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In vitro cytotoxicity of ranpirnase (onconase) in combination with components of R-CHOP regimen against diffuse large B cell lymphoma (DLBCL) cell line*

Działanie cytotoksyczne onkonazy w skojarzeniu z lekami schematu R-CHOP na komórki linii chłoniaka rozlanego z dużych komórek B (DLBCL)*

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Ranpirnase (onconase; ONC) is an endoribonuclease obtained from the frog *Rana pipiens*. This enzyme exhibits anticancer properties mediated by degradation of cellular RNA and induction of apoptosis. In this study we assessed cytotoxicity of ONC in combination with currently used anticancer drugs on a human diffuse large B-cell lymphoma (DLBCL)-derived cell line (Toledo). Cytotoxic activity was measured by the exclusion of propidium iodide assay while apoptosis was assessed using the annexin-V binding method. Additionally, flow cytometry was used to assess the decline of mitochondrial potential and to determine activation of caspases 3, 8 and 9. It was observed that *in vitro* treatment with ONC in combination with rituximab, mafosfamide, vincristine, doxorubicin, and dexamethasone (drugs corresponding with elements of R-CHOP regimen) resulted in increased cytotoxicity. As a result ONC showed marked cytotoxicity against Toledo cells. Importantly, in combination of ONC with drugs imitating the R-CHOP regimen, this effect was significantly intensified. The main mechanism responsible for this event was induction of apoptosis along a mitochondrial dependent pathway. In conclusion, these data indicate that further preclinical and eventually clinical studies assessing activity of ONC+R-CHOP treatment are warranted.

Key words: **Onconase • R-CHOP regimen • diffuse large B-cell lymphoma • DLBCL • Toledo cell line**

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most frequent type of lymphoma in the Western world [27]. DLBCL is a heterogeneous group with distinct pathological subtypes that have different clinical outcome and expected treatment response. DLBCL has an extremely aggressive natural history, with a median survival of less than one year in patients without any therapy. Response to the treatment was seen to significantly improve after addition of rituximab (RIT; monoclonal anti-CD20 antigen antibody) to standard chemotherapy with cyclophosphamide, vincristine, doxorubicin, and prednisolone (CHOP regimen). This combination led to a major increase in the response rate and overall survival in first line treatment of patients with DLBCL [4]. Chemo-sensitive patients can be still cured by second line treatment with high-dose therapy with subsequent autologous stem cell transplantation (ASCT). Despite significant improvement, there is still a relapsed or refractory group with poor prognosis, even among transplant-eligible patients who need new therapeutic approaches.

Ribonucleases (RNases), which have been extensively studied since the 1960s, are a group of enzymes well known for their cytotoxic ability to degrade RNA and inhibit protein synthesis. As a whole, this superfamily of secretory enzymes is responsible for operating at the crossroads of transcription and translation. In 1972, Moore, Stein, and Anfinsen were awarded a Nobel prize for their work on RNase A. After enormous early enthusiasm, RNases were almost dismissed in terms of their clinical use until quite recently when they attracted attention again due to the discovery of their remarkable and complex biological activities [17,18]. For instance, they are able to promote angiogenesis [24], possess immunosuppressive and antitumor activity, and demonstrate aspermatogenic and embryotoxic effects [5].

In the early 1970s it was observed by Shogen and Yoon that the extract from embryos of the Northern leopard frog has a strong cytotoxic activity [28]. Twenty years later, it was found that cytotoxicity of the extract is due to the presence of a protein initially named Pannon [6] then ranpirnase (onconase; ONCONASE®; ONC) and subsequently amphinase from the first letters of *Rana pipiens* RNase [8].

Of particular interest are numerous observations that ONC acts strongly synergistically when combined with several different antitumor drugs. This was observed *in vitro* in combination with tamoxifen or trifluoperazine [21] as well as lovastatin [22] in treatment of pulmonary carcinoma A549 cells or pancreatic adenocarcinoma ASPC-1 cells. In further studies increased toxicity was observed when ONC was used in combination with vincristine [26], interferons [30], ionizing radiation [16], tumor necrosis factor α [7], differentiation-inducing agents [12], and with cepharanthine [14]. Interestingly, in combination with vincristine, ONC has shown high toxicity even against multi-drug resistant cells [26].

ONC was the first RNase among all those used in clinical trials. It was tested in phase I and phase II human clinical trials in treatment of numerous solid tumor cancers, for instance lung and pancreatic cancers [19,20]. It appeared that ONC was associated with a prolonged survival time in both groups of patients. The drawback of this treatment was that renal toxicity was dose limiting, but on the other hand, it was completely reversible if the treatment was discontinued.

Thus far there have been no reports on activity of ONC in combination with standard cytostatic treatment against DLBCL cells. In the present study we observed that ONC has marked toxicity against DLBCL-derived cells. Importantly, we show that combination of ONC with drugs imitating the standard R-CHOP regimen significantly increased the cytotoxic effect in comparison with ONC or R-CHOP tested separately.

MATERIAL AND METHODS

Cell cultures

Experiments were performed *in vitro* on a diffuse large B cell (DLBCL)-derived TOLEDO cell line (American Type Culture Collection; ATCC, Manassas, VA, USA). Cell cultures were maintained in 25 ml dishes (Nunc, Roskilde, Denmark), in concentration of 0.2×10^6/ml. Viability of TOLEDO cells before culturing was 95% or more. Cell cultures were maintained in RPMI 1640 medium conta-

ining 10% (v/v) heat inactivated fetal calf serum (FCS) and antibiotics (streptomycin 50 µg/ml, penicillin 50 IU/ml; Life Technologies, Scotland), at 37°C, 5% CO_2, fully humidified.

Drug administration settings

At the 0 h time point and after 48 h PBMC were subjected to a simultaneous assessment of viability and apoptosis. Cell were incubated for 48 h with ONC, the drug kindly provided by TAmiR Biotechnology, Inc, Somerset, NJ, USA. The drug was used in doses of 10 µg/ml–100 µg/ml. Based on pilot experiments, the lowest dose of ONC inducing significant cytotoxicity in comparison with untreated controls was chosen (10 µg/ml). Optimal doses of other drugs were chosen in a similar way. They were rituximab (RIT; 20 µg/ml), mafosfamide (MAF; 1 µg/ml), doxorubicin (DOX; 5 µg/ml), vincristine (VIN; 0.01 µg/ml) and dexamethasone (DEX; 20 µg/ml). MAF was used in this *in vitro* study as a biologically active analogue of cyclophosphamide. Combinations of those drugs with ONC were assessed: ONC+ RIT, ONC+ MAF, ONC+ DOX, ONC+ VIN, ONC+ DEX, ONC+ RIT+ MAF+ DOX+ VIN+ DEX (=ONC+R-CHOP). Each experiment was repeated at least 6 times.

Assessment of drug cytotoxicity

Cytotoxicity (cell viability) was assessed based on ability of cells to exclude the cationic fluorochrome propidium iodide (PI). After incubation with drugs, cells were washed twice in PBS and then re-suspended in 0.5 ml of 10 µg/ml PI solution in PBS. After 10 minutes of staining (room temperature, in the dark) cell fluorescence was measured by flow cytometry, using the FL3 (red) standard fluorescent filter. The cells were identified based on their forward- and side-light scatter properties and live cells were defined as PI-negative. Cytotoxic index (CI) was calculated as a percentage of PI-positive cells in drug-treated samples.

Assessment of apoptosis

Annexin-V assay. The rate of apoptosis was determined by Annexin-V assay after 72 h of incubation with the drugs. In brief, after incubation cells were washed twice with cold PBS and then re-suspended in 100 µl of binding buffer, containing 2 µl of FITC conjugated Ann-V and 10 µl of 10 µg/ml PI (Becton-Dickinson, San Jose, CA, USA). Next, the samples were incubated for 15 minutes, at room temperature, in the dark. The fluorescence was measured immediately after staining by flow cytometer using FL1 (green, Ann-V) and FL3 (red, PI) standard fluorescent filters. Apoptotic index (AI) was calculated as the percentage of Annexin-V-positive cells in drug-treated samples.

Detection of activated caspases. Caspase-3 activation was detected using FITC-conjugated monoclonal rabbit anti-active caspase-3 antibody (BD Pharmingen, San Diego, CA, USA). After incubation cells were fixed and permeabilized using Cytofix/CytopermTM (BD Pharmingen, San Diego, CA, USA) solution (20 minutes, on ice), then washed twice and re-suspended in Perm/WashTM buffer (BD Pharmingen, San Diego, CA, USA). The antibody was added in the amount of 60 µl per 300 µl of cell suspension (30 minutes incubation, at room temperature). The fluorescence was measured directly after staining and washing in Perm/WashTM buffer by flow cytometry using FL1 filter for detecting the green fluorescence of anti-active caspase-3 antibody.

Synthetic fluorochrome labeled fluoromethyl ketone peptides that are cell permanent and bind to the active catalytic site of caspase proteases were developed by Immunochemistry Technologies LLC (Bloomington, MN, USA). Commercially available FAM-LETD-FMK FLICA™ Caspase 8 Assay Kit and FAM-LEHD-FMK Reagent-9 FLICA™ Caspase 9 Assay Kit were used for assessment of caspase-8 and caspase-9 activation. According to the insert-protocol it was initially prepared as a 150x concentrated solution in dimethyl sulfoxide (DMSO; SIGMA Aldrich, St. Louis, MO, USA). Aliquots were stored at -20°C, protected from light. Directly before use, they were diluted in PBS (1:5), and then added to 300 µl of culture to obtain a 10 mM concentration of FAM-LETD-FMK (caspase-8 detection) or FAM-LEDH-FMK (caspase-9 detection), respectively, for the final hour of incubation. Cultures were terminated by washing the cells twice (5 minutes, 140xg) with the "wash buffer" (component of both kits). After centrifugation, the pellets were re-suspended in 1 ml of wash buffer and the samples were placed on ice. Cell green fluorescence derived from FAM--LETD-FMK or FAM-LEDH-FMK was measured during the next 15 minutes by flow cytometry.

Fluorescence measurements

All fluorescence measurements were performed by flow cytometry (FACScan; Becton-Dickinson, San Jose, CA, USA), using standard emission filters: green (l=530±20 nm; FL1), orange (l=560-600 nm; FL2) and red (l>600 nm; FL3), where necessary. Ten thousand cells per sample were calculated routinely in all experiments.

Statistics

For the statistical analysis of data, the range of the measured variable, means and standard deviations (SD) were calculated, using statistical software (STATISTICA v.7.0, Tulsa, OK, USA). The data are presented as a median or mean ± SD values. The differences between values were evaluated with Student's t-test. P values less than 0.05 were considered statistically significant.

RESULTS

Cytotoxic effects

After 48 h of treatment with 10 µg/ml of ONC the mean CI was 25.4% (vs. control p=0.002); similar cytotoxicity was induced by MAF (26.7%; vs. control p=0.001). VCR

showed the highest activity, even at a very low dose (36%, vs. control p<0.001). RIT, DEX, and DOX induced the following mean CIs: 12.9%, 10.2% and 15.6%, respectively (vs. control all – p<0.05) (Figure 1).

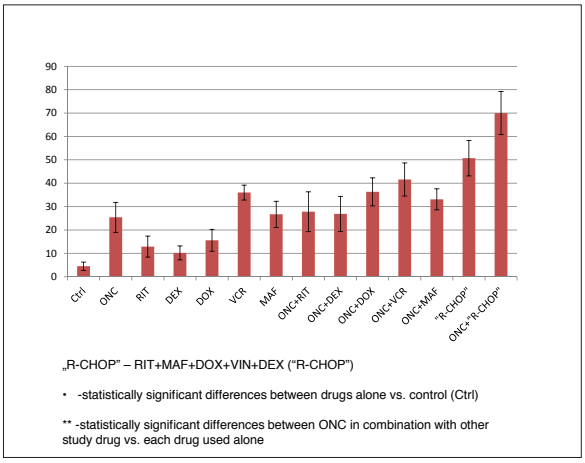

Fig. 1. Cytotoxicity of onconase (ONC) alone and in combinations with rituximab (RIT), mafosfamide (MAF), doxorubicin (DOX), vincristine (VIN) and dexamethasone (DEX) as measured by propidium iodide (PI) assay in Toledo cells, after 48 h of incubation

The combinations ONC+RIT, ONC+DEX, ONC+DOX exerted a statistically significant increase in cytotoxicity compared to RIT, DEX and DOX alone (p=0.006, p=0.0005 and p=0.0006, respectively). The effect of ONC+DOX was significantly higher also compared to ONC alone (p=0.018). The combination ONC+VCR exerted significant cytotoxicity only in comparison to ONC (p=0.003). Combined treatment with ONC+MAF did not markedly increased cytotoxicity of single drugs (Figure 1).

Most importantly, the combination ONC+R-CHOP (RIT+MAF+DOX+VCR+DEX) showed significantly higher CI than R-CHOP (p=0.007) (Figure 1).

Proapoptotic effects

Ann-V/PI assay showed proapoptotic activity of ONC against DLBCL cells. After 48 h of treatment the mean AI was 11.3% (vs. control - p=0.031). DOX, VCR and MAF induced significant levels of apoptosis: 14.4%, 21.1% and 17.1%, respectively (vs. control – p=0.012, p=0.019 and p=0.022, respectively). RIT and DEX did not trigger significant apoptosis (vs. control – p>0.05) (Figure 2).

The pro-apoptotic effect induced by combinations ONC+RIT and ONC+DEX was markedly higher than RIT and DEX alone (p=0.024 and p=0.008, respectively). Mean AI in samples treated with ONC+DOX was significantly higher than both ONC (p=0.012) and DOX (p=0.005) used separately. ONC+VCR combination induced apoptosis significantly higher only in comparison to ONC (p=0.005) (Figure 2).

Finally, ONC in combination with R-CHOP drugs induced significantly higher mean AI than R-CHOP (p=0.002) (Figure 2).

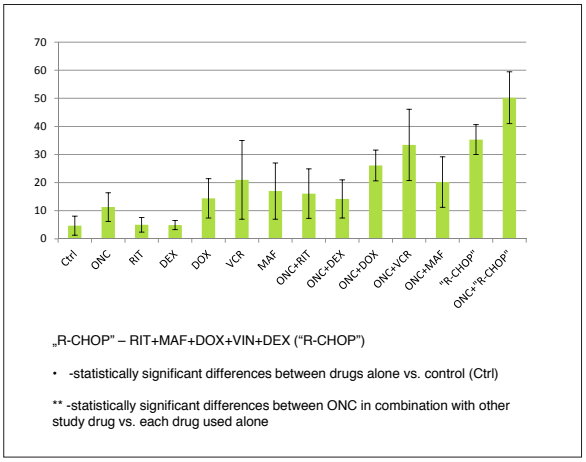

Fig. 2. Proapoptotic effects of onconase (ONC) alone and in combinations with rituximab (RIT), mafosfamide (MAF), doxorubicin (DOX), vincristine (VIN) or dexamethasone (DEX) as measured by annexin-V/propidium iodide (Ann-V/PI) assay in Toledo cells, after 48 h of incubation

Drug-induced caspase activation

ONC significantly activated caspase-3 in Toledo cells (vs. control – p=0.001). Also DOX, VCR, MAF and DEX used alone in the established doses induced marked caspase-3 activation (vs. control – p=0.0006, p=0.0009, p=0.036 and p=0.042, respectively). RIT used in the established dose did not significantly trigger caspase-3 (Figure 3).

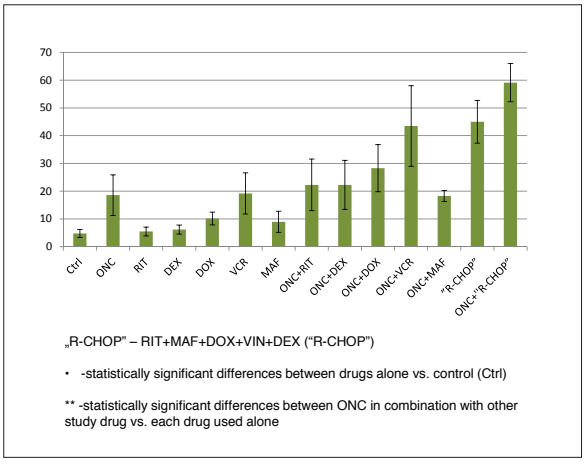

Fig. 3. Caspase-3 activation in Toledo cells by onconase (ONC) alone and in combinations with rituximab (RIT), mafosfamide (MAF), doxorubicin (DOX), vincristine (VIN) or dexamethasone (DEX). Results of 48 h incubation

The combinations ONC+RIT, ONC+DOX and ONC+DEX significantly activated caspase-3 (vs. RIT – p<0.001, DOX – p<0.003 and DEX – p<0.003, respectively) (Figure 3). ONC+VCR p<0.004

The combination ONC+R-CHOP significantly increased the percentage of caspase-3-positive in comparison to R-CHOP used separately (p=0.028). Interestingly, R-CHOP also triggered activation of both caspase-8 and caspase-9 (p=0.002 and p=0.003, respectively). Combination of ONC and R-CHOP further intensified this effect (Figure 4).

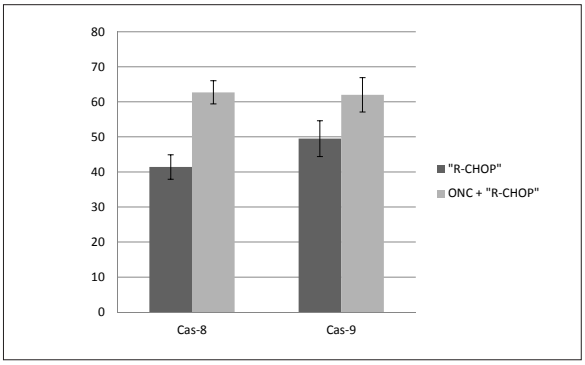

Fig. 4. Caspase-8 (Cas-8) and caspase-9 (Cas-9) activation in Toledo cells by combined treatment with rituximab (RIT), mafosfamide (MAF), doxorubicin (DOX), vincristine (VIN) and dexamethasone (DEX) (R-CHOP combination) vs. Onconase (ONC)+ R-CHOP treatment. Results of 48 h incubation

DISCUSSION

Nowadays, in patients with DLBCL treated with R-CHOP as a first line immunochemotherapy the cure rate is approximately 50%. Despite these advances, half of the patients will experience an early relapse, partial response, or are refractory after the initial treatment. In this context, innovative agents and new treatment strategies are warranted. A novel promising therapy in DLBCL lymphoma potentially can be provided by RNases, which have an entirely different mechanism of action than the chemotherapeutics currently used in the treatment.

The supposed antitumoral and immunosuppressive mechanism of ONC involves apoptosis in the majority of neoplastic cells [13]. In previous studies, it was observed that ONC selectively alters expression of genes involved in apoptosis with decreased expression of anti-apoptotic BCL-2 as well as increased expression of pro-apoptotic BAX [2]. In this study, we assessed the pro-apoptotic effect of ONC in combination with currently used anticancer drugs and the data were similar. Ann-V/PI assay showed pro-apoptotic activity of ONC after 48 h of treatment with mean AI that was 11.3% (vs. control – $p=0.031$). Moreover, the pro-apoptotic effect induced by combinations ONC+RIT and ONC+DEX was markedly higher than RIT and DEX alone ($p=0.024$ and $p=0.008$, respectively). Mean AI in samples treated with ONC+DOX was significantly higher than both ONC and DOX used separately. Last but not least, ONC in combination with R-CHOP induced significantly higher mean AI than ONC ($p<0.001$) and R-CHOP when used alone ($p=0.002$).

It is believed that programmed cell death is mostly induced by the mitochondrial pathway involving caspase-9 as the initiator [11]. As a result, the role of executioner is attributed to caspase-3 and caspase-7. In this pathway neither caspase-8 nor the tumor-suppressor protein p53 is required. Our studies demonstrated elevated activation of caspase-3 with ONC alone and in the majority of combinations (ONC+RIT, ONC+DOX, ONC+VCR and ONC+DEX). What is more, synergy in caspase-3 activation in the combined treatment ONC+R-CHOP was significant. Our results were in agreement with data presented by Grabarek et al. [11].

It was reported that ONC is more toxic to neoplastic cells than to normal cells in both *in vitro* and *in vivo* studies. The mechanism responsible for this phenomenon is still unclear and several explanations have been proposed. According to one ONC is more selectively internalized by tumor compared to normal cells due to differences in charge of the plasma membrane [15]. Specifically, in neoplastic cells the level of sialic acid, rich in gangliosides, is markedly higher, which contributes to electro-negativity of the plasma membrane [9]. Because ONC is strongly cationic it has greater electrostatic affinity to the anionic surface and thus is more avidly internalized by tumor cells. Another proposed mechanism takes into an account different intracellular routes for ONC in neoplastic versus normal cells with different respective rates of RNA degradation, especially in fast growing tumors [25,32].

The cytotoxic effect mentioned above has already been investigated in several studies. It was observed that ONC acts strongly synergistically when combined with numerous well-known antitumor agents. Synergism in the cytotoxicity was reported when ONC was used in combination with either tamoxifen or trifluoperazine on human lung carcinoma or pancreatic adenocarcinoma cell lines [21]. After this study, a series of subsequent investigations were performed. All of them revealed synergism or additive effects of ONC in combination with a variety of other widely used agents such as lovastatin [22], vincristine [26], tumor necrosis factor α [7], or interferons [26]. The striking feature is that the observed synergisms were in combinations with different antitumor agents, with completely distinct mechanisms of action. The data of the present studies are consistent with the above-mentioned observations. We observed the apparent enhancement of cytotoxicity for RIT, DEX, VCR, and R-CHOP towards DLBCL cells when combined with ONC. Of particular interest is the finding that the combination ONC+R-CHOP showed significantly higher CI than both ONC and R-CHOP implemented separately. Although it may be an interesting therapeutic target for this group of patients, thus far, there are no similar data with which to compare in the published literature.

The mechanism by which ONC sensitizes tumor cells to other drugs and thus is responsible for the synergistic or additive effects, as discussed, is not fully understood either. The evidence that ONC down-regulates and prevents activation of nuclear factor kappa B (NFκB), known to have anti-apoptotic and pro-survival properties [3], prompted the authors to propose this mechanism [10,31]. Specifically, by targeting NFκB ONC makes cells more sensitive to most agents that induce apoptosis. On the other hand, the observation that ONC targets intracellular siRNA [1,34] suggests that it may prevent the induction of RNA interference, which is often induced in cells treated with anticancer drugs, protecting them from the treatment [29].

Recently, there were attempts to bind ONC with antibody in order to increase toxicity selectively towards tumor cells. Neoplastic cells from B-cell non-Hodgkin's lymphomas, including DLBCL, display on their surface the CD22 antigen. Fusion of a human monoclonal antibody against CD22 with ONC will increase binding to tumor cells. Moreover, this protein displayed enhanced specificity, potency and decreased systemic toxicity in experiments on mice [23].

The further direction of anticancer therapy is thought to involve aggressive protocols of chemotherapy in combination with targeted therapies. As discussed above, there is pre-clinical evidence that ONC, as a new class of adjunct drugs, may significantly enhance therapeutic activity of standard chemotherapeutic treatments of various tumors including DLBCL. Such an approach may be a promising direction in further multi-drug clinical application. The full therapeutic potential and mechanism of action still remain to be elucidated.

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