Rhein Induces Apoptosis through Induction of Endoplasmic Reticulum Stress and Ca$^{2+}$-dependent Mitochondrial Death Pathway in Human Nasopharyngeal Carcinoma Cells

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Abstract. Apoptosis is a physiological mechanism for eliminating malignant cells, including cancer cells, without eliciting damage to normal cells or surrounding tissues. Here, we report that rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), a major constituent in the rhizome of rhubarb, induced apoptosis of human nasopharyngeal carcinoma (NPC) cells. Rhein induced apoptosis in NPC cells as demonstrated by increased nuclear condensation and DNA fragmentation. Moreover, for the first time in NPC cells it was demonstrated that the pathway involved in rhein-induced apoptosis is caspase-dependent, presumably through the endoplasmic reticulum (ER) stress pathway, as shown by an increase in the levels of glucose-regulated protein 78 (GRP 78), PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6) and CCAAT/enhancer-binding protein homologous protein (CHOP) as well as the activation of caspase-3, -8, -9 and -12. This increased susceptibility to ER stress-induced apoptosis may be due to an increased accumulation of reactive oxygen species (ROS). Rapid accumulation of calcium (Ca$^{2+}$) and a decrease in the mitochondrial membrane potential (MMP) were also observed. Cytochrome c and apoptosis-inducing factor (AIF) were released upon treatment with rhein. Taken together, these results suggest that ER stress and Ca$^{2+}$-dependent mitochondrial death pathway may be involved in rhein-induced apoptosis in NPC cells.

Cancer is a major public health problem worldwide because of its significantly high rates of morbidity and mortality. Nasopharyngeal carcinoma (NPC) is a rare tumor arising from the epithelium of the nasopharynx (1). It occurs with high frequency in Asian populations, especially among Chinese people (2, 3). The incidence of NPC among people in Hong Kong is about 15 to 25 per 100,000 (4). Of note, the average age of NPC patients is usually ten years younger than those of patients with other head and neck cancers. Clinically, this cancer exhibits a high incidence of lymph node spread as well as distant metastasis that contribute to its poor prognosis (5).

Use of chemopreventative agents is now regarded as a promising strategy against cancer development (6) and many natural or dietary substances have been shown to inhibit carcinogenesis in vitro and in vivo (7). Apoptosis is a physiological mechanism for eliminating malignant cells or cancer cells without eliciting damage to normal cells or surrounding tissues (8-10). Thus, induction of apoptosis in target cells is a key mechanism by which anticancer therapy works (8). Rhein is isolated from the rhizome of rhubarb (Rheum palmatum. or Rheum tanguicum maxim), which is a Chinese medicinal plant used traditionally for treatment of chronic liver disease in mainland China (11). In vivo experimental studies show that rhein inhibits the growth of tumor cells in rat liver (12). In addition, it has also been shown to induce apoptosis in several cancer cell lines such as human colon adenocarcinoma cells, promyelocytic leukemia cell (HL-60), and cervical cancer Ca Ski cells (13-15). These results suggest that rhein should have a great

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potential serving as a cancer chemopreventative agent. However, the molecular mechanisms of its anti-cancer activity are still poorly understood.

The endoplasmic reticulum (ER) is the principal organelle for the synthesis of proteins, as well as being a regulator of intracellular Ca\(^{2+}\) homeostasis. An imbalance between the rate of protein synthesis and the folding capacity of the ER induces ER stress. Cells initially adapt to the accumulation of unfolded proteins by inducing the expression of ER-resident molecular chaperones such as glucose-regulated protein 78 (GRP 78) (16, 17). However, excessive and prolonged stress leads cells to apoptosis associated with the induction of the CCAAT/enhancer-binding protein homologous protein (CHOP) (18). The current view is that three ER transmembrane proteins, activating transcription factor 6 (ATF6), inositol-requiring 1 (IRE1) and PKR-like ER kinase (PERK) are considered to be sensors of ER stress (19). In the case of PERK, it is responsible for the increased phosphorylation of the translation elongation initiation factor 2α (eIF2α) for the inhibition of synthesis of new proteins after exposure to ER stress (19-21). It has been shown that prolonged ER Ca\(^{2+}\) depletion triggers ER stress and activates PERK (22). X box binding protein 1 (XBP1), a key transcription factor of the unfolded protein response (UPR), is activated by ATF6 and an active form of XBP1 is activated by IRE1-mediated splicing of its mRNA in response to ER stress (23). Previous reports show that increases in the intracellular level of Ca\(^{2+}\) was accompanied by the activation of the ER resident caspase-12 (24). Moreover, Cu\(^{2+}\) released from the ER enters mitochondria leading to depolarization of the inner membrane and cytochrome c release (25, 26). The results also indicate that ER stress may lead to cell death via accumulation of ROS (27). ROS induction has also been shown to activate the down-stream apoptosis cascade or induce the alteration of mitochondrial proteins, such as the voltage-dependent anion channel (VDAC) and/or the adenine nucleotide translocase (ANT) that can induce the pro-apoptotic mitochondrial membrane permabilization (28, 29). Furthermore, ER and mitochondria cross-talk apoptotic pathways induced by ER stress resulted in the activation of caspase-9, -12 and/or cytochrome c release from mitochondria (30-32).

In the present study, we investigated the possible involvement of the ER stress and mitochondrial death pathway in rhein-induced NPC apoptosis.

Materials and Methods

**Human NPC cell lines.** The nasopharyngeal carcinoma-derived cell line NPC-039 was cultured routinely in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (33). WI-38 (human embryonic fibroblast, lung-derived cell line) cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and cultured in minimum essential medium (MEM) supplemented with 10% FBS. Both cell lines were grown in 10 cm tissue culture dish at 37°C in a humidified incubator containing 5% CO\(_2\).

**Chemicals and reagents.** Rhein, propidium iodide (PI), Tris-HCl, 4’-6-diamidino-2-phenylindole (DAPI) and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and potassium phosphate were purchased from Merck Co. (Darmstadt, Germany). DMEM, FBS, penicillin-streptomycin, trypsin-EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3, and -9 activity assay kits were purchased from OncoImmunin, Inc (Gaithersburg, MD, USA). Inhibitors of caspase-3 (Z-DQMD-FMK) was purchased from Calbiochem (San Diego, CA, USA) and was dissolved in DMSO. Western blotting Luminol reagent was purchased from Santa Cruz Co. (La Cruz, CA, USA).

**Isolation of peripheral blood mononuclear cells (PBMC).** Isolation of PBMC was performed by concentration on a Ficoll-Hypaque (Sigma, St Louis, MO, USA) gradient according to the protocol provided by the manufacturer. Briefly, heparinized venous blood (5 ml) was layered over Ficoll-Hypaque and centrifuged at 375 xg for 30 min. Cells at the interface were removed and washed three times with PBS.

**Antibodies.** ATF-6α, ATF-6β, GRP 78, PERK, CHOP, and AIF antibodies were purchased from Santa Cruz Co. (La Cruz, CA, USA) and cytochrome c and caspase-12 antibodies were purchased from BD PharMingen (San Diego, CA, USA). Anti-mouse, -goat and -rabbit IgG peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratory (West Grove, Pennsylvania, USA).

**Cell viability assay.** Cells were seeded at a density of 1x10\(^5\) cells per well into 12-well plate before drug treatment. Drugs were added to medium at various times (12, 24, 36 and 48 h) and concentrations (120, 140, 160, 180 and 200 μM). Cells were harvested and stained with 10 μg/ml of propidium iodide (PI) for the determination of cell viability. Samples were analyzed on a fluorescence-activated cell sorter (FACS; BD Biosciences, Franklin Lakes, NJ, USA). Cell Quest software was applied to analyze the results (BD Biosciences, Franklin Lakes, NJ, USA).

**DAPI staining for the analysis of nuclear morphological changes.** Cells were seeded at a density of 1x10\(^5\) cells per well into 12-well plates. Cells were cultured with 180 mM rhein or 180 mM rhein and caspase-3 inhibitor at 37°C for 36 h. After treatment, cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 and stained with 1 mg/ml of DAPI for 10 min at room temperature (RT). Cells were then washed twice with PBS. The morphological changes were observed using an Olympus fluorescence microscope (Olympus 1 X 71, Inc., Melville, NY, USA).

**Comet assay.** This assay was performed as described elsewhere (34). Briefly, treated cells or control cells were pelleted and resuspended in 0.5% low melting point agarose at 37°C then layered on a frosted microscope slide previously coated with a thin layer of 0.5% normal melting agarose and kept for 5 min at 4°C. After solidification, slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 10.5), 1% Triton X-100 and 10% DMSO for 24 h. After lysis, slides were washed with 300 mM NaCl. The slides were immersed for 30 min in an alkaline solution of 300 mM NaOH at 4°C, and then dried. A single cell was used as a source of DNA to form a comet. The comets were stained with 10 μg/ml of DAPI and scored for tail moment using an image analysis system (Comet Assay I,astic, UK).
Western blot analysis. Rhein-treated cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol and 0.1% bromophenol blue) containing a cocktail of protease inhibitors. Protein concentration was determined by the Bradford method (BioRad, Laboratories, Richmond, CA, USA) as described in the manufacturer’s instructions. GRP 78, PERK, ATF6α, ATF6β, CHOP and caspase-12 were analyzed by electrophoresis on a 10% SDS-PAGE gel and then transferred to a PVDF membrane (Millipore, Immobilon-P; Bedford, MA, USA). Immunoblot analysis was performed by blocking overnight with a phosphate-buffered saline (PBS) buffer containing 3% skimmed milk and then visualized by staining with horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit or donkey anti-goat antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA) and Western Blotting Luminol Reagent. γ-Tubulin was used as internal control for sample loading.

Detection of cytochrome c and AIF release. Cytosolic protein extracts were prepared by the method of Zuppinin et al. (35). Cells were homogenized in ice-cold buffer containing 75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose and 190 µg/ml digitonin. The cytosolic fractions were then centrifuged at 13,000 xg for 5 min at 4°C. The assay for the release of AIF was performed as described elsewhere (36). Cells were first lysed in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES (pH 7.5)) and homogenized for 30 to 40 strokes with a Dounce homogenizer (Dounce; Bellco Glass Co., Vineland, NJ, USA). Nuclei and unbroken cells were removed by centrifugation at 500 xg for 5 min at 4°C. The supernatants were centrifuged at 10,000 xg for 30 min at 4°C, and the resulting supernatants were stored as the cytosolic fraction. Twenty µg of total proteins underwent electrophoresis on a 12% SDS-polyacrylamide gel and transferred onto a PVDF membrane. The membrane was incubated with rabbit anti-cytochrome c antibody at 4°C overnight. After incubation with goat anti-rabbit horseradish peroxidase antibody, the signal was visualized by Western Blotting Luminol Reagent.

Assay for caspase-3, -8 and -9 activity. The activities of caspase-3, -8 and -9 were measured using the PhiPhiLux G1D2, CaspaLux L1D2 and CaspαLux M1D2 kits (OncoImmunin, Inc., College Park, MD, USA), respectively, according to the protocol provided by the manufacturer. Briefly, rhein-treated NPC cells were incubated with PhiPhiLux Fluorogenic caspase substrate at 37°C for 1 h. After incubation, samples were washed three times with PBS. Subsequently, 500 µl of ice-cold flow cytometry dilution buffer was added to this reaction mixture and cells were analyzed by FACS as described elsewhere (37).

Detection of reactive oxygen species (ROS). ROS production was measured as described elsewhere (37). The NPC cells (1x10⁵ cells) were treated with or without 180 µM rhein for 0.5, 1, 2, 3 and 6 h to detect the changes of ROS. After treatment, cells were harvested and washed twice with PBS. Cells were then re-suspended in 500 µl of 2,7-dichlorodihydrofluorescein diacetate (10 µM) and incubated at 37°C for 30 min. The level of ROS of the NPC cells was examined by flow FACS.

Detection of the intracellular Ca²⁺ level. Intracellular Ca²⁺ levels were determined as described elsewhere (38). Briefly, rhein-treated NPC cells were incubated with 3 µg/ml of Indo 1/AM (Calbiochem; La Jolla, CA, USA) and incubated at 37°C for 30 min. After washing twice with assay buffer, cells were suspended in assay buffer. The level of Ca²⁺ of the NPC cells was determined by flow cytometry (FACS Calibur, BD Bioscience, Fanklin, NJ, USA).

Measurement of mitochondrial membrane potential (MMP). MMP was determined by measuring the retention of the dye 3,3'-dihexyloxacarbocyanine (DiOC₆) (39). Briefly, rhein-treated NPC cells were incubated with 4 mol/l of DiOC₆ for 30 min at 37°C. Cells were then pelleted by centrifugation at 1,000 rpm for 10 min. The cell pellet was re-suspended and washed twice with PBS. The MMP was determined by flow cytometry (FACS Calibur, BD Bioscience, Fanklin, NJ, USA).

Statistical analysis of data. Statistical calculations of the data were performed using an unpaired Student’s t-test and ANOVA analysis. Statistical significance between control and experimental groups was set at p<0.05.

Results

Apoptosis is induced in NPC cells by rhein. Since rhein was found to inhibit tumor growth and to induce apoptosis in several cancer cell lines (12-14), the first study served to determine whether rhein exhibited cytotoxicity on NPC cells. To quantitate the effect of rhein on cell growth, cell viability was determined by PI staining and FACS analysis. As shown in Figure 1 (panel A), NPC cells exhibited a clear dose- and time-dependent growth inhibition with rhein. In contrast, human peripheral blood mononuclear cells (PBMC) and normal fibroblasts show no significant reduction in viability following treatment with rhein (Figure 1, panels B and C). Figure 2A illustrate that apoptotic bodies increased with increasing concentrations of rhein up to 180 µM after 48 h of exposure to rhein. Furthermore, after 12, 24, 36 and 48 h of 180 µM rhein treatment apoptotic bodies were found but the control cells were not affected (Figure 2B). These data suggest that rhein-treated NPC cells undergo cell death and exhibit morphological features suggestive of apoptosis.

To further investigate whether the induction of cell death by rhein could be linked to apoptosis in NPC cells, single cell gel electrophoresis (Comet assay) was performed and nuclear morphological changes examined. As expected, rhein-treated cells showed well-formed comets while the control cells did not demonstrate any comet-like appearance (Figure 3A). NPC cells also showed a remarkable change in nuclear morphological changes examined. As expected, rhein-treated cells showed well-formed comets while the control cells did not demonstrate any comet-like appearance (Figure 3A). NPC cells also showed a remarkable change in nuclear morphology after treatment with rhein (Figure 3B), whereas the caspase-3 inhibitor (Z-DQMD-FMK) had significant effect on blocking nuclear morphological change.
Based on the above results, it was indicated that rhein could induce apoptosis in NPC cells.

Rhein-induced ER stress provokes CHOP expression. To investigate whether apoptosis is induced by ER stress, we evaluated the translation levels of ER stress genes in NPC cell lines exposed to rhein. First, we analyzed the expression of GRP 78 protein which is an ER-resident molecular chaperone whose expression serves as a good marker of ER stress (40). Immunoblot analysis showed that GRP 78 expression was augmented after rhein treatment (Figure 4A). Consistently, ER stress sensors ATF6 and PERK were also significantly induced after 6 h treatment with rhein (Figure 4B). It has been shown that induction of ER stress-associated apoptosis factor CHOP is regulated by ATF6 and PERK. Here, a marked increase in CHOP protein levels was also observed after 12 and 24 h in rhein-treated NPC cells (Figure 4C). These results suggest that rhein induces cell apoptosis through an ER stress pathway.

ER stress markers are activated by rhein. To explore whether rhein induces apoptosis by activation of caspase, we used flow cytometry to analyze the activity of caspase-3, a marker of caspase-dependent apoptosis (41). Compared with control cells, rhein treatment caused the activation of caspase-3 (Figure 5A). In order to confirm the induction of ER stress in NPC cells, we next examined caspase-8 and -9 activities. Caspase-8 and -9 were significantly activated at 6 h and continued to 36 h (Figure 5B and C). The changes in the caspase-8 and -9 activities at 12 h may have resulted from the activation of ER stress-induced CHOP (Figure 4C) and caspase-3 (Figure 5A). Western blot was then performed to examine the cleavage of procaspase-12 during rhein-induced apoptosis of NPC cells. Figure 5D shows that the cleaved form of caspase-12, p42/35, was noted at 12 h after rhein treatment and increased up to 36 h. These results indicate that the ER resident caspase-12 plays an important role in rhein-induced apoptosis.
Cross-talk between ER stress- and mitochondrial-mediated cell apoptosis. We investigated whether rhein treatment affects the cytosolic Ca\(^{2+}\) level, MMP and the cytochrome c level. The levels of cytosolic Ca\(^{2+}\), ROS and MMP were determined by flow cytometric assay. As shown in Figures 6A and 6B, the levels of Ca\(^{2+}\) and ROS increased in NPC cells after being treated with rhein, and the levels of Ca\(^{2+}\) were maintained in the following treatment periods up to 6 h. Rhein-induced apoptosis of NPC cells was also accompanied by a significant decrease in MMP (Figure 6C).

Western blot results showed that cytochrome c was significantly increased in the cytosol in a time-dependent manner (Figure 7A) and AIF was released at the same time (Figure 7B), suggesting that rhein is sufficient to induce the release of cytochrome c and AIF but not Endo G (data not shown). Thus these results indicate that the mitochondrial death pathway may also be involved in rhein-induced apoptosis in NPC cells.

Discussion

Despite the possible involvement of rhein in the apoptosis of human cancer cells, the pathway of rhein-induced apoptosis has not been fully elucidated. In the present paper, we demonstrated that rhein induces nuclear morphological changes and reduces the percentage of viable NPC cells through apoptosis via ER stress and the Ca\(^{2+}\) dependent mitochondrial death pathway. Here, we provide evidence that ER stress induced by rhein was correlated with the augmented expression of GRP 78 and CHOP as well as the cleavage of procaspase-12. Indeed, NPC cells
exposed to rhein demonstrated a dramatic increase in mitochondrial dysfunction, including the loss of MMP and the release of cytochrome c and AIF.

Two pathways of caspase-dependent apoptosis have been identified. The first is death receptor-mediated caspase-8 activation. The activated caspase-8 then initiates the activity of downstream caspase-3 (42). The other is the intrinsic pathway, in which the release of cytochrome c from mitochondria to the cytosol during the early stages of apoptosis triggers the activation of procaspase-9 initiating

Figure 5. The ER stress-specific caspase cascade is activated during rhein treatment. (A-C) Rhein induced the activation of caspase-3, -8 and -9 in NPC cells treated with 180 μM of rhein. The caspase activities were measured as described in materials and methods. The results are shown as means±SD of the three independent experiments. (D) Rhein induced the cleavage of caspase-12 in NPC cells treated with 180 μM of rhein. Cytosolic protein extracts were subjected to Western blot with antibody to caspase-12.
an apoptosome formation composed of Apaf-1, dATP, procaspase-9 and cytochrome c (43). Apoptosome formation leads to the activation of executioner caspase-3, -6 and -7 (44, 45). Previous studies have demonstrated that MMP is a key factor for apoptosis induction (46), triggering the release of cytochrome c into the cytosol and the occurrence of downstream caspase activation (47). It was shown that the release of Endo G and AIF from the mitochondria in response to proapoptotic stimuli occurs in a caspase-dependent manner (36).
In our present study, the release of cytochrome c was observed quickly after cells were exposed to rhein (Figure 7A). Indeed, a significant amount of AIF was released (Figure 7B). Unlike AIF, the release of Endo G was unaffected (data not shown). The rapid loss in MMP of NPC cells upon treatment with rhein was significantly correlated with the release of cytochrome c (Figures 6C and 7A). Therefore, these findings indicate that rhein-induced mitochondrial membrane permeabilization is sufficient to induce the release of cytochrome c and AIF.

The release of cytochrome c, which occurred at the same time as the processing of caspase-3, -8 and -9, suggests that cytochrome c release is a preceding event for the activation of caspase cascades. Rhein-induced nuclear morphological change was completely inhibited by preincubation with a caspase-3 inhibitor (Figure 3B). Taken together, our results suggest that the rhein-induced apoptosis process occurred in a caspase-dependent manner. Interestingly, processing of caspase-8 by rhein was also observed in the present assay. A recent study also demonstrated similar findings that rhein-induced caspase-8 activation may be associated with apoptosis of human cervical cancer CaSki cells (15). Although no evidence supports the direct action of rhein in the activation of caspase-8, it is speculated that rhein may activate caspase-8 by elevating the expression of Fas ligand. Caspase-12, an ER resident caspase, is activated by ER stress and can lead to the cleavage and activation of caspase-9 (48, 49). Activated caspase-9 consequently leads to the activation of the caspase cascade, such as caspase-3 (50). Caspase-8 is a key component of the Fas death receptor pathway leading to cell apoptosis (51), whereas caspase-9 activation is associated with the mitochondrial apoptotic pathway. In several experiments, it was found that induction of apoptosis in cancer cells by anticancer drugs was mediated by up-regulation of Fas ligand and Fas (52, 53). Accordingly, we cannot exclude the possibility that rhein activates caspase-8 via a Fas death receptor pathway. Further studies are required to elucidate the mechanism.

Cellular Ca$^{2+}$ homeostasis is critical for maintaining normal cell function; depletion of ER Ca$^{2+}$ stores causes growth arrest and apoptosis (54). In this regard, a previous report has shown that an increase in the level of intracellular Ca$^{2+}$ is thought to converge on mitochondrial dysfunction, which in turn results in the overproduction of ROS (55). Moreover, experimental data indicated that ER stress causes accumulation of ROS leading to cell death, the generation of ROS being derived from the ER and mitochondria (27). The relationship between ROS or Ca$^{2+}$ and apoptosis has been broadly investigated in several cancer cell lines (37, 56). Little is known about the role of ROS and Ca$^{2+}$ in the induction of apoptosis caused by rhein. Our results show that rhein increases the production of ROS and intracellular Ca$^{2+}$. Despite the accumulation of high concentrations of ROS after cells were treated with rhein, the cells were able to survive with low levels of intracellular Ca$^{2+}$ (Figures 6A and B). Therefore, it is unclear whether or not rhein may interact directly with a Ca$^{2+}$ channel that is responsible for the final demise of the cell which leads to prevent its opening. Moreover, it could be that they may prevent the signaling mechanism between high ROS levels and the opening of the Ca$^{2+}$ channel.

It was reported that GSH levels in cells are associated with ROS levels and loss in cellular GSH up to 85% of the control level causes only a 5- to 10-fold increase in levels of ROS (57). The interesting point is that a greater GSH loss may stimulate mitochondria to produce a 100-fold increase in ROS which causes cell death (57). Whether or not rhein can act as an antioxidant needs further investigation.

The present chemotherapy agents for cancer are usually toxic to normal cells, often resulting in punishing side-effects such as temporary hair loss, nausea and vomiting. In this paper, we show that rhein significantly inhibited the growth of and induced apoptosis of NPC cells, without toxicity to the human PBMC and normal fibroblasts. These data highlight its potential as a treatment for NPC.

In summary, this is the first report of rhein-induced apoptosis in human NPC cells. Rhein-induced apoptosis is an ER stress and mitochondria-dependent pathway which also involves Ca$^{2+}$ levels in these cells. Understanding the molecular mechanism involved in rhein-induced apoptosis may provide essential information for the development of novel therapies to treat nasopharyngeal carcinoma.
References


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