# Mild hyperthermia predisposes tumor cells to undergo apoptosis upon treatment with onconase

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**Abstract.** Onconase (ONC), (ranpirnase) a cytotoxic ribonuclease isolated from amphibian oocytes and early embryos targeting tumor cells *in vitro* and *in vivo*, is currently in a confirmatory Phase IIIb clinical trial for unresectable malignant mesothelioma where it demonstrates antitumor activity with relatively minor overall toxicity to patients. Since hyperthermia has been shown to be synergistic with certain antitumor modalities, the aim of the present study was to explore whether the cytotoxic effects of ONC can be enhanced under conditions of mild hyperthermia. Treatment of human lymphoblastoid TK6 cells with 2 or 5 $\mu$g/ml of ONC at 40˚C for 24 or 48 h led to 64-200% enhancement in incidence of apoptosis assessed by frequency of cells showing the presence of activated (cleaved) caspase-3 or activated serine proteases, compared to treatment at 37.5˚C. The incidence of apoptosis at 40˚C in the absence of ONC was unchanged compared to 37.5˚C, for up to 48 h. Although at 41˚C in absence of ONC the incidence of apoptosis was elevated compared to 37˚C the cytotoxicity of ONC was further enhanced and the overall pro-apoptotic effect was above the level of additive effects of ONC plus that of 41˚C-hyperthermia. While the mechanism of the observed enhancement of ONC cytotoxicity is currently under investigation, the findings suggest that a combination of ONC and mild hyperthermia should be explored to increase effectiveness of ONC in cancer treatment.

## Introduction

Onconase (ONC) (ranpirnase) is a germ plasm ribonuclease isolated from oocytes or early embryos of Northern Leopard frog *(Rana pipiens)* (1-3). This protein is the smallest member (~12,000 MW) of pancreatic RNase A superfamily and it displays weak ribonucleolytic activity (reviewed in refs. 4-8). ONC suppresses proliferation and is cytotoxic to several tumor cell lines of different lineage (9-15). It also inhibits growth of certain tumors in mice (16) and is synergistic with many chemotherapeutic agents (11,13,15-18). ONC is currently in Phase IIIb confirmatory clinical trial for unresectable malignant mesothelioma (UMM) where it demonstrates antitumor activity with relatively minor overall toxicity to the patient (4,19).

The cytostatic effect of ONC, seen after 24 h of the treatment, manifests as an arrest in $G_{0/1}$ phase of the cell cycle and is followed by apoptosis during which caspases and serine proteases are being activated (9,16,20-22). The $G_{0/1}$ arrest of histiomonocytic lymphoma U-937 cells was shown to be mediated by downregulation of cyclin D3, upregulation of the inhibitors of cyclin-dependent kinases (CKIs) $p27^{KIP1}$, $p16^{INK4A}$ and $p21^{WAF1/CIP1}$ and hypophosphorylation of pRb (20). It is unknown whether this mechanism of cytostasis induced by ONC is generic, common to other cell types, or only to U-937 cells.

ONC binds to receptors on plasma membrane of the sensitive cells and is internalized by a dynamin-independent endocytic pathway (14,23,24). The ribonucleolytic activity of ONC is essential for its cytostatic and cytotoxic effects, and it was reported that within the target cells tRNA was preferentially degraded (14,26). It has been postulated, therefore, that these effects of ONC are mediated by degradation of intracellular tRNA which leads to overall suppression of the rate of protein synthesis (14,25,26).

Several observations, however, cannot be explained by such a mechanism. The upregulation of CKIs by ONC (20), for example, would be incompatible with the mechanism based on indiscriminate suppression of translation by this drug. Furthermore, suppression of protein synthesis appears to play no role in induction of apoptosis by ONC (25). Also, the patterns of cytostatic and cytotoxic effects of ONC on different cell types are quite different compared to the effects of classic inhibitors of transcription or translation such as actinomycin D or cycloheximide (27,28). All these observations suggest that tRNA cannot be the sole target of ONC. We proposed, therefore, that at least in part, ONC may exert its effects by targeting RNAs that play a role in regulation of the translation (siRNA, miRNA, microRNAs) by the RNA interference (RNAi) mechanism (29). It is intriguing that this protein, repeatedly infused during treatment of patients, has no significant immunogenic activity (5,19). Perhaps the enhancement of activation-induced apoptosis of lymphocytes

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by ONC may be responsible for the suppression of immunogenic response (30).

Hyperthermia alone has been used to treat some forms of cancer (reviewed in ref. 31). More frequently, however, hyperthermia is being combined with chemo- or radiotherapy. Depending on the nature of the chemotherapeutic agent and tumor type the antitumor effect of such combinations varies, but often is enhanced in comparison to the effect of chemotherapeutic agents alone (31-38). The aim of the present study was to explore whether the cytotoxic effect of ONC can be enhanced under condition of mild hyperthermia, when thermal effects on cells viability are either not detectable or minor. Towards this aim we tested the effect of hyperthermia on the potency of ONC to induce apoptosis of human lymphoblastoid TK6 cells.

## Materials and methods

*Cells and culture conditions*. Human B cell lymphoblastoid TK6 cells were kindly provided by Dr Howard Liber of Colorado State University, Fort Collins Co. (39). The cells were grown in 25 ml Falcon flasks (Becton Dickinson Co., Franklin Lakes, NJ) in RPMI-1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin and 2 mM L-glutamine (all from Gibco/BRL Life Technologies, Inc., Grand Island, NY) at 37.5, 40 or 41˚C in an atmosphere of 5% $CO_2$ in air, as described in the figure legends. ONC (Alfacell Corp., Bloomfield, NJ) was dissolved in phosphate-buffered saline (PBS) at concentration of 1 mg/ml to obtain stock solution, which was freshly made. Some cultures were treated with a stock solution of ONC to obtain final ONC concentrations 2 or 5 $\mu$g/ml; parallel control cultures were treated with equivalent volumes of PBS. At the onset of the experiments, there were ~$2x10^5$ cells per ml in cultures and the cells were at an exponential and asynchronous phase of growth. The cultures were consistently screened for possible contamination with Mycoplasma microorganisms. Other details of cell culturing are presented elsewhere (40).

*Detection of caspase-3 activation and apoptosis by fluorescence microscopy*. Cells grown in the absence or presence of ONC at different temperatures were rinsed with PBS and then fixed in 1% methanol-free formaldehyde in PBS (on ice, 15 min) followed by 70% ethanol at 4˚C. After being washed with PBS, the cells were immersed in 0.2% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in a 1% (w/v) bovine serum albumin (BSA) solution in PBS for 30 min. The cells were then incubated in 100 $\mu$l of 1% BSA containing 1:100 diluted anti-cleaved (activated) caspase-3 rabbit polyclonal Ab (Cell Signaling Technology, Beverly, MA) overnight at 4˚C, washed twice with PBS and then incubated in 100 $\mu$l of 1:200 diluted Alexa Fluor® 488 conjugated goat anti-rabbit IgG (H+L) (Invitrogen/Molecular Probes, Carlsbad, CA) for 30 min in room temperature in the dark. The cells were then counter-stained either with 10 $\mu$g/ml of propidium iodide (PI; Invitrogen/Molecular Probes) in the presence of 100 $\mu$g of RNase A (Sigma) for 30 min for analysis by flow cytometry, or with 1 $\mu$g/ml of 4',6-diamidino-2-phenylindole (DAPI) and 10 $\mu$g/ml of sulforhodamine 101 in PBS for 5 min for inspection by fluorescence microscopy. Cellular morphology and fluorescence was examined under a Nikon Microphot FXA microscope, utilizing a 40X objective.

*Detection of serine protease(s) activation*. An unprotected analog of the serine protease inhibitor, N-tosyl-L-phenylalanine chloromethylketone (TPCK; TFCK using current amino-acid symbols) has been modified to obtain 5(6)-carboxyfluoresceinyl-L-phenylalanylchloromethyl ketone (FFCK); the chymotrypsin-like serine protease active center ligand that contains the fluorescent tag (41,42). This reagent was obtained from Immunochemistry Technologies (Bloomington, MN) as part of a 2-color (dual label) caspase-serine protease detection kit. The reagent stored in the dark at -80˚C was dissolved initially in DMSO to yield a 50 mM concentration. Aliquots were then made from this stock solution and stored frozen at -20˚C protected from light. An additional dilution in DMSO was made to get the FFCK reagent down to 10 mM concentration prior to its use for cell labeling. FFCK was then included into culture medium at 10 $\mu$M final concentration and the cells were incubated in its presence for 30 min at 37˚C; PI was added for the final 5 min of incubation. Dilution of the reagent into aqueous (cell culture) media was done just prior to cell exposure to preserve the labile chloromethyl ketone reactivity of FFCK reagent. Other details of serine protease labeling with FFCK are presented elsewhere (41,42).

*Flow cytometry*. Cellular green (Alexa Fluor 488), or FFCK, and red (PI) fluorescence emission was measured using either a FASCcan (Becton-Dickinson, San Jose, CA), or EPICS/Elite (Coulter-Beckman, Miami, FL) flow cytometers, each employing 488 nm laser as excitation source. The green and red fluorescence from each cell were separated and quantified using the standard optics and CELLQuest (Becton-Dickinson) or MultiCycle (Phoenix Flow Systems, San Diego, CA) software, respectively. Cells (10,000) were measured per each sample. Each experiment was run in duplicate and the experiments were repeated at least twice. Other details of the fluorochrome-cell labeling and flow cytometric analysis were presented before (40-44).

## Results

Fig. 1 shows morphological appearance of cells growing in the absence or presence of 2 $\mu$g/ml of ONC at 37.5 or 40˚C for 24 h. Cell growth at 40˚C in the absence of ONC had no apparent effect on their morphology and did not significantly suppress their proliferation, as evident from the presence of mitotic figures (Fig. 1B). Exposure of cells to ONC at 37.5˚C led to the appearance of relatively few apoptotic cells characterized by cell shrinkage, highly condensed chromatin and nuclear fragmentation (Fig. 1C, arrows). The frequency of apoptotic cells was distinctly higher in cultures containing ONC but maintained at 40˚C compared to cultures treated with ONC at 37.5˚C (Fig. 1D). The cells characterized by morphological changes typical of apoptosis expressed activated caspase-3 (Fig. 2).

Fig. 3 illustrates effects of ONC alone, 40˚C-hyperthermia and combined effects of ONC and hyperthermia on induction of apoptosis of TK6 cells after 24 h of the treatment. The

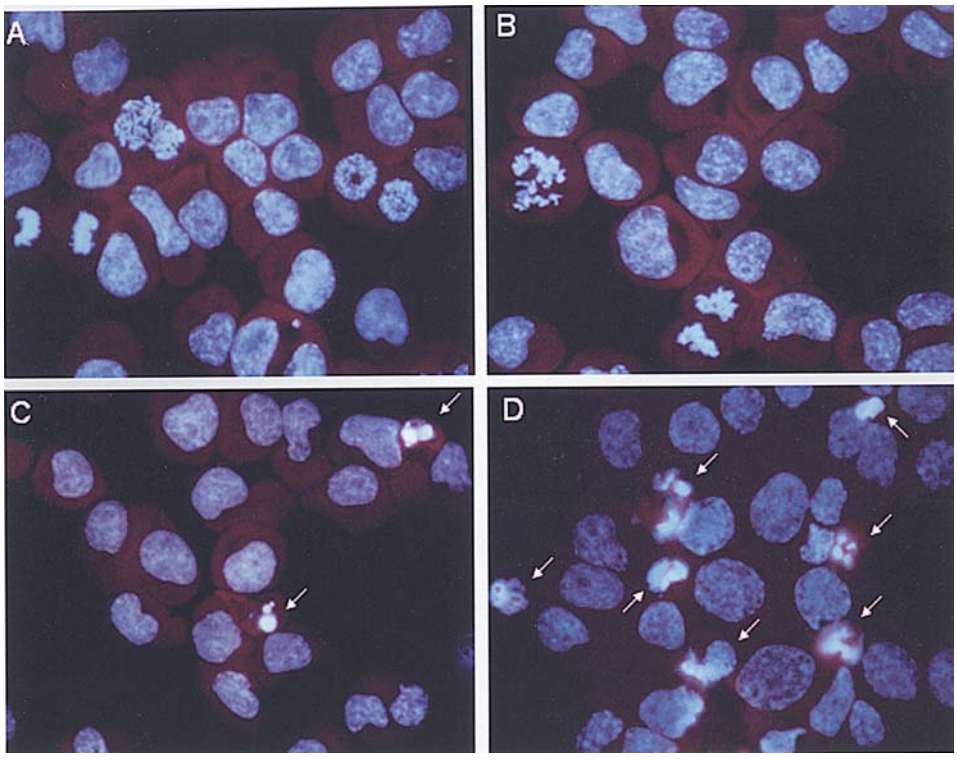


Figure 1. Morphology of TK6 cells, growing at 37.5˚C (A), at 40˚C (B), treated with 2 $\mu$g/ml of ONC at 37˚C (C) and treated with 2 $\mu$g/ml of ONC at 40˚C (D). The cells were maintained in culture at 37.5 or 40˚C without or with ONC for 24 h then were deposited on slides by cytocentrifugation, fixed, stained with DAPI and sulforhodamine 101 and examined by fluorescence microscopy as described (46). Note characteristic cell shrinkage, chromatin condensation and nuclear fragmentation of apoptotic cells (marked by arrows).

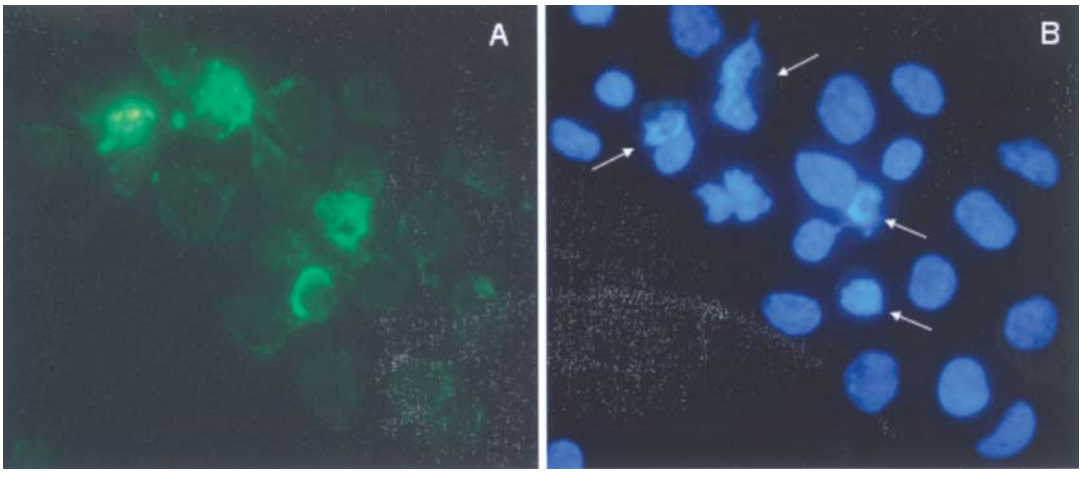


Figure 2. Activation of caspase-3 in TK6 cells grown at 40˚C in the presence of 2 $\mu$g/ml of ONC for 24 h. The cells were deposited on slides by cytocentrifugation, fixed and the presence of activated (cleaved) capase-3 was detected immunocytochemically using Alexa Fluor® 488 conjugated secondary Ab (A); cellular DNA was counterstained with DAPI (B). Note that apoptotic cells characterized by condensed chromatin (B, arrows) express activated caspase-3.

data are shown as scatterplots representing individual cells expressing activated caspase-3 vs. DNA content, measured by flow cytometry. It is quite evident that cell growth at 40˚C in the absence of ONC did not lead to any increase in the percentage of apoptotic cells as compared to 37.5˚C. The presence of minor subpopulation (6%) of cells expressing activated caspase-3 at 37.5 and 40˚C reflects the background level of spontaneous apoptosis observed in cultures of TK6 cells (40). A modest increase in percentage of apoptotic cells (to 13 or 15%, respectively) was apparent in cultures treated with 2 or 5 $\mu$g/ml of ONC at 37.5˚C. However, in cultures treated with 2 or 5 $\mu$g/ml of ONC at 40˚C the percent of apoptotic cells was increased to 39 and 49%, respectively, well above the level seen in the cultures treated with ONC at 37˚C.

The percentage of cells with activated caspase-3 in cultures exposed to ONC at 37.5 and 40˚C for the duration of 24 or

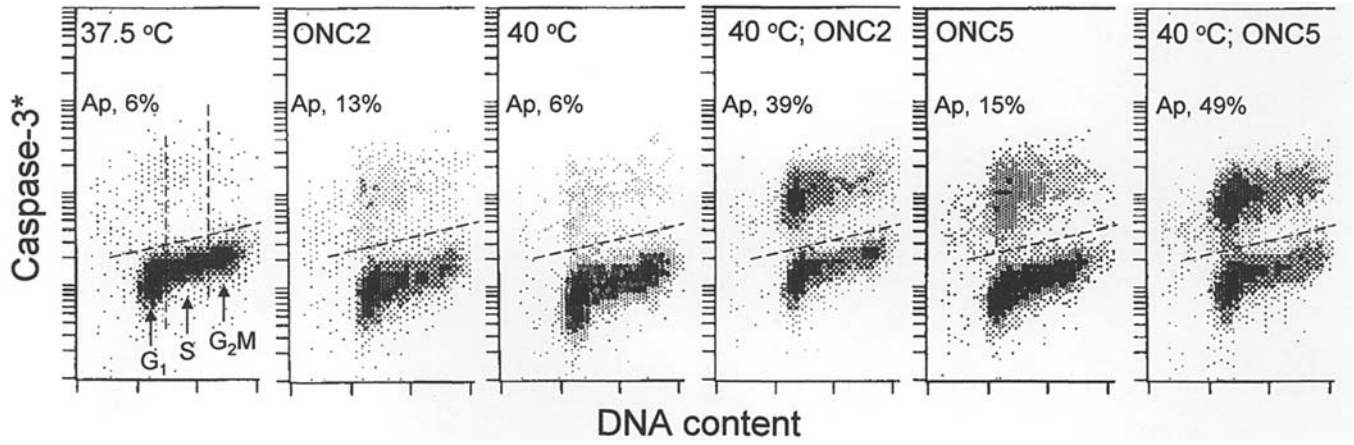


Figure 3. Induction of apoptosis of TK6 cells by ONC in cultures maintained at 37.5 and 40˚C for 24 h. Bivariate distributions (scatterplots) showing cellular DNA content vs. the presence of activated (cleaved) caspase-3 detected immunocytochemically of the untreated cells growing at 37.5˚C (37.5˚C), treated with 2 or 5 $\mu$g/ml of ONC at 37.5˚C (ONC2, ONC5, respectively) or treated with 2 or 5 $\mu$g/ml of ONC at 40˚C (40˚C, ONC2; 40˚C, ONC5; respectively). The percent of apoptotic cells (Ap) expressing activated caspase-3 (above the threshold is marked with a dashed line) is shown in each panel.

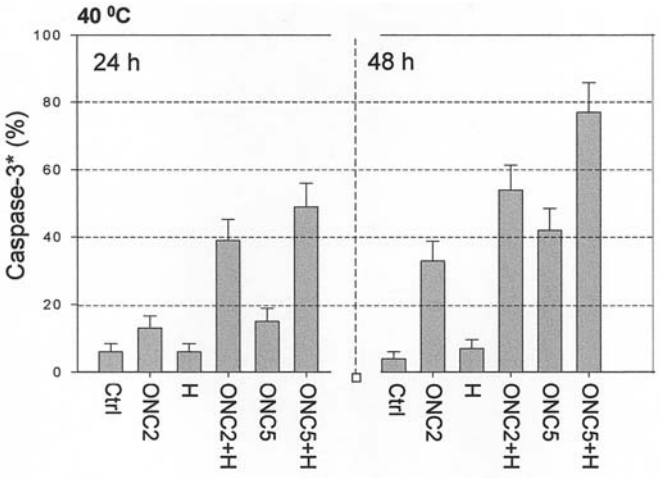


Figure 4. Induction of apoptosis of TK6 cells by ONC in cultures maintained at 37.5 and 40˚C for 24 or 48 h. The bars show percentage of cells expressing activated caspase-3 following growth at 37.5˚C in the absence of ONC (Ctrl), at 37.5˚C in the presence of 2 or 5 $\mu$g/ml of ONC (ONC2 or ONC5, respectively) or grown at 40˚C in the absence (H) or presence of 2 or 5 $\mu$g/ml of ONC (ONC2+H or ONC5+H, respectively). The bars show SD estimated based on Poisson statistical distribution.

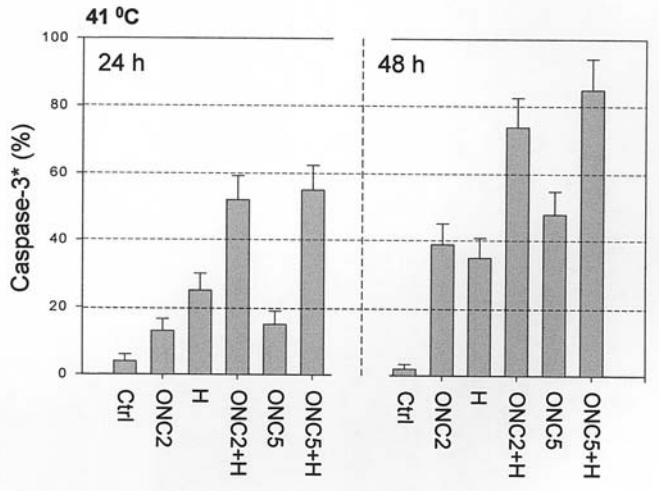


Figure 5. Induction of apoptosis of TK6 cells by ONC in cultures maintained at 37.5 and 41˚C for 24 or 48 h. The bars show percentage of cells expressing activated caspase-3 following growth at 37.5˚C in the absence of ONC (Ctrl), at 37.5˚C in the presence of 2 or 5 $\mu$g/ml of ONC (ONC2 or ONC5, respectively) or grown at 41˚C in the absence (H) or presence of 2 or 5 $\mu$g/ml of ONC (ONC2+H or ONC5+H, respectively). The bars show SD estimated based on Poisson statistical distribution.

48 h is shown in Fig. 4 as bar graphs. The bars representing cells after 24 h of the treatment report the raw data illustrated in Fig. 3. Compared to cells treated for 24 h the cytotoxic effects observed after 48 h of exposure to ONC were more pronounced. Thus, at 37.5˚C apoptotic cells were present at a higher frequency after 48 h than after 24 h. This was apparent both at 2 and 5 $\mu$g/ml concentration of ONC (33 and 42% vs. 13 and 15%, respectively). In contrast, cell growth at 40˚C in the absence of ONC for 48 h did not lead to any further increase in percentage of apoptotic cells compared to 24 h (7 vs. 6%). In the presence of 2 or 5 $\mu$g/ml of ONC at 40˚C, however, the percentage of apoptotic cells was distinctly higher after 48 h than after 24 h (54 and 77% vs. 39 and 49%, respectively).

Fig. 5 shows the results of the experiment designed as the one presented in Fig. 3 but carried out at 41˚C rather than at 40˚C. It is quite evident that unlike 40˚C, the 41˚C-hyperthermia itself was cytotoxic, inducing apoptosis of 25 and 45% of cells after 24 and 48 h, respectively. Similar as at 40˚C, however, the effects of treatment of cells with ONC at 41˚C after 24 h were greater than additive. After 48 h of treatment with 2 or 5 $\mu$g/ml of ONC at 41˚C a large majority of cells (74 or 85% cells) expressed activated caspase-3.

We observed before that during the ONC-induced apoptosis of HL-60 cells serine proteases were activated concurrently with activation of caspases (21). In the present study, therefore, we tested whether apoptosis induced by a combination of ONC and mild hyperthermia can also lead to activation of serine proteases. As before (21), the probe (FFCK) used to detect serine proteases activation was a fluorochrome-tagged analog of TPCK (TFCK according to the current amino-acid abbreviations), the permeant and specific ligand-inhibitor

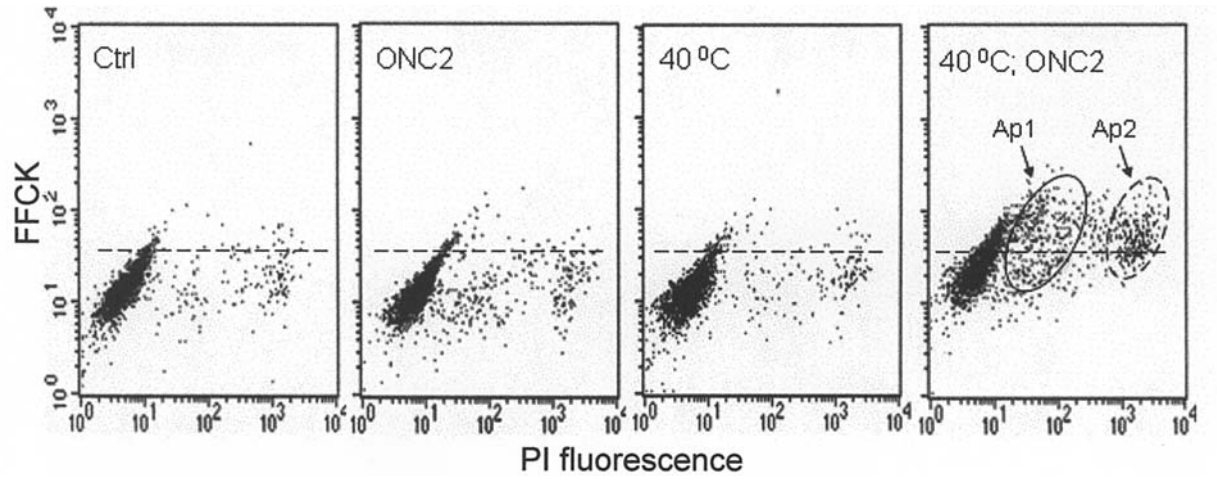


Figure 6. Activation of serine proteases in TK6 cells induced to apoptosis by treatment with ONC and hyperthermia. Bivariate distributions displaying intensity of propidium iodide (PI) fluorescence vs. fluorescence of FFCK, the carboxyfluorescein-tagged inhibitor-ligand of active center of chymotrypsin-like serine proteases (41,42). The cells were growing at 37.5˚C (Ctrl), treated with 2 $\mu$g/ml of ONC at 37.5˚C for 24 h (ONC2), growing at 40˚C for 24 h (40˚C), or treated with 2 $\mu$g/ml of ONC at 40˚C for 24 h (40˚C; ONC2), respectively. The increased PI fluorescence reports increasing permeability of plasma membrane to this fluorochrome and allows one to distinguish early- (Ap1) and late- (Ap2) apoptotic cells (44). The maximal level of FFCK binding below which >97% of cells in the untreated 37.5˚C culture (Ctrl) were located in the scatterplot is marked in each panel with dashed line. The increased FFCK binding, particularly of Ap2 cell population indicates activation of serine proteases (41,42).

of active enzymatic center of chymotrypsin-like proteases (41,42,45). The elevated FFCK fluorescence reports binding of this probe to the enzymatic center of proteases and thereby their activation (21,41,42). Concurrently, integrity of cellular plasma membrane was probed by a brief exposure of cells to PI. This cationic fluorochrome is normally excluded from live cells, to a large degree is excluded also from cells at early stage of apoptosis, but it easily penetrates plasma membrane and strongly labels cells with compromised plasma membrane integrity such as cells at late stage of apoptosis or dying by necrosis (46). As shown in Fig. 6 the assay was able to identify live vs. apoptotic cells and among the latter to distinguish early- (Ap1) vs. late- (Ap2) apoptotic cells. The data are consistent with the morphological observations (Fig. 1), and with the findings based on detection of activated caspase-3 (Figs. 2 and 3), that cell growth at 40˚C had no effect on their viability while treatment with ONC at 40˚C induced distinctly higher frequency of apoptotic cells than treatment with ONC at 37˚C. Compared with live cells (Ctrl) apoptotic cells, particularly at later phase of apoptosis (Ap2) had a higher level of FFCK fluorescence, indicative of activation of serine protease(s) in these cells (21,41,42).

## Discussion

The presented data indicate that induction of apoptosis of TK6 cells by ONC was enhanced under conditions of mild hyperthermia. Apoptosis was manifested by classical changes in cell morphology and activation of caspase-3 (Figs. 1 and 2), both considered the hallmarks of apoptotic mode of cell death (46). The presence of activated serine protease(s) was also apparent (Fig. 5), consistent with the earlier study that ONC-induced apoptosis triggers activation of these proteases as well (21).

Particularly interesting are findings showing increased incidence of apoptosis in cultures treated with ONC at 40˚C, i.e. at the temperature at which, in the absence of ONC, the cells were totally unaffected. Namely, we were unable to see any significant effect of 40˚C-hyperthermia alone, not only on incidence of apoptosis of TK6 cells (Figs. 1-3), but also on their progression through the cell cycle (data not shown). The data, thus, are encouraging in terms that they suggest that the antitumor effectiveness of ONC may be increased under conditions of very mild hyperthermia that can be tolerated by the patient. The whole-body hyperthermia at 40˚C, or even at 41˚C, is considered to be a safe therapy that does not lead to any serious or sustained organ dysfunction (47).

As mentioned in the Introduction, hyperthermia increases the therapeutic response and often is synergistic with cytotoxic drugs or radiation (31-38). The possibilities of a combination of hyperthermia with other antitumor modalities are expanding, and are being driven by the development of new approaches providing improved control of thermal delivery to the tumor, such as mediated by cancer imaging and nanoparticles of gold or magnetite (48-50). Since ONC is already in clinical trials where it shows therapeutic activity against malignant mesothelioma (5,19), it could be expected that its effectiveness may still be improved by combination with hyperthermia. Experiments are currently in progress on the effect of mild hyperthermia in combination with ONC on mesothelioma cells and comparing it with normal, non-tumor cells of different lineage *in vitro*.

The mechanism that may be responsible for the observed enhancement of cytotoxic activity of ONC under conditions of mild hyperthermia is unknown. As mentioned, ONC is taken up by the cells by the endocytic pathway (14,23,24) and as an active ribonuclease is enzymatically targeting intracellular RNA (14,28). It is thus possible that hyperthermia accelerates the endocytic process of ONC internalization and thereby its effectiveness. It is also possible that the enzymatic activity of ONC in terms of a rate of RNA digestion within the target cells, such as kinetics of other enzymes, is accelerated at higher temperature and this contributes to its enhanced cytotoxic activity. Of course, both mechanisms may play a role concurrently.

We have previously postulated that the mechanism of ONC antitumor action may involve targeting RNAi (29). The actual direct target may be microRNAs (miRNAs), whose destruction could activate translation of particular genes otherwise silenced by these mRNAs. The observed upregulation of some gene products such as CKIs by ONC would be consistent with this mechanism (20). Also consistent with this mechanism are our recent findings on up- and down-regulation by ONC of specific sets of genes in mesothelioma cell lines (unpublished data). In addition, it is conceivably that ONC may mimic activity of Dicer, a member of the RNase III enzyme family, by cleaving large transcripts and generating miRNAs with gene silencing properties (51).

The targeting RNAi may be the mechanism responsible for higher effectiveness of ONC towards tumor as compared to normal cells. It was recently shown that development of many tumors is associated with early alterations at the level of miRNA genes (52-57). These genes are often located at the genome hot spots associated with cancer. There is a growing body of evidence that miRNAs are extensively involved in pathogenesis not only of leukemias or lymphomas but also of solid tumors and they promote neoplastic growth by controlling the expression of protein-coding tumor suppressors and oncogenes (56). Thus, by targeting RNAi, ONC may be more effective in suppressing growth of tumor than normal cells.

Is there any evidence that ONC may target RNAi in frog eggs or developing embryos and could this be its physiological function? Unfortunately, little is known about the possible role of ONC of embryo development or its targets within an egg or embryo. It has been proposed that ribonucleases of RNase A superfamily having anti-pathogen activities may be involved in innate host defense (58). Within the framework of this function microbial RNA would be their primary target. However, RNAi plays a critical role in gene regulation during development (59-63). Abundance of ONC in developing embryo is clearly consistent with its possible role in RNAi-mediated gene regulation during embryogenesis.

RNases homologous to ONC are present in eggs of a variety of amphibian species (5). It is intriguing to note that the sex of amphibians is determined by the characteristic temperature at which the egg is incubated (64-66). The mechanism of sex determination appears to depend on the modulation of expression of particular genes, which varies at the temperatures favoring either males or females (66). Our present data indicate that effects of ONC are temperature-dependent and one of the possible mechanisms for the observed effect is the variation in ONC enzyme kinetics with temperature. Therefore, we speculate that if ONC or its homologues play a role in gene regulation via RNAi mechanism, the sex determining genes are among the regulated genes.

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