IN VITRO STUDY OF SYNERGISM BETWEEN ⁹⁰YTTRIUM-EDTA AND CHOP REGIMEN IN RAMOS CELLS

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ABSTRACT: To demonstrate the in vitro synergistic antiproliferation between ⁹⁰Yttrium-EDTA and standard treatment for lymphoma-CHOP regimen including cyclophosphamide, doxorubicin, and vincristine. Ramos cell line, CD20-bearing lymphoma cells, was used as representative for aggressive type non-Hodgkin's lymphoma. Cells were treated with chemotherapy alone or with sequential therapy of chemotherapy and 2 or 4 µCi/ml ⁹⁰Yttrium-EDTA for 24 hours. After further 72 and 96 hours incubation in standard condition, changes in proliferation were determined by Rezasulin. Antiproliferation IC₅₀ and IC₉₀ of each drug were estimated and dose modifying factors were calculated. The results showed that ⁹⁰Yttrium at 2 and 4 µCi/ml had no antiproliferative effect. Sequential use of ⁹⁰Yttrium and cyclophosphamide or vincristine inhibited cell proliferation over 60 or 80%, respectively. On the contrary, IC₅₀ due to combined ⁹⁰Yttrium-doxorubicin was not significantly different from those treated with doxorubicin alone. Besides, no viable cell was found after treatment with sequential of ⁹⁰Yttrium-cyclophosphamide, doxorubicin, and vincristine. This in vitro study suggests that sequential combination of ⁹⁰Yttrium and the standard CHOP regimen provide more benefit for aggressive lymphoma than chemotherapy alone.

Keywords: antilymphoma drugs, Cyclophosphamide, Doxorubicin, Non-Hodgkin's lymphoma, Vincristine, ⁹⁰Yttrium

INTRODUCTION: Diffuse large B-cell lymphoma (DLBCL) is the most common type non-Hodgkin's lymphoma (NHL), classified as an intermediate grade and aggressive type. Approximately 75% of patients presented with bulky stage II, or stage III-IV associated with worse prognosis on diagnosis. The introduction of standard treatment-CHOP regimen including cyclophosphamide, doxorubicin, vincristine, and prednisone - turned DLBCL to a curable disease. However, its long-term survival is still unpredictable due to the heterogeneity in molecular pathology. Besides, most patients suffered from serious side effects during chemotherapy administration. CD20 is a transmembrane receptor responsible for cell cycle progression, signal transduction in B-lymphocyte. Its expression is restricted to pre-B-cell stage to late stage of plasmacytoid immunoblast. Currently, CD20 protein has been accepted as an effective target for B-cell depletion in hematologic cancer. ⁹⁰Yttrium ibritumomab tiuxetan (Y2B8), a radioimmunoconjugate, was approved as target therapy for NHL by United State Food and Drug Administration Committee in 2002. Its molecule composed of a beta radiation-emitter (⁹⁰Yttrium) and a murine immunoglobulin specific to CD20 (ibritumomab) as its carrier; both are linked to a linker-tiuxetan. It is generally safe except the most common delayed and reversible dose-limiting cytopenia. Due to the crossfire effect of radioisotope ⁹⁰Yttrium and the specificity to CD20+ cancer cells, Y2B8 provides more advantage over its allied antibody, rituximab, in treating bulkier tumors or non-responsive tumors. Y2B8 is now considered as an alternative to CHOP regimen and rituximab for aggressive lymphoma. Unfortunately, unexpected low overall response rate (44%) was reported in a study of 104 DLBCL patients treated with Y2B8 alone as a second-line therapy. Some studies suggested that the response rate to Y2B8 inversely correlates with prior treatment. Another trial performed in relapsed or refractory cases demonstrated that early treatment of Y2B8 promised more durable and higher overall response rate. All available retrospective data revealed that patients who have been treated with Y2B8 can tolerate later therapies, such as chemotherapy, high dose chemotherapy regimen, stem-cell transplantation, rituximab, and even retreatment with radioimmunoconjugate. These trials suggest the logical of developing a sequential treatment protocol of Y2B8 and chemotherapy in

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DLBCL patients that might provide higher response rate. This study was therefore aimed to determine the *in vitro* synergism in anti-proliferation between $^{90}$Yttrium and standard antilymphoma - CHOP regimen in Ramos cell line as representative for aggressive DLBCL non-Hodgkin’s lymphoma.

**MATERIALS AND METHODS:**

**Cell culture**

Ramos cells (RA1) (ATCC Code CRL-1596, LN 3953138.), a human CD20-expressing lymphoma cell line, were incubated in RPMI 1640 [Gibco] medium with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate, in standard incubation environment composing 5% carbon dioxide in air atmosphere at 37°C according to manufacturer’s recommendation. Cells were replenished with fresh complete media every 2-3 days to maintain cell density between $2 \times 10^5$ and $1 \times 10^6$ cells/ml. Lymphoma cells in the exponential growth phase, confirmed with over 90% survival by Trypan blue exclusion assay, were used in all experiments.

**Drugs and chemicals**

Cyclophosphamide injection 20 mg/ml (Baxter, LN 7H547), Doxorubicin injection 2 mg/ml (Pfizer, LN CN12D), Vincristine injection 1 mg/ml (Pharmacia, LN CXZ 7F), $^{90}$Yttrium-EDTA, Trypan blue solution, and Rezasulin powder. All powdered drugs were reconstituted with sterile distilled water according to manufacturer’s procedure. Serial 10-fold dilutions of each drug in incomplete media were prepared freshly before each experiment to provide at least 5 different doses of each combination.

**The *in vitro* study of drug synergism**

All data were generated from 3 repeated experiments which performed in triplicate. Cells were harvested and resuspended in 96 well culture plates at high density of $1 \times 10^6$ cells/ml similarly to lymphomic cells-riched environment.

Cells were treated with each drug alone ($^{90}$Yttrium-EDTA, cyclophosphamide, doxorubicin, and vincristine) for 24 hours or with combination of chemotherapy and $^{90}$Yttrium-EDTA as 2 different sequentials: $^{90}$Yttrium-EDTA followed by antilymphoma drug, and vice versa. In either sequence, the second agent was administered 3 hours after the first agent without washing out between the doses. At the end of 24 hours incubation time with drugs since the addition of the first agent, cells were washed twice with incomplete media. $^{90}$Yttrium-EDTA at two fixed doses, 2 and 4 µCi/ml (estimated from ref.) 12, were used, providing 4 treatment arms for each drug. Cells treated with incomplete media were used as control. At least 5 of 10-fold dilutions of each drug were prepared for each experiment to create dose-growth inhibitory effect curve calculated from cell viability based on Rezasulin assay13. Then, $IC_{50}$ and $IC_{90}$ values were determined using curve fitting method14,15 estimated by the program ORIGIN PRO 8. The synergy in antiproliferation were evaluated as % growth inhibition and dose modification factors (DMF) as follows.

**Determination of growth inhibition:**

Each 45 µl of Ramos cells at density $1 \times 10^6$ cells/ml was plated into 96 well plate. Cells were then incubated with 5 µl of each drug alone or drug combination for 24 hours as described above. Cells were then washed twice with incomplete media and further incubated with 200 µl fresh complete media for additional 72 or 96 hours. At the end of incubation period, cells were incubated with Rezasulin 20 µg/ml (final concentration) for at least 5 hours in standard condition for cell culture. The absorbance was determined at the wavelength 570/600. The percentages of growth inhibition were calculated from this following equation:

$$\% \text{ growth inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of treated}}{\text{Absorbance of control}} \times 100$$

**Calculation of Dose modification factors (DMF):**

Dose modification factors (DMF) were estimated as follows16.

$$DMF = \frac{IC_{50} \text{ drug alone}}{IC_{50} \text{ combined therapy}} \times \text{surviving fraction} \left( ^{90} \text{Yttrium treated alone} \right)$$

Synergism was indicated by the value of DMF larger than 1. The increase in DMF suggested the higher degree of synergism.
Statistical analysis

Reliable correlation coefficient due to regression analysis was set at > 0.9. In vitro study parameters were presented as mean ± standard deviation or other appropriate statistics according to data distribution. One way or two way ANOVA, where appropriate, is used to confirmed statistical difference at 95% significance level.

RESULTS:

Growth inhibition after treated with each drug alone

$^{90}$Yttrium at concentration higher than 10 µCi/ml showed antiproliferation to Ramos cells with slightly difference between 72 and 96 hours incubation period (Figure 1). Those cells incubated for 96 hours show less inhibitory effect than those incubated for 72 hours. This probably resulted from re-proliferation of some viable cells due to Ramos’s doubling time of 20 hours and short half-life of $^{90}$Yttrium. This implied that $^{90}$Yttrium at 2 and 4 µCi/ml had no antiproliferation to Ramos cells. Repeated experiment also confirmed that $^{90}$Yttrium at 2 and 4 µCi/ml did not inhibit cells proliferation. Cyclophosphamide at 20 - 200 µg/ml showed statistical difference between those incubated for 72 and 96 hours. Both IC$_{50}$ and IC$_{90}$ assessed after 96 hours (19.54 µg/ml and 0.48 mg/ml, respectively) were lower than those observed after 72 hours incubation time (268.19 µg/ml and 1.27 mg/ml, respectively). No significant difference in antiproliferation profile due to doxorubicin, and vincristine when compared between 72 and 96 hours. Their IC$_{50}$ and IC$_{90}$ were demonstrated in Figure 1.

Figure 1 Inhibition of proliferation of Ramos cell, density 1x10$^6$ cells/ml, evaluated at 72 and 96 hours after treating with each of $^{90}$Yttrium, cyclophosphamide, doxorubicin, and vincristine for 24 hours

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$IC_{50}$ after 72 hours incubation = 49.69 µCi/ml
$IC_{50}$ after 96 hours incubation = 40.61 µCi/ml

$IC_{90}$ after 72 hours incubation = 0.48 mg/ml
$IC_{90}$ after 96 hours incubation = 49.69 µCi/ml

$IC_{50}$ after 72 hours incubation = 0.12 µg/ml
$IC_{50}$ after 96 hours incubation = 0.06 µg/ml

$IC_{90}$ after 72 hours incubation = 0.41 µg/ml
$IC_{90}$ after 96 hours incubation = 0.19 µg/ml

$IC_{50}$ after 72 hours incubation = 19.54 µg/ml
$IC_{50}$ after 96 hours incubation = 268.19 µg/ml

$IC_{90}$ after 72 hours incubation = 1.27 mg/ml
$IC_{90}$ after 96 hours incubation = 0.48 mg/ml

$IC_{50}$ after 72 hours incubation = 3.13 ng/ml
$IC_{50}$ after 96 hours incubation = 0.59 ng/ml

$IC_{90}$ after 72 hours incubation = 5.40 ng/ml
$IC_{90}$ after 96 hours incubation = 1.80 ng/ml

$IC_{50}$ after 72 hours incubation = 0.001 1E-05 1E-04 0.001 0.01 1 10 100
$IC_{50}$ after 96 hours incubation = 0.01 0.1 1 1000

$IC_{90}$ after 72 hours incubation = 0.59 ng/ml
$IC_{90}$ after 96 hours incubation = 3.13 ng/ml

$IC_{90}$ after 72 hours incubation = 1.80 ng/ml
$IC_{90}$ after 96 hours incubation = 5.40 ng/ml
Growth inhibition after treated with drug combinations

Concerning cyclophosphamide, it seemed that combined treatment with 90Yttrium provided higher degree of inhibition comparing with treatment with cyclophosphamide alone (Figure 2). The larger difference was shown when cells were incubated for 96 hours without any significance difference between two sequential or between 2 and 4 µCi/ml 90Yttrium. On the contrary, in those incubated for 72 hours, treatment with 90Yttrium -> cyclophosphamide provided higher degree of inhibition than the other sequential. Over 60% inhibitory effect was observed after 96 hours incubation time among cells treated with combined drug.

Similar inhibition profile was revealed among cells treated with doxorubicin alone or combined with 90Yttrium (Figure 3). Correspondingly, all calculated DMF could not demonstrate favorable synergism with 90Yttrium. This implies no or little synergistic effect between doxorubicin and 90Yttrium. The combination of vincristine- 90Yttrium in any sequence exhibits strong antiproliferation to Ramos cells since over 80% inhibition was found in every treatment arm (Figure 4). Complete inhibition of cell proliferation was confirmed in another experiment of combined therapy of 90Yttrium and part of CHOP regimen including cyclophosphamide, doxorubicin, and vincristine using IC50 level of each drug. There was no viable cell left after 72 or 96 hours incubation period.

To summarize, combination therapy of 90Yttrium and cyclophosphamide, lower than 0.2 µg/ml, and vincristine demonstrated strong synergy in antiproliferation to Ramos cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC50 (µg/ml)</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide alone</td>
<td>268.19</td>
<td></td>
</tr>
<tr>
<td>2 µCi/ml Yt → Cyclophosphamide</td>
<td>219.48</td>
<td>1.22</td>
</tr>
<tr>
<td>4 µCi/ml Yt → Cyclophosphamide</td>
<td>382.31</td>
<td>0.70</td>
</tr>
<tr>
<td>Cyclophosphamide → 2 µCi/ml Yt</td>
<td>688.52</td>
<td>0.39</td>
</tr>
<tr>
<td>Cyclophosphamide → 4 µCi/ml Yt</td>
<td>958.94</td>
<td>0.27</td>
</tr>
<tr>
<td>Cyclophosphamide alone</td>
<td>19.54</td>
<td></td>
</tr>
<tr>
<td>2 µCi/ml Yt → Cyclophosphamide</td>
<td>cannot assess</td>
<td></td>
</tr>
<tr>
<td>4 µCi/ml Yt → Cyclophosphamide</td>
<td>cannot assess</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide → 2 µCi/ml Yt</td>
<td>cannot assess</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide → 4 µCi/ml Yt</td>
<td>cannot assess</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2 Inhibition of proliferation of Ramos cell, density 1x10⁶ cells/ml, evaluated at 72 and 96 hours after treating with 90Yttrium combined with cyclophosphamide for 24 hours
After 72 hours incubation period

**Figure 3** Inhibition of proliferation of Ramos cell, density 1x10⁶ cells/ml, evaluated at 72 and 96 hours after treating with ⁹⁰Yttrium combined with doxorubicin for 24 hours

- **Drug alone**
- **⁹⁰Yttrium 2 μCi/ml → Drug**
- **⁹⁰Yttrium 4 μCi/ml → Drug**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC₅₀ (μg/ml)</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin alone</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>2 μCi/ml Yt → Doxorubicin</td>
<td>0.24</td>
<td>0.50</td>
</tr>
<tr>
<td>4 μCi/ml Yt → Doxorubicin</td>
<td>0.22</td>
<td>0.57</td>
</tr>
<tr>
<td>Doxorubicin → 2 μCi/ml Yt</td>
<td>0.13</td>
<td>0.93</td>
</tr>
<tr>
<td>Doxorubicin → 4 μCi/ml Yt</td>
<td>0.22</td>
<td>0.55</td>
</tr>
</tbody>
</table>

After 96 hours incubation period

**Figure 4** Inhibition of proliferation of Ramos cell, density 1x10⁶ cells/ml, evaluated at 72 and 96 hours after treating with ⁹⁰Yttrium combined with vincristine for 24 hours

- **Drug alone**
- **⁹⁰Yttrium 2 μCi/ml → Drug**
- **⁹⁰Yttrium 4 μCi/ml → Drug**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC₅₀ (ng/ml)</th>
<th>DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vincristine alone</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>2 μCi/ml Yt → Vincristine</td>
<td>cannot assess</td>
<td></td>
</tr>
<tr>
<td>4 μCi/ml Yt → Vincristine</td>
<td>cannot assess</td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>Vincristine → 4 μCi/ml Yt</td>
<td>cannot assess</td>
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</tr>
</tbody>
</table>

**Figure 3**
- Drug alone
- ⁹⁰Yttrium 2 μCi/ml → Drug
- ⁹⁰Yttrium 4 μCi/ml → Drug

**Figure 4**
- Drug alone
- ⁹⁰Yttrium 2 μCi/ml → Drug
- ⁹⁰Yttrium 4 μCi/ml → Drug
CONCLUSION AND DISCUSSION: Ramos cell line is a CD20-rich lymphoma cell derived from Burkitt’s lymphoma, another serious lymphoma similar to diffuse large B-cell lymphoma (DLBCL). Even though it expresses less amount of CD20, it exhibits general properties of B-lymphocyte similarly to DLBCL cells17).

Due to specific characteristics of $^{90}$Yttrium ibritumomab tiuxetan (Y2B8) designing as a systemic target therapy for B-lymphoma cells and its instability7), $^{90}$Yttrium-EDTA is more appropriate for in vitro study. The difference between these two molecules is Y2B8’s specificity to CD20 containing lymphoma cells. However, since this study was performed in vitro with CD20 containing cell line, the effect of $^{90}$Yttrium apply directly to the cells can estimate the effect of Y2B8 on in situ lymphoma cells.

Lymphomas are very radiosensitive tumors. Radiotherapy alone or combined with chemotherapy has long been used to treat these diseases18). Low dose rate radiotherapy (LDR RT) results in fewer ionizing events per minute than high dose rate radiotherapy (HDR RT). This effect is partially attributed to G2/M arrest that results in apoptosis. Some tumor cells may be more susceptible to the low-dose-rate radiation used in radioimmunotherapy than to the high-dose-rate radiation as in external beam radiotherapy19). Various cells are susceptible to LDR RT differently. Some appeared to die via apoptosis while the others have cellular necrosis20). Generally, dose rates of 2–3 cGy/hr counteract proliferation, while exposing to higher dose rates for a few days, could cause 2 or 3 logs of cell kill21). Therefore, benefit of radioisotope particularly at low dose is long term cell destroying effects rather than short term cytotoxic effect. This supports that $^{90}$Yttrium > 10 $\mu$Ci/ml exhibited dose-dependently antiproliferative effect. Paradoxically, pre-treatment with $^{90}$Yttrium might result in unhealthy cells that may easily die or be destroyed by following chemotherapy.

Two fixed dosage of 2 and 4 $\mu$Ci/ml $^{90}$Yttrium used in this study were primarily estimated from its allied derivative, $^{131}$Iodine tositumomab, in another study22). Both concentrations were considered subtherapeutic for antiproliferative effect as shown in this study. However, most experiments did not show the significant difference between two dosages. This may be due to too close levels. Therefore, lower concentration of $^{90}$Yttrium should be tried. Two opposite sequentials of drug combination were chosen since the order of drug administration might provide different effect. Theoretically, due to the crossfire effect and the high penetrative effect of $^{90}$Yttrium, pre-treatment with isotope may provide more benefit than the vice versa treatment. However, no significant difference in two sequentials was observed in any drug pairs.

Large difference was shown in the combination of $^{90}$Yttrium and cyclophosphamide comparing between different incubation times, similarly to the experiment on cyclophosphamide alone. The higher degree in antiproliferation may be due to the immunosuppressive effect of cyclophosphamide that suppresses cell growth20). Therefore, less remaining cells to face with radioisotope when comparing to the effect of other drug pairs. The high degree of synergism is also revealed with the combination with vincristine. Similar to cyclophosphamide but via different mechanism of action, vincristine reduces number of healthy cells in the wells resulting in more effect of radioisotope on remaining cells23). Doxorubicin showed minimal synergy at high level $^{90}$Yttrium assessed after 96 hours. This maybe resulted from doxorubicin producing reactive oxygen species (ROS) that consume much surrounding oxygen. Hypoxic environment usually desensitize radiotherapy23). As conclusion, this in vitro study suggests the possible benefit from combination therapy of $^{90}$Yttrium and CHOP regimen.

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REFERENCES: