ORIGINAL PAPER

Apoptosis induction in human breast cancer (MCF-7) cells by a novel venom L-amino acid oxidase (Rusvinoxidase) is independent of its enzymatic activity and is accompanied by caspase-7 activation and reactive oxygen species production

[3]. This is achieved via two major pathwaysÑthe extrinsic pathway that occurs through death receptors present in the outer membrane of the cell, and the intrinsic pathway, which is a mitochondria-dependent pathway [3]. Any critical defect in the apoptotic signaling pathways may result in uncontrolled proliferation and growth of cells which may ultimately lead to cancer, and the use of chemotherapeutic agents to induce apoptosis in cancer is one of the effective ways to overcome this deadly disease. However, despite the development of new therapies, acquired multidrug drug resistance in cancer cells has become one of the major impediments against successful treatment. Therefore, new anticancer drugs capable of targeting cancer through multiple mechanisms can provide a signiÞcant therapeutic advantage.

Snake venoms show promise in the treatment of several diseases, including cancer [4, 5]. Among the different components, L-amino acid oxidase (LAAO, E.C.1.4.3.2) is a well-studied, important component of snake venom which inhibits growth of mammalian cancer cells by induction of apoptosis and inhibition of angiogenesis, suggesting its potential as a lead compound for anticancer drug development [5, 6]. However, there is signiÞcant controversy regarding the anticancer mechanism(s) of snake venom LAAO; it has been shown that the cytotoxic

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returned to 37 C for an additional 24 h. The cells treated with only growth medium served as a control. The adherent cells were harvested by trypsinization and combined with non-adherent cells, washed in PBS and DNA was prepared from the pelleted cells following the procedure described by Herrmann et al. [17]. For the quantitative DNA fragmentation assay, the cells were cultured and treated with Rusvinoxidase as above. Following lysis of cells, the lysate was centrifuged at 11,400 g for 10 min to separate the fragmented DNA (supernatant) from the intact chromatin (pellet). Both fractions were treated with 1.0 ml of 0.5 M trichloroacetic acid (TCA) overnight at 4 C. The next day, both mixtures were centrifuged and the pelleted DNA was treated with 160 µl of 5 % TCA at 90 C for 15 min [18]. The DNA content of both fractions was estimated at

demonstrated that this protein has signiÞcant similarity with LAAOs isolated from other snake venoms, especially with those isolated from other viperid venoms (Table 2).

Biochemical characterization of Rusvinoxidase

Rusvinoxidase was yellow in color due to presence of ßavin adenine dinucleotide, and it exhibited LAAO speciÞc activity of 23.8 U/mg protein. The LAAO activity diminished progressively after storage at 4 C yet cytotoxic activity of Rusvinoxidase was not affected (supplementary Fig S3). The LAAO enzyme activity of Rusvinoxidase following two freeze and thaw cycles was completely abolished and after six cycles of freeze and thaw, the enzyme activity could not be regained after incubation for 24 h at 37 C; again, its cytotoxic property remained unaffected. Rusvinoxidase did not show protease (azocaseinolytic, Þbrinolytic, Þbrinogenolytic), phospholipase A_2, TAME- or BAEE-esterase activities.

Rusvinoxidase signiÞcantly inhibits proliferation of MCF-7 breast cancer cells

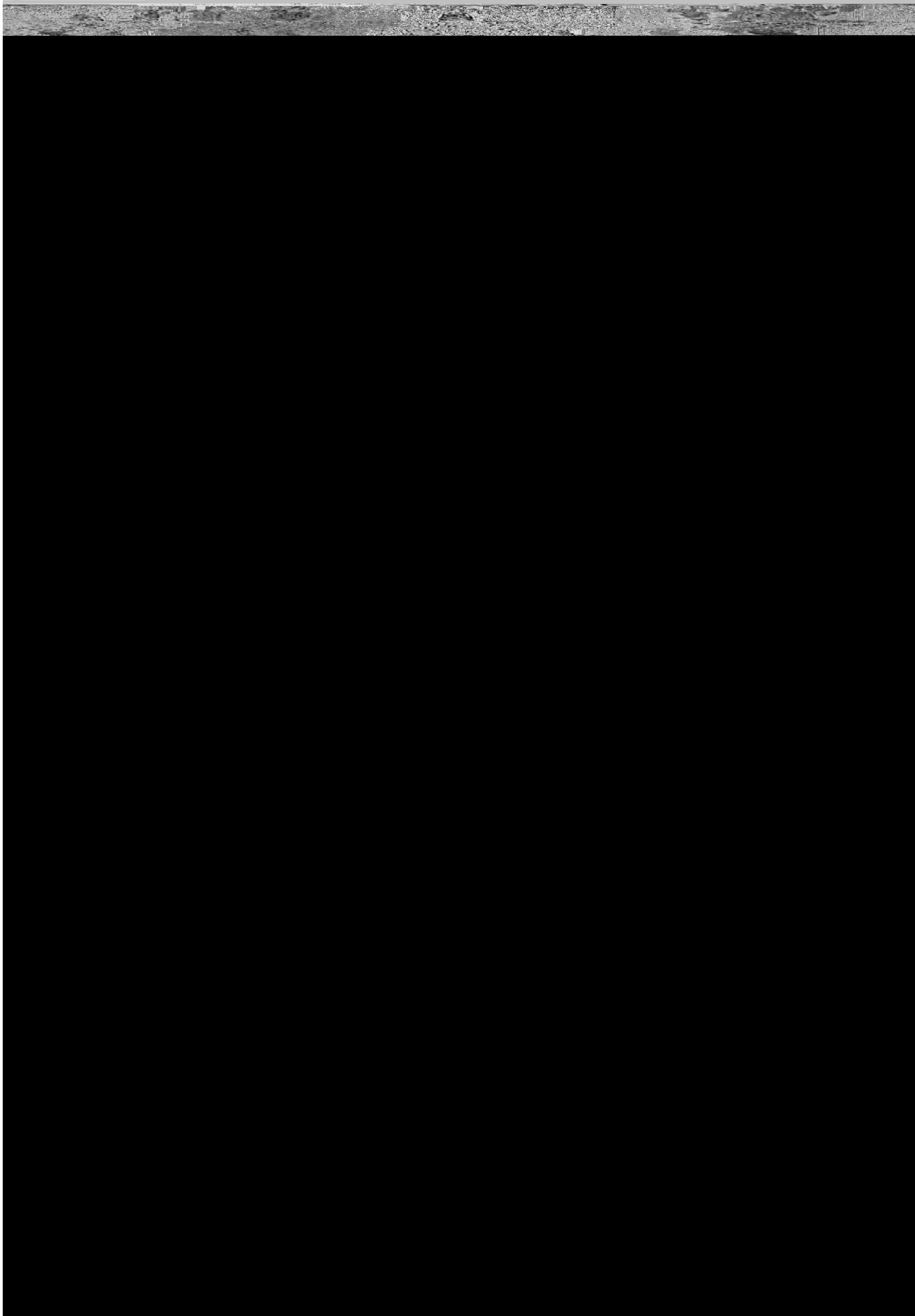
Rusvinoxidase demonstrated signiÞcantly higher ($p < 0.05$) dose-dependent cytotoxic activity toward MCF-7 cells compared with the commercial anticancer drug cytosine β-D-arabinofuranoside (AraC), an antitumor agent which selectively inhibits DNA synthesis (Fig. 2). From regression analysis, the IC_{50} value of Rusvinoxidase towards MCF-7 cells, after 24 h incubation, was 5.5 µg/ml (83 nM). After 24 h of treatment with Rusvinoxidase at a dose of 10 µg/ml (* 2 9 IC_{50}), MCF-7 cell viability was zero (Fig. 2). Following a 24 h treatment with Rusvinoxi9000244a

membrane integrity, and shrinkage of cells; apoptotic body formation was detected in a dose-and time-dependent manner (Fig. 3a). Twenty-four hour incubation of MCF-7 cells with 2 9 IC_{50} of Rusvinoxidase resulted in pronounced apoptosis, and apoptotic cells had undergone secondary necrosis (Fig. 3a). The changes in nuclear morphology of treated breast cancer cells were evident from chromatin condensation and formation of apoptotic cells (Fig. 3a). Further, using Hoechst 33258 staining, dose- and time-dependent apoptosis induction in MCF-7 cells by Rusvinoxidase was observed (Fig. 3b). In addition to being dependent on Rusvinoxidase concentration, percent apoptosis of MCF-7 cells induced by Rusvinoxidase increased with exposure time (Fig. 3c).

Incubation of MCF-7 cells with Rusvinoxidase resulted in an increase in DNA fragmentation of cancer cells as compared to controls (Fig. 4a). This result confirmed that apoptosis was accompanied by DNA fragmentation in MCF-7 cells after exposure to Rusvinoxidase. Using the APO-BrdUTuNEL assay, a routine method to quantify the extent of apoptosis induction by anticancer agents, Rusvinoxidase was found to induce apoptosis (DNA fragmentation) dose-dependently in treated cancer cells as compared with control cells (Fig. 4b).

Rusvinoxidase induces apoptosis in MCF-7 cells via activation of caspases-8, 9 and 7

Increase or decrease in caspase-9, caspase-8, caspase-7 and caspase-3 expression as compared to control (untreated) cells was determined by release of chromophore/fluorophore from their respective chromogenic/fluorogenic substrates by cell lysates of Rusvinoxidase-treated (at 1 9 IC_{50} dose) MCF-7 cells. Caspase-9 activity of MCF-7 cells marginally increased after 60 min of treatment with Rusvinoxidase, and peak activity for caspase-8 and caspase-9 was attained 1 and 3 h after treatment, respectively (Fig. 5). Thereafter, a gradual decrease in the level of these enzymes was observed; however, the caspase-9 activity in Rusvinoxidase-treated MCF-7 cells was significantly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b Fig. 3 a Dose- and time-dependent morphological changes induced by Rusvinoxidase in MCF-7 cells. Light micrographs were obtained after ethidium bromide-acridine orange staining (9 60 magniÞcation). b Dose- and time-dependent nuclear changes induced by Rusvinoxidase in MCF-7 cells. The cells were observed under a light microscope after Hoeschst 33258 staining (9 60 magniÞcation). The white arrows indicate cells with membrane blebbing and shrunken nuclei, the black (solid) arrows show chromatin condensation, and black dashed arrows indicate secondary cellular necrosis. c Quantitation of dose and time-dependent apoptosis induction in MCF-7 cells by Rusvinoxidase. The percentage of apoptotic cells (control as well as Rusvinoxidase-treated) after Hoeschst 33258 staining was counted from four random microscopic Þelds at 9 60 magniÞcation for each treatment. Data represent mean ± SD of three determinations. SigniÞcant differences with respect to controls are indicated by different letters *p \ 0.05; **p \ 0.01

higher than the baseline value (untreated MCF-7 cells) after 24 h of treatment (Fig. 5). Treatment of MCF-7 cells with Rusvinoxidase resulted in a time-dependent decrease in the level of caspase-3 compared to the same enzyme activity displayed by control (untreated) MCF-7cells (Fig. 5). Conversely, extracts of MCF-7 cells treated with

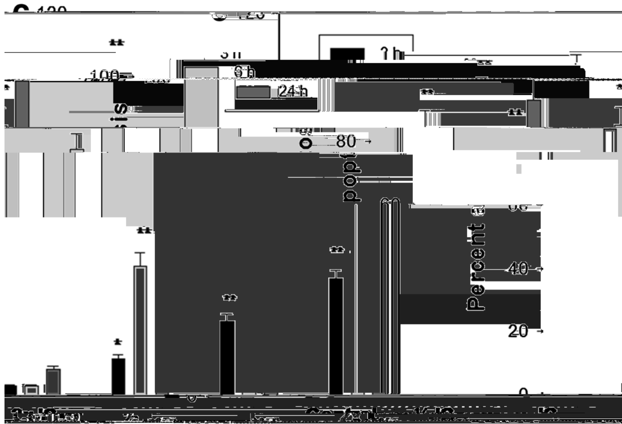


Fig. 3 continued

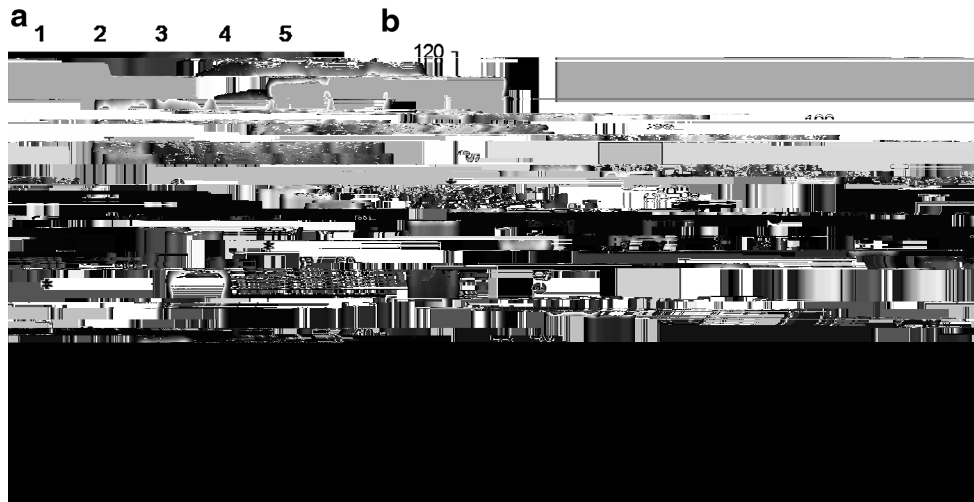
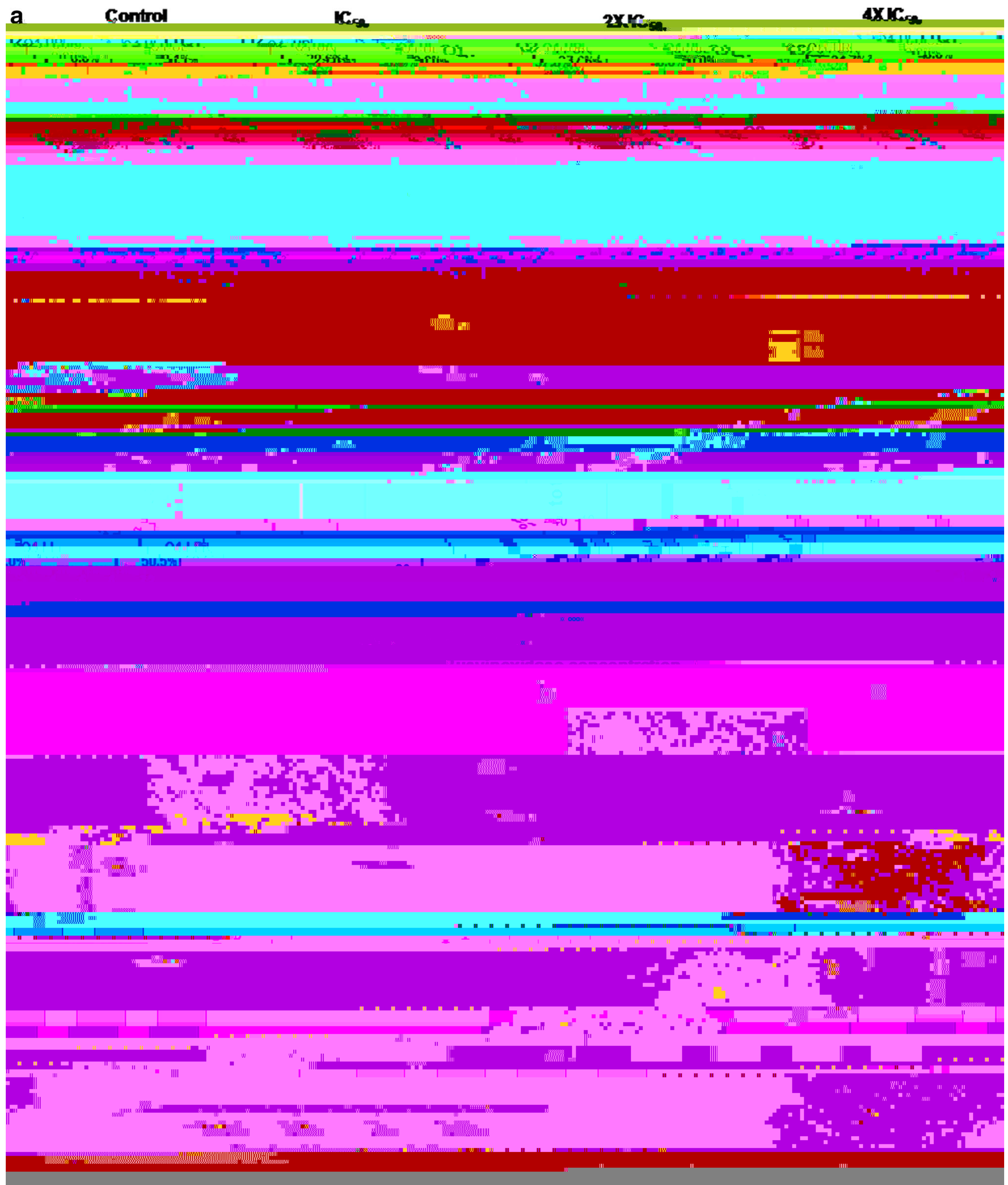


Fig. 4 a DNA fragmentation analysis in Rusvinoxidase-treated MCF-7 cells. The MCF-7 cells (1 9 10^6 cells/well) were incubated with an IC_{50} dose of Rusvinoxidase (83 nM) or growth medium (control) for 6 or 24 h at 37 C in a humidiÞed CO_2 incubator. After exposure, DNA fragmentation was analyzed by agarose gel electrophoresis. Lane 1 1 kb DNA ladder, lane 2 DNA from control MCF-7 cells (6 h); lane 3 DNA from Rusvinoxidase-treated MCF-7 cells (6 h); lane 4 DNA from control MCF-7 cells (24 h); lane 5 DNA from Rusvinoxidase-treated MCF-7 cells (24 h). b Analysis of apoptosis (DNA fragmentation) by APO-BrdUTuNEL assay. MCF-7 cells (1 9 10^6 cells/well) were plated in a 24-well plate and incubated with different doses (½ IC_{50}, IC_{50}, and 2

Rusvinoxidase exhibited a time-dependent increase in caspase-7 activity after 3 h of treatment and the peak value was observed 6 h post treatment (Fig. 5). Thereafter, a progressive decrease in caspase-7 activity was observed, although its activity remained significantly higher ($p < 0.05$) in Rusvinoxidase-treated MCF-7 cells compared to control (untreated MCF-7) cells 24 h after treatment (Fig. 5).

Effect of Rusvinoxidase on the level of cellular glutathione and catalase activity in MCF-7 cells

Rusvinoxidase at its IC_{50} value depleted the cellular glutathione (GSH) of treated-MCF-7 cells in a time-dependent manner as compared to baseline values (considered as 100 %) shown by control MCF-7 cells (Fig. 6). Total glutathione levels in Rusvinoxidase-treated MCF-7 cells were lowest at 3 h post-treatment; however, glutathione levels progressively increased with increasing treatment time beyond 3 h (Fig. 6). At 18 h glutathione levels were ~at 85 % of baseline and they returned to baseline values 24 h after Rusvinoxidase-treatment (Fig. 6). Treatment of MCF-7 cells with Rusvinoxidase resulted in a similar time-dependent decrease in catalase activity of cell-free extract compared to the same activity of control cells (consid-


a
Control
2X IC50
4X IC50

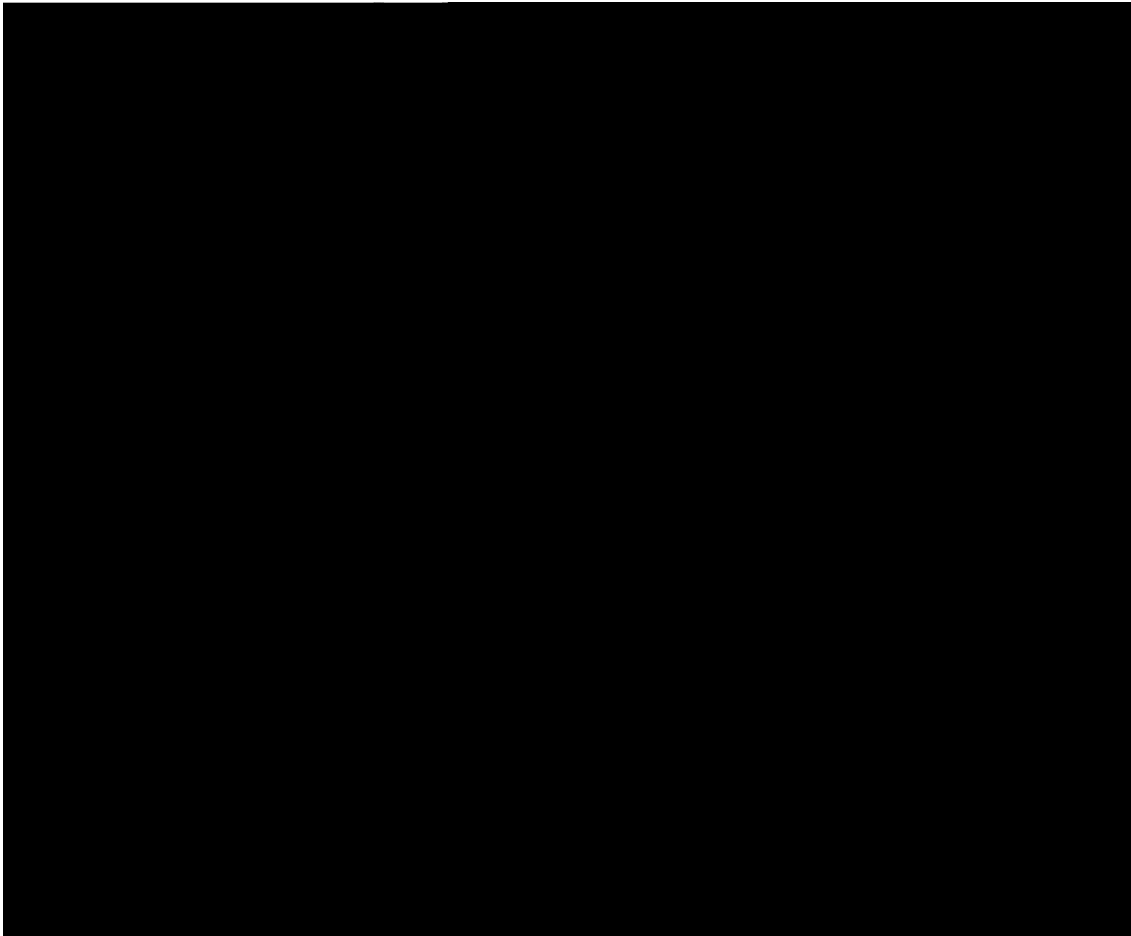


Fig. 8 a Time-dependent expression of pro-(Bax, Cyt C), and anti-apoptotic (Bcl-XL, Hsp90, Hsp70) proteins, and cytochrome c in Rusvinoxidase-treated (9 IC_{50}) MCF-7 cells. After treatment of the cells for the indicated time period, the 50 g of cell extracts were separated by 12 % SDS-PAGE and the expressions of proteins were detected by Western blot analysis using antibodies against the targeted proteins. b The expression of pro- and anti-apoptotic proteins was normalized to b-actin using ImageJ software and the Þgures show the mean ±

demonstrated that expression of the anti-apoptotic proteins HSP-70, HSP-90 and Bcl-XL decreased with time; conversely, an increase in the expression of the apoptotic protein Bax was observed (Fig. 8a, b). The release of cytochrome c from the mitochondria to the cytosol of MCF-7 cells was detected as early as 60 min after exposure to Rusvinoxidase but was most apparent by 6 and 24 h after treatment (Fig. 8a, b).

Rusvinoxidase does not show in vivo toxicity in a mouse model

Rusvinoxidase at 4.0 g/g body weight (i.p.) was not lethal to NSA mice nor did it induce any behavioral changes/adverse effects in treated animals.

Discussion

Snake venom LAAOs are thermolabile proteins; an exception to this rule was shown by an LAAO isolated from *Naja naja oxiana* venom [21] which retains enzymatic activity after repeated freezing and thawing. Repeated freezeÐthaw cycles led to a loss in enzymatic activity of Rusvinoxidase, but this did not compromise its cytotoxic properties, indicating that signiÞcant differences in substrate-speciÞc properties exist among various members of this family of venom enzymes. The molecular mass of Rusvinoxidase is lower than the molecular masses of LAAO isoenzymes (60Ð63 kDa) isolated from venom of *D. r. russelii* of eastern India origin [8]. This suggests geographic variation in LAAO enzymes occurs in RVVs.

mRNA of catalase, the major antioxidant defense systems
in cells, to down-regulate the excess ROS production [

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