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Research paper

Cellular mechanism of resistance of human colorectal adenocarcinoma cells against apoptosis-induction by Russell's Viper venom L-amino acid oxidase (Rusvinoxidase)

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ABSTRACT

The present study highlights the cellular mechanism of resistance in human adenocarcinoma (Colo-205) cells against apoptosis induction by Rusvinoxidase, an L-amino acid oxidase purified from Russell's Viper venom (RVV). The significantly lower cytotoxicity as well as apoptotic activity of Rusvinoxidase towards Colo-205 cells (compared to MCF-7 breast cancer cells) is correlated with lower depletion of cellular glutathione content and increased down-regulation of catalase activity of Colo-205 cells following Rusvinoxidase treatment. Exposure to Rusvinoxidase subsequently diminished reactive oxygen species (ROS) production and failed to impair mitochondrial membrane potential, resulting in apoptosis induction resistance in Colo-205 cells. Further, higher expression levels of caspase 8, compared to caspase 9, indicate that Rusvinoxidase preferentially triggers the extrinsic pathway of apoptosis in Colo-205 cells. A time-dependent lower ratio of the relative expression of Bax and Bcl-xL (pro- and anti-apoptotic proteins) in Colo-205 cells, compared to our previous study on MCF-7 cells, unambiguously supports a higher cellular resistance mechanism in Colo-205 cells against Rusvinoxidase-induced apoptosis.

1. Introduction

The control of cell proliferation, differentiation and development includes a strongly regulated apoptotic (programmed cell death) mechanism that is characterized by chromatin condensation, DNA fragmentation and the activation of cellular caspases [1,2]. Apoptosis is achieved via two major pathways: (a) the extrinsic pathway, occurring through cell surface death receptors (e.g., Fas/APO-1), and (b) the intrinsic pathway, which is a mitochondria-dependent apoptotic pathway. Any defect in the apoptotic process results in uncontrolled cell proliferation and growth, ultimately leading to cancer. Therefore, chemotherapeutic agents that induce apoptosis represent an effective approach to fight cancers. However, despite the development of new therapies against cancers, acquired multi-drug resistance in cancer cells has become one of the major impediments against successful treatment.

Among the pharmacologically active proteins and polypeptides of snake venom, L-amino acid oxidases (LAAOs, E.C.1.4.3.2) are well studied, important components of snake venom that can inhibit growth of mammalian cancer cells by induction of apoptosis and inhibition of angiogenesis, suggesting their potential as novel anticancer therapeutics [3]. Studies from our laboratory have shown that a non-toxic LAAO (Rusvinoxidase) purified from venom of *Daboia russelii* induces apoptosis in MCF-7 cells by both the extrinsic (death-receptor) and intrinsic (mitochondrial) signaling pathways, though the latter pathway predominates [1]. However, Rusvinoxidase demonstrated significantly less cytotoxicity toward colorectal adenocarcinoma (Colo-205) cells under identical experimental conditions. Colon cancer is responsible for a large number of cancer deaths worldwide and is the second leading cause of cancer in the United States. Therefore, in the present study, we investigated the cellular mechanism of resistance in Colo-205 cells

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against apoptosis-induction by Rusvinoxidase.

2. Materials and methods

2.1. Cell proliferation assay

The cytotoxic L-amino acid oxidase (Rusvinoxidase) was purified from the venom of *Daboia russelii* as described previously [1]. Human colorectal adenocarcinoma cells (Colo-205; ATCC CCL-222) were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, and maintained in 75 cm^2 flasks. All cell assays described below were conducted at 37 °C in a humidified 5% CO_2 incubator. Sub-cultivation was performed according to ATCC instruction using trypsin-EDTA (0.05% trypsin and 0.02% EDTA). For *in vitro* cytotoxicity assays, 100 μl cell aliquots, at a concentration of 1×10^5 cells/ml in complete growth media, were plated in a 96-well cell culture plate and incubated with various dilutions of Rusvinoxidase (0–80.0 μg/ml) for 24 h at 37 °C. Inhibition of cell proliferation post-addition of Rusvinoxidase was assayed by a MTT [3-(4, 5-dimethylthiazolyl-2)-2, 5- diphenyl-2H-tetrazolium bromide]-based method [1,2,4]. Different concentrations of the anticancer drug cytosine-β-D-arabinofuranoside hydrochloride (Sigma) were added to separate wells as a positive control. Cells treated with growth medium only was the negative control, and medium without cells was used for blanks. Effects of each treatment were compared with values obtained from a standard curve of control Colo-205 cells. In another set of experiments, 100 μl of 1×10^6 cells/ml were treated with graded concentrations of Rusvinoxidase (2.5–20 μg/ml) at 37 °C for 24 h, and the cells were then photographed under a phase-contrast microscope (Olympus CKX41).

2.2. Cellular and nuclear morphological changes

To study morphological changes induced by Rusvinoxidase, 5×10^5 cells in growth medium were seeded in 24 well plates and allowed to adhere overnight at 37 °C. The next day, the medium was replaced with fresh medium, and cells were treated with different doses of Rusvinoxidase (0–80.0 μg/ml) or the anticancer drug cytosine-β-D-arabinofuranoside-HCl (positive control) for different time periods (0–24 h). Following treatment, both the floating and attached cells (following trypsinization) were collected. The control cells were treated with 1× PBS. Cells were washed with PBS, re-suspended in culture medium and then stained by adding 5 μl each of ethidium bromide and acridine orange (each at 10 mg/ml in PBS). After 15 min of incubation at 37 °C, the cells were washed twice with PBS and then observed and photographed under 400× magnification using an Olympus D21 camera attached to an Olympus CKX41 inverted microscope.

Rusvinoxidase induced nuclear damage in Colo-205 was observed via Hoechst 33258 (10 mg/ml) staining [1]. The percentage of apoptotic cells was counted from four randomly selected microscopic fields at 400× magnification.

2.3. Rusvinoxidase-induced DNA fragmentation assay

For the qualitative DNA fragmentation assay, Colo-205 cells were plated at a density of 1×10^6 cells/well and allowed to adhere overnight. The next day, medium was replaced with fresh medium containing different doses of Rusvinoxidase (½ IC_{50}, IC_{50}, 2 × IC_{50}) or 1× PBS (negative control) and incubated for an additional 24 h at 37 °C. The attached cells were harvested by trypsinization, combined with floating cells, and washed with PBS, and the quantitative DNA fragmentation assay was performed as described previously [1]. Briefly, after lysing the cells with RIPA buffer, 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, then both mixtures were centrifuged at 10,000 × g for 10 min, and the pelleted DNA was treated with 160 μl of 5% TCA at 90 °C for 15 min [5]. The DNA content of both fractions was estimated at 260 nm using a NanoDrop 2000 spectrophotometer. The percent fragmentation was determined by the following equation:

$$\text{Percent DNA fragmentation} = \frac{\text{DNA in the supernatant}}{\text{Total DNA recovered in the supernatant and pellet}} \times 100$$

2.4. Assessment of total reduced glutathione and catalase activity

Total reduced glutathione was estimated as described by Zargan et al. [6]. Briefly, Colo-205 cells were seeded in a 24-well plate at a density of 1×10^6 cells/well and then allowed to adhere to the plate overnight at 37 °C, 5% CO_2. Medium was then replaced with new medium containing an IC_{50} dose (9.8 μg/ml, 170 nM) of Rusvinoxidase or growth medium alone (control) and then incubated for an additional 0–24 h. After a defined time interval, both the floating and adhered cells were collected and a cell lysate was prepared as described under qualitative DNA fragmentation analysis [1]. The cell lysates were sonicated for 10 min, centrifuged at 10,000 g for 10 min and then protein content of the supernatant was determined by Bio-Rad Protein Assay Dye Reagent. To an equal volume of supernatant, 10% (w/v) ice-cold TCA was added and the mixture was allowed to stand on ice for 20 min to precipitate protein. After centrifuging the mixture at 10,000 g for 10 min, the resulting supernatant was collected. The supernatant (20 μl) was mixed with 75 μl lysis buffer, 55 μl Tris buffer (pH 8.5) containing 0.02 M EDTA and 25 μl of 20 mM DTNB [5,5-dithio bis (2-N benzoic acid)] and the absorbance of the supernatant was read at 412 nm against a blank containing TCA instead of a sample. Total glutathione content was expressed as μg GSH/mg protein using a molar extinction coefficient of 13600 [6]. Catalase activity was assayed as described previously [1]. Results were expressed as micromoles of hydrogen peroxide consumed/min/mg protein, based on the molar extinction of hydrogen peroxide as 43.6 at 240 nm.

2.5. Assay for caspase activity

For caspase activity assay, 1×10^6 Colo-205 cells were treated with one IC_{50} dose (9.8 μg/ml; 170 nM) of Rusvinoxidase or growth medium (control) for various time periods (0–24 h), and at the end of treatment, cells were lysed with RIPA lysis buffer. The cell lysate was centrifuged at 10,000 g for 10 min at 4 °C and the protein content of the supernatant was determined. Ten microliters of the supernatant was mixed with 1 mM chromogenic substrate (50 μl) for caspase-3 (Ac-Asp-Met-Gln-Asp-pNA) or caspase-9 (Ac-Leu-Glu-His-Asp-pNA) and the total volume of reaction mixture was adjusted to 375 μl with 100 mM HEPES (pH 8.0) containing 100 mM NaCl [7]. After incubation

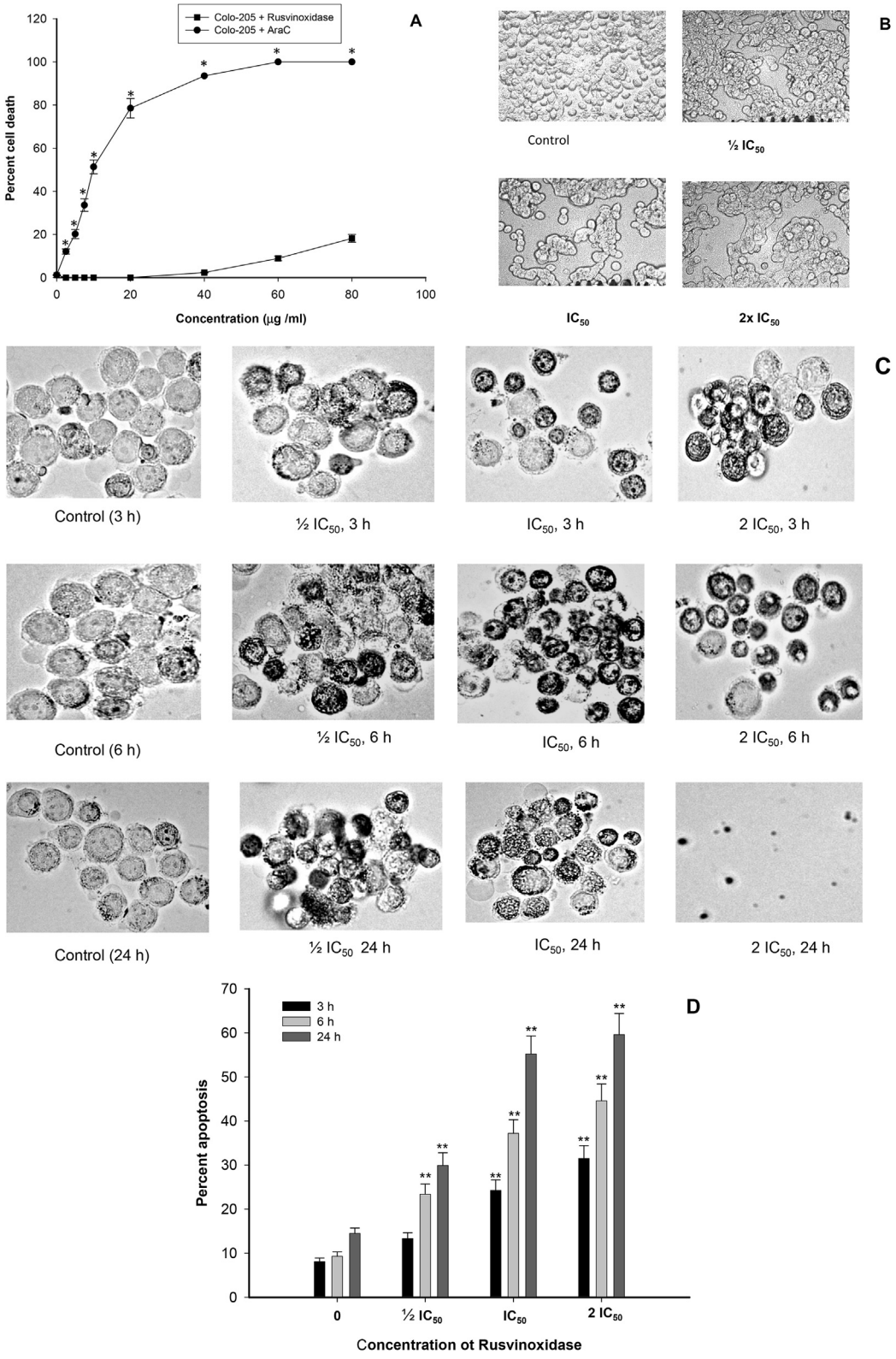

Fig. 1. A. Dose-dependent cytotoxicity of Rusvinoxidase and cytosine-β-D-arabinofuranoside hydrochloride (AraC) towards Colo-205 cells (1×10^5 cells/ml) after 24 h incubation at 37 °C, 5% CO_2. Data represent means ± standard deviation (SD) of three determinations. Significance of difference with respect to AraC under identical experimental conditions: $^*p < 0.01$. **B.** Phase-contrast microscopic observation of Colo-205 cells treated with different concentrations of Rusvinoxidase for 24 h at 37 °C. **C**. Concentration- and time-dependent nuclear morphological changes induced by Rusvinoxidase in Colo-205 cells after Hoechst 33258 staining (400× magnification). **D.** Quantitation of dose- and time-dependent apoptosis induction in Colo-205 cells by Rusvinoxidase. The percentage of apoptotic cells (control as well as Rusvinoxidase-treated) after Hoechst 33258 staining was counted from four random microscopic fields at 60× magnification for each treatment. Data represent mean ± SD of three determinations. Significant differences with respect to controls: **, $p < 0.01$.

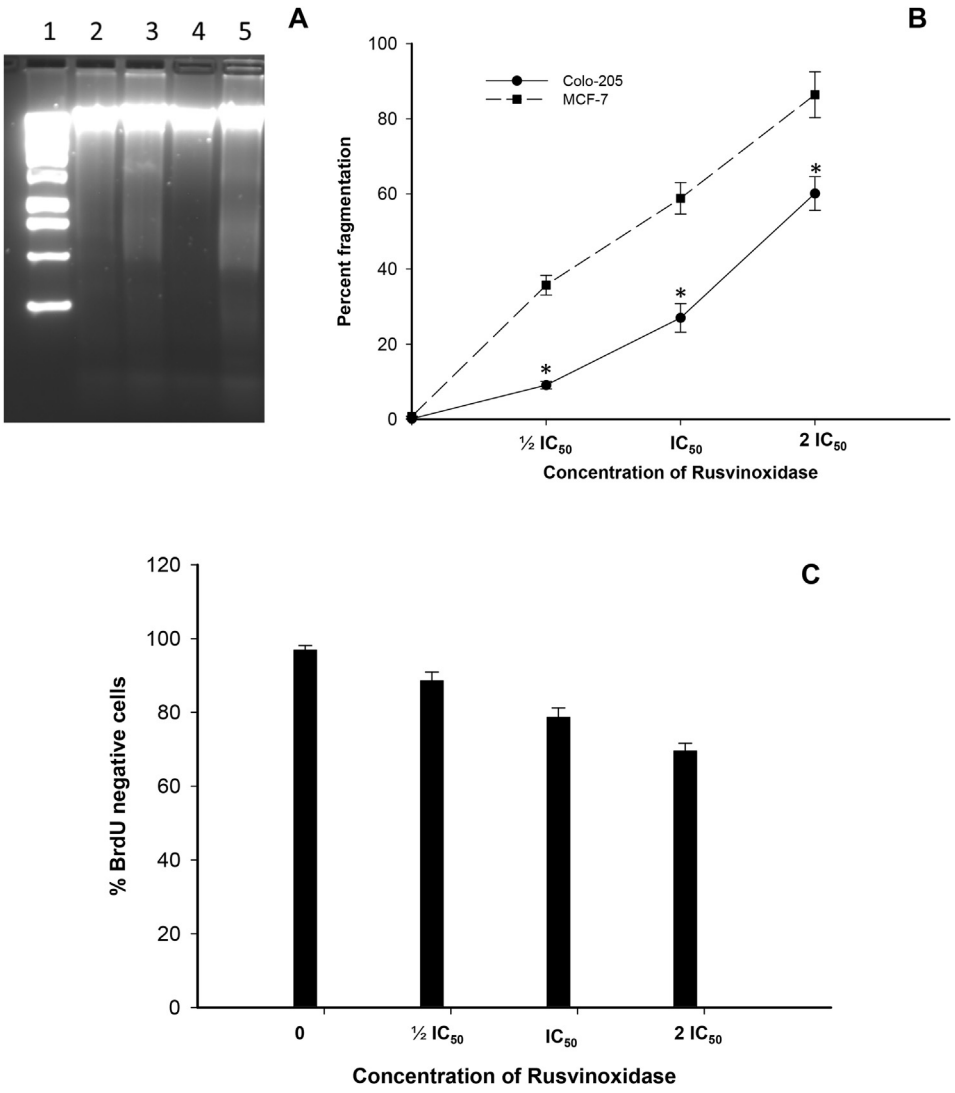

Fig. 2. **A** Dose-dependent DNA fragmentation analysis in Rusvinoxidase-treated (170 nM) Colo-205 cells. Lane 1, 1 kb DNA ladder; lane 2, DNA from control Colo-205 cells; lanes 3–5, DNA isolated from $1 \times IC_{50}$, $½\ IC_{50}$, and $2 \times IC_{50}$ doses of Rusvinoxidase-treated Colo-205 cells. **B**. A comparison of DNA fragmentation between Colo-205 and MCF-7 cells under identical treatment conditions. Values are mean ± SD of three determinations. Significance of difference with respect to MCF-7 cells: *, $p < 0.01$. **C**. Flow-cytometric analysis of by APO-BrdU TuNEL assay. Values are means ± SD of three determinations.

for 15 min at 37 °C, the reaction was terminated by addition of 75 μl of 50% (v/v) acetic acid and the liberation of 4-nitroaniline was determined at 405 nm against a reagent blank. The units of caspase activity (amidolytic activity) was defined as μmoles of 4-nitroaniline released per minute by the enzyme under the assay condition [7].

2.6. Assay for ROS generation and change in mitochondrial transmembrane potential

Intracellular reactive oxygen species levels generated upon Rusvinoxidase exposure to 1×10^6 cells were measured using the non-fluorescent compound 2′,7′-dichlorofluorescein-diacetate (DCFH-DA). The cells were incubated with different doses of Rusvinoxidase for 4 h and thereafter both the attached and floating cells were collected and washed twice with PBS, pH 7.4. The cells were then incubated with 10 μM DCFH-DA at 37 °C for 30 min in the dark followed by washing twice with chilled PBS. The fluorescence intensity of the fluorescent derivative 2′,7′-dichlorodihydrofluorescein (DCF) produced by intracellular reactive oxygen species was analyzed through a Flow Cytometer (BD AccuriTM C6 Cytometer) with excitation and emission at 480 nm and 530 nm, respectively [5].

The Rusvinoxidase-induced change in mitochondrial transmembrane potential (MMP) in Colo-205 cells was determined using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (MitoProbe™ JC-1 Assay kit, Sigma) as described previously [1]. After exposure of 1×10^6 cells to one IC_{50} dose of Rusvinoxidase for 4 h, the cells were treated with JC-1 or carbonyl cyanide m-chlorophenylhydrazone (CCCP; positive control) following the instructions of the manufacturer, and the fluorescence intensity was determined by flow cytometry using an excitation at 488 nm and emission at 525 nm. Data was presented as percentage of cells with altered MMP. Rusvinoxidase induced apoptosis on Colo-205 1×10^6 cells/well was determined by APO-BrdU™ TUNEL Assay Kit [1]. The samples were analyzed by flow cytometry within 3 h after staining with propidium iodide.

2.7. Immunoblot analysis of expression of pro- and anti-apoptotic proteins

For analyzing the expression of pro- and anti-apoptotic proteins,

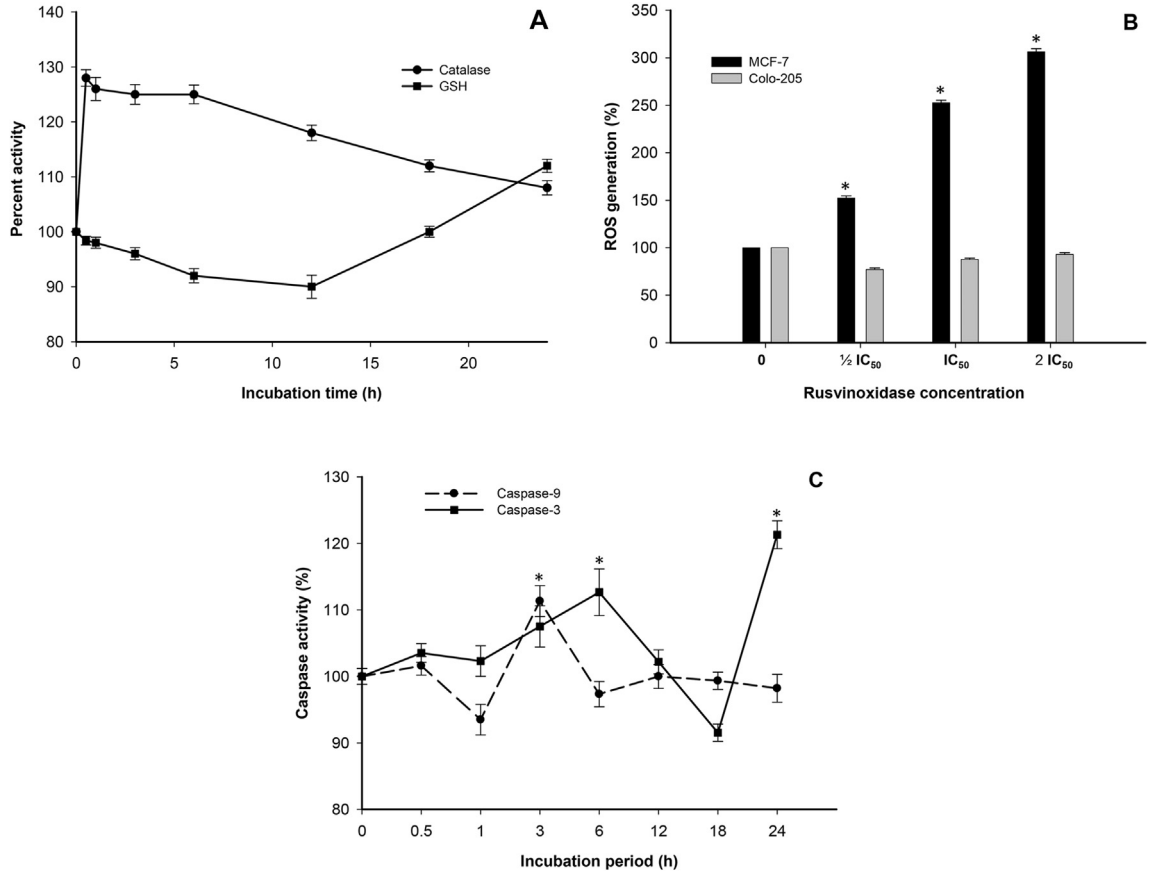

Fig. 3. A. Assessment of time-dependent depletion in GSH content and decrease in catalase activity of Colo-205 cells treated with 170 nM (IC_{50} dose) Rusvinoxidase. Data represent means ± SD of three determinations. Significant differences with respect to respective controls (100% activity) *, $p < 0.01$. **B.** A comparison of ROS production in Colo-205 and MCF-7 cells treated with different concentrations of Rusvinoxidase for 4 h at 37 °C. The ROS production by control cells was considered as 100% and other values were compared to that. Values are means ± SD of three determinations. Significance of difference with respect to Colo-205 cells: *, $p < 0.01$. **C**. Analysis of kinetics of activation of caspase-3 and caspase-9 in Colo-205 cells after Rusvinoxidase (IC_{50} value) treatment. The activity of cells treated with growth medium (control) at each time point was considered as baseline and other values were compared to that. Values are means ± SD of three determinations. Significance of difference compared to control: *, $p < 0.05$.

1×10^6 Colo-205 cells were incubated with Rusvinoxidase ($1 \times IC_{50}$ dose) or growth medium (control) for 0–24 h, and both floating and adhered cells were collected and washed with PBS, pH 7.4. Cells were lysed on ice in 200 μl of RIPA lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich, USA), centrifuged at 10,000 g for 20 min at 4 °C. The protein content of supernatant was estimated by the Bradford method [8] and 50 μg of protein from each sample was run in 12.5% NuPAGE Novex® Bis–Tris mini gels (Invitrogen). The proteins were electrophoretically transferred to a nitrocellulose membrane and nonspecific sites were blocked by incubating the membrane with 5% (W/V) dry skimmed milk in 20 mM TBSt (Tris buffered saline, pH 7.4) containing 0.1% Tween-20 (Sigma–Aldrich, St. Louis) for 1 h at room temperature. After washing the membrane with TBSt, it was incubated with primary antibodies (1:750 dilution) against Bcl-XL, Bax, caspase-8, cytochrome-c, or β-actin (internal control) for 1 h at room temperature and then overnight at 4 °C. The following day, the membrane was washed with TBSt and then incubated with horseradish peroxidase-conjugated mouse anti-rabbit secondary antibodies (1:3000) for 1 h at room temperature and the Western blot was developed with SuperSignalTM Western Blot Enhancer reagent (Fisher Scientific). The signal was detected with a CCD camera was analyzed through Quantity one 1-D analysis software (VersaDoc™ Imaging System, BioRad, USA).

Student's *t*-test using Sigma Plot 11.0 for Windows (version 10.0) was used to test the significance of differences between controls and experimental values. A p-value of ≤ 0.05 was considered statistically significant.

3. Results and discussion

3.1. Rusvinoxidase showed significantly less cytotoxicity towards Colo-205 cells compared to MCF-7 cells

The dose-dependent cytotoxicity of Rusvinoxidase, as well as cytosine-β-D-arabinofuranoside, toward Colo-205 cells is shown in Fig. 1A. A comparison with our previous work [1] showed that the cytotoxic effect of Rusvinoxidase was more pronounced in MCF-7 cells compared to Colo-205 cells; however, cytotoxicity of Rusvinoxidase was significantly higher ($p < 0.05$) compared to commercial anticancer drug cytosine-β-D-arabinofuranoside (Fig. 1A). From the regression equation, the IC_{50} value of Rusvinoxidase towards Colo-205 cell was determined to be 9.8 μg/ml (~170 nM), which is ~1.8 fold higher than the IC_{50} value of Rusvinoxidase towards MCF-7 cells (5.5 μg/ml) under identical experimental conditions; however, both are significantly less than the IC_{50} values of the anticancer drugs oxoplatin (9) and cytosine-β-D-arabinofuranoside (Fig. 1A). Nevertheless, unlike the MCF-7 cells [1], the Colo-2015 cells did not detach from the culture flasks 24 h after treatment with one IC_{50} dose of Rusvinoxidase.

The dose-dependent reduction in the population, and the induction of apoptosis, in Colo-205 cells after Rusvinoxidase treatment were also evident from phase-contrast microscopic

observation of changes in cell morphology, as demonstrated by loss of cell membrane integrity and clumping of cells (Fig. 1B). It was reported that MCF-7 cells treated with a 2 × IC_{50} value of Rusvinoxidase at 24 h had undergone secondary necrosis [1]; nevertheless, the same phenomenon was not observed for Colo-205 cells under the same experimental conditions. The changes in nuclear morphology of treated cancer cells were evident from chromatin condensation and formation of apoptotic cells (Fig. 1C). By Hoeschst 33258 staining, the dose- and time-dependent apoptosis induction in Colo-205 cells by Rusvinoxidase was determined (Fig. 1D). It was observed that the percentage of apoptotic MCF-7 cells was more pronounced [1] compared to induction of apoptosis in Colo-205 cells (Fig. 1D) following Rusvinoxidase treatment. This likely indicates that the apoptosis-induction potency of Rusvinoxidase against MCF-7 cells is significantly higher compared to Colo-205 cells, and that Colo-205 cells exhibit a higher level of resistance to Rusvinoxidase.

DNA fragmentation is one of the hallmarks of apoptosis, and fragmentation in Colo-205 cells post-Rusvinoxidase treatment (Fig. 2A and B) was significantly less than that observed for MCF-7 cells [1]. The flow-cytometric analysis (APO-BrdU TuNEL assay) indicated that 24 h after treatment, Rusvinoxidase dose-dependently augmented apoptosis induction (DNA fragmentation) in Colo-205 cells compared with apoptosis in control cells (Fig. 2C); however, under identical experimental conditions, Rusvinoxidase induced significantly less apoptosis in Colo-205 cells when compared to MCF-7 cells.

3.2. Rusvinoxidase decreases the level of cellular glutathione and enhances the catalase activity of Colo-205 cells

Rusvinoxidase at its IC_{50} value progressively depleted the cellular glutathione (GSH) of Colo-205 cells from 3 to 12 h post treatment as compared to baseline values of control (untreated) cells (Fig. 3A). However, under identical experimental conditions, depletion in cellular GSH was significantly greater ($p < 0.05$) in Rusvinoxidase-treated MCF-7 cells (1) as compared to Colo-205 cells (Fig. 3A). Furthermore, Colo-205 GSH levels returned to baseline values after 18 h of treatment, with a significant increase in cellular GSH level observed 24 h after treatment (Fig. 3A). Conversely, in Rusvinoxidase-treated MCF-7 cells, GSH levels were found to be less than what was observed in control MCF-7 cells 24 h after treatment, suggesting that lower depletion of cellular glutathione by Colo-205 cells (compared to MCF-7 cells) may be a mechanism to counteract apoptosis-induction by Rusvinoxidase in Colo-205 cells [2].

Rusvinoxidase (IC_{50} value) augmented Colo-205 catalase activity within 30 min of incubation, and this enhanced level of catalase activity was maintained up to 6 h following treatment (Fig. 3A). Thereafter, a steady decline in catalase activity in Rusvinoxidase-treated Colo-205 cells was observed; however, activity levels were above the baseline value of catalase activity displayed by control cells after 24 h (Fig. 3A). In contrast, and under identical treatment conditions, an opposite effect was observed for catalase activity in MCF-7 cells. Treatment of MCF-7 cells with Rusvinoxidase resulted in a gradual decrease in catalase activity of cell-free extract compared to activity levels of control cells [1]. This higher catalase activity in Colo-205 cells treated with Rusvinoxidase is consistent with induction of catalase activity as a protective mechanism against reactive oxygen species (ROS) produced in these resistant cancer cells.

The intrinsic apoptotic pathway has been shown to be associated with key mitochondrial events such as depolarization of the mitochondrial membrane through generation of ROS [2, 9–11]. Under normal cellular conditions, production of excess ROS is down-regulated by antioxidant enzymes such as catalase, as well as by non-protein antioxidants such as GSH, which provides electrons for glutathione peroxidase to reduce H_2O_2 to H_2O [12,13]. Therefore, following exposure to Rusvinoxidase, Colo-205 cells increase catalase activity, as well as counteract the decreasing cellular GSH level (a hallmark of apoptosis). This ultimately results in diminished ROS production within the cytoplasm (see below), and promotes resistance to apoptosis induction [2,10,11].

3.3. Mitochondrial transmembrane potential and ROS generation

In order to explore the mechanism of apoptosis induction by Rusvinoxidase on Colo-205 cells, ROS generation and disruption of mitochondrial membrane potential by Rusvinoxidase was investigated. The JC-1 dye is permeable to intact mitochondrial membranes and consequently, it demonstrates membrane potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green wavelengths (~530 nm) to red wavelengths (~590 nm). Therefore, a decrease in the red/green fluorescence intensity ratio indicates mitochondrial depolarization. Flow cytometric analysis of mitochondrial membrane depolarization (using the MitoProbe™ JC-1 assay) demonstrated that at the doses tested, Rusvinoxidase failed to impair the mitochondrial membrane potential of Colo-205 cells (data not shown). However, studies from our laboratory have shown that, in a dose- and time-dependent manner, Rusvinoxidase enhanced the mitochondrial membrane depolarization of MCF-7 cells [1]. These contrasting results in mitochondrial membrane depolarization potential may indicate a differential mode of action or sensitivity of Colo-205 and MCF-7 cells toward Rusvinoxidase. Interestingly, exposure to Rusvinoxidase resulted in a decrease in ROS production in Colo-205 cells (Fig. 3B). Conversely, MCF-7 cells undergoing apoptosis demonstrated a dose-dependent increase in ROS production at 4 h of Rusvinoxidase treatment [1]. The opposite effect that was observed in ROS production for Rusvinoxidase-treated Colo-205 cells is well correlated with the time-dependent change in the levels of catalase enzyme activity and cellular glutathione contents in Colo-205 (present study) and MCF-7 cells [1].

Studies on anticancer drug resistance mechanisms in human cancer cell lines have found a marked increase in glutathione synthesis as well as enhanced stability of catalase mRNA, the major antioxidant defense systems in cells that down-regulate excess ROS production [14,15]. Studies from our laboratory have also shown that Ruviprase, a pro-apoptotic peptide from *D. r. russelii* venom, time-dependently decreases the cellular glutathione content and enhances the ROS production in MCF-7 cells, inducing apoptosis [2]. However, the marked increase in cellular GSH production of MCF-7 cells at 6 h of Ruviprase treatment indicated that the cells are likely to counteract Ruviprase-induced apoptosis [2], an observation that is similar to the response shown by Rusvinoxidase-treated Colo-205 cells in the present study. Therefore, significant enhancement of catalase activity up to 12 h post treatment, followed by a sharp increase in cellular glutathione content post exposure, may be a significant mechanism in Colo-205 cells to counteract the potential of Rusvinoxidase to induce apoptosis [14–16].

3.4. Effect of Rusvinoxidase on activation of caspases

Rusvinoxidase demonstrated differential activation of caspases in Colo-205 cells (present study) when compared to MCF-7 cells [1]. At its IC_{50} value, Rusvinoxidase induced a gradual increase in activation of caspase-9 and caspase-3 in Colo-205 cells in a time-dependent manner (Fig. 3C). Caspase-9 activity of Colo-205 cells reached peak values at 3 h of exposure to Rusvinoxidase, and

thereafter the level of caspase-9 was same as that of control (untreated) cells. The activation of caspase-9 is associated with the mitochondrial apoptosis pathway [17]. Caspase-9 activity of Rusvinoxidase-treated Colo-205 cells was less enhanced compared to MCF-7 cells under the same conditions [1], indicating the mitochondrial apoptotic pathway is induced at low levels in Rusvinoxidase-treated Colo-205 cells.

Caspase-3 activation in Colo-205 cells exposed to Rusvinoxidase was slightly enhanced at 6 of treatment; thereafter, caspase-3 activity of Colo-205 cells started to decline and reached its baseline value after 12 h of treatment (Fig. 3C). Nevertheless, at 24 h of exposure to Rusvinoxidase, caspase-3 activity of Colo-205 cells increased significantly. The activation of pro-caspase-3 to caspase-3, the executer caspase of apoptosis, is associated with activation of caspase-9, and to a larger extent of caspase-8 [18].

3.5. Colo-205 shows resistance to Rusvinoxidase-induced apoptosis by changing the relative expression of pro- and anti-apoptotic proteins

It is well known that the extent of expression of anti- and pro-apoptotic proteins post treatment with anticancer agents is one of the crucial mechanisms responsible for the decisive fate in the apoptotic process of treated cancer cells. The cells undergo apoptosis via regulation of expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-xL, and the relative expression of both Bcl-xL and Bax determines cell survival or death [19]. Investigation of time-dependent expression of these pro- and anti-apoptotic proteins in Colo-205 cells (following exposure to one IC_{50} dose of Rusvinoxidase) demonstrated inhibition of expression of the anti-apoptotic protein Bcl-xL after only 24 h of exposure, whereas the expression of pro-apoptotic protein Bax was induced after 12 h of treatment (Fig. 4A). The fold-changes in the expression of these pro- and anti-apoptotic proteins are shown in Fig. 4B. A time-dependent higher ratio of the relative expression of Bax and Bcl-xL (pro- and anti-apoptotic proteins) in MCF-7 cells [1] compared to Colo-205 cells (Fig. 4C) unequivocally suggests a higher level of cellular resistance mechanisms against Rusvinoxidase-induced apoptosis in Colo-205 cells compared to MCF-7 cells [1].

The release of cytochrome *c* from mitochondria to the cytosol in Rusvinoxidase-treated Colo-205 cells did not significantly increase after 12 h of treatment (Fig. 4A and B), and by 24 h the release of cytochrome *c* increased only 1.4 fold (Fig. 4B). In comparison, at 24 h of treatment, Rusvinoxidase enhanced the release of mitochondrial cytochrome *c* in MCF-7 cells by nearly 4 fold, which likely resulted in earlier as well as significantly higher expression of caspase-9 in this cell line [1], relative to Colo-205 cells (Fig. 3C).

Our previous study has shown that Rusvinoxidase can induce apoptosis in MCF-7 cells by both the extrinsic and intrinsic pathways, and the intrinsic pathway is more significant in Rusvinoxidase-treated MCF-7 cells [1]. However, in Colo-205 cells, the significantly higher expression of caspase-8 (~6 fold) (Fig. 4A and B) compared to caspase-9 (~1.15 fold) (Fig. 3C) indicates that Rusvinoxidase preferentially triggers the extrinsic pathway of apoptosis in these cells [20].

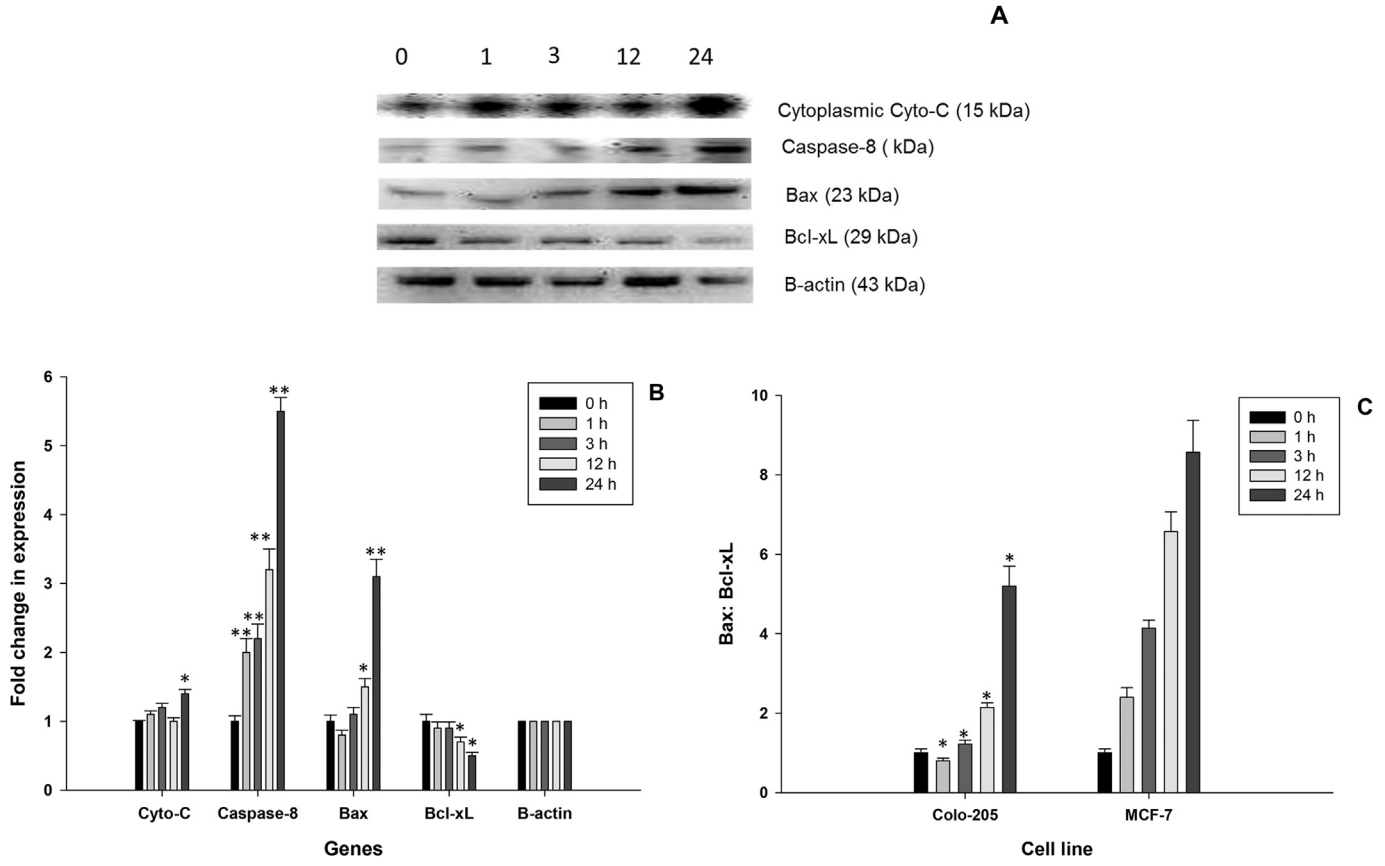

Fig. 4. A. Time-dependent expression of pro-(Bax), and anti-apoptotic (Bcl-xL) proteins, cytochrome *c*, and caspase-8 in Rusvinoxidase-treated ($1 \times IC_{50}$) Colo-205 cells. **B.** The expression of pro- and anti-apoptotic proteins was normalized to β-actin using ImageJ software and the figures show the means ± SD of normalized values for 3 different experiments. Significance of difference compared to control: *, $p < 0.05$; **, $p < 0.01$. **C.** A comparison of relative expression of pro-(Bax) and anti-apoptotic (Bcl-xL) proteins in MCF-7 and Colo-205 cells. Values are means ± SD of three determinations. Significance of difference compared to MCF-7 cells at each time point: *, $p < 0.05$.

Our work with the venom of *D. r. russelii* has demonstrated that snake venoms contain a variety of proteins with potent biological activities [1,2,7], many of which have therapeutic potential or serve as model compounds that can be utilized in novel drug development. Because venoms represent a co-opting and repurposing of normal regulatory enzymes and ligands, it is not surprising that animal venom toxins can be harnessed to yield new therapeutic drugs. Our investigations into the mechanisms of action of Rusvinoxidase reveal that even well-studied venom components, such as LAAOs [21,22], can yield new and useful information for the design of novel anti-cancer therapeutics. This work also highlights the important variation in sensitivities of different cancer cell lines to apoptosis-stimulating compounds, such as snake venom LAAOs.

4. Conclusions

In summary, the current study demonstrates distinct differences in the mechanism of Rusvinoxidase-induced apoptosis between Colo-205 and MCF-7 cell lines. The higher apoptotic resistance in Colo-205 cells following Rusvinoxidase treatment is attributed to a reduction of cellular glutathione levels, significant increases in the catalase activity, and a subsequent decrease in ROS production. Further, the relative expression of pro-apoptotic (Bax) and anti-apoptotic (Bcl-xl) proteins strongly suggests that the mechanism of Colo-205 resistance against Rusvinoxidase-induced apoptosis likely includes differential expression of these two proteins.

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