphate buffer, pH 7.2 for 10 min at 25°C. The reaction was stopped by adding 1 μ l of 10 M phosphoric acid. To 25 μ l of this solution 1 mM ABTS (2,2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and 1 U horseradish peroxidase was added in 225 μ l of 100 mM potassium phosphate buffer, pH 5.0. Absorption was measured photometrically at 405 nm (reference 690 nm). The H₂O₂ concentrations in cell cultures were determined by removing 100 μ l aliquots of the supernatant at the indicated time points and treatment with 40 μ l of 20 mM N-ethylmaleimide in 200 mM sodium acetate, pH 5.5 for 10 min at room temperature to block interfering thiols. Horseradish peroxidase reaction of the supernatant and H₂O₂ standards was performed as described above.

Validation of RNAi by realtime PCR

A total of 20 000 HeLa cells/well were seeded in a 96-well plate one day prior to transfection. Transfection was performed with 0.25 μ g siRNA directed against Prx I (AAGGCGAUGAAGGCAUCUCGDTdT), Lamin A/

C (CUGGACUCCAGAAGAACAAdTdT), and Luciferase (CUUACGCU-GAGUACUUCGAdTdT) as control and 2 μ l transmessenger per well using the transmessenger transfection kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. For the APIT treatment, transfections were conducted in triplicates. At 24 h after transfection, cells were splitted and grown for additional 48 h before fresh medium with or without APIT was added for 6 h. Assay conditions, which led to a 50–70% reduction of the metabolic activity of treated cells, were chosen for RNAi experiments. In parallel, RNA from about 50 000 cells was isolated using the RNeasy[®] 96 BioRobot[®] 8000 system (Qiagen) 48 h after transfection. The relative amount of mRNA was determined by realtime PCR using Quantitect[™] SYBR[®] Green RT-PCR Kit from Qiagen following manufacturer's instructions. The expression level of Prx mRNA was normalized against the internal standard GAPDH. The following primers were used: Prx I 5': CTGTTATGCCAGATGGTCAG, Prx I 3': GATACCAAAGGAATGTT-CATG, Lamin A/C 5': CAAGAAGGAGGGTGACCTGA, Lamin A/C 3': GCATCTCATCCTGAAGTTGCTT, GAPDH 5': GGTATCGTGAAG-GACTCATGAC, GAPDH 3': ATGCCAGTGAGCTTCCCGTTACG.

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