ผลตอบสนองขั้นต้นและความปลอดภัยของการให้ยายิเทรียม ไอบริทูโมแมบ ไทยูซีแทน ตามด้วยไซโคลฟอสฟาไมด์ ดอกโซรูบิซิน วินคริสตีน และ เพรดนิโซโลน สำหรับผู้ป่วยมะเร็งต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์

นางสาวกฤตติกา ตัญญะแสนสุข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชวิทยา (สหสาขาวิชา)
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PRIMARY RESPONSE AND SAFETY OF ^{90}YTTRIUM IBRITUMOMAB TIUXETAN FOLLOWED BY COMBINED CHEMOTHERAPY OF CYCLOPHOSPHAMIDE, DOXORUBICIN, VINCRISTINE, AND PREDNISOLONE FOR DIFFUSE LARGE B-CELL LYMPHOMA

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmacology
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2007

Thesis Title	PRIMARY RESPONSE AND SAFETY OF ^{90}YTTRIUM IBRITUMOMAB TIUXETAN FOLLOWED BY COMBINED CHEMOTHERAPY OF CYCLOPHOSPHAMIDE, DOXORUBICIN, VINCRISTINE, AND PREDNISOLONE FOR DIFFUSE LARGE B-CELL LYMPHOMA
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กฤตติกา **ตัญญะแสนสุข : ผลตอบสนองขั้นต้นและความปลอดภัยของการให้ยายิเทรียม ไอบริทูโมแมบ ไทยูซีแทน ตามด้วยไซโคลฟอสฟาไมด์ ดอกโซรูบิซิน วินคริสตีน และ เพรดนิโซโลน สำหรับผู้ป่วยมะเร็งต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์.** (PRIMARY RESPONSE AND SAFETY OF ^{90}YTTRIUM IBRITUMOMAB TIUXETAN FOLLOWED BY COMBINED CHEMOTHERAPY OF CYCLOPHOSPHAMIDE, DOXORUBICIN, VINCRISTINE, AND PREDNISOLONE FOR DIFFUSE LARGE B-CELL LYMPHOMA) อ. ที่ปรึกษา : รศ. พญ. สุมนา ชมพูทวีป, อ.ที่ปรึกษาร่วม: ผศ. นพ. อุดมศักดิ์ บุญวรเศรษฐ์, .105 หน้า.

การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาการเสริมฤทธิ์ของ ยิเทรียม ไอบริทูโมแมบ ไทยูซีแทน กับยารักษามะเร็งต่อมน้ำเหลืองที่ใช้กันอยู่ โดยแบ่งการวิจัยเป็น 2 ระยะ ระยะแรกเป็นการศึกษาถึงการเสริมฤทธิ์ความเป็นพิษและฤทธิ์ด้านการเติบโตของเซลล์โดยใช้เซลล์รามอส ค่าความเข้มข้นที่ทำให้เกิดผลร้อยละ 50 ที่ลดลง และ ค่า drug modification factor (DMF) ที่สูงกว่า 1 จะบ่งบอกถึงการเสริมฤทธิ์ แม้ว่า ยิเทรียมที่ 2 และ 4 ไมโครคูรี่ต่อมิลลิลิตรจะไม่มีผลต่อเซลล์ แต่พบการเสริมฤทธิ์พิษต่อเซลล์เมื่อให้ร่วมกับดอกโซรูบิซิน (DMF 3.00-4.18), อีโทโปไซด์ (DMF 1.96-2.36) และเมื่อให้ยิเทรียมตามด้วยฟลูดาราบีน (DMF 2.99-3.52) การให้ร่วมกับไซโคลฟอสฟาไมด์ ไซตาราบีน (< 0.2 ไมโครกรัมต่อมิลลิลิตร) และ วินคริสตีน จะยับยั้งการเติบโตของเซลล์ได้ถึงร้อยละ 60-100 และพบการเสริมฤทธิ์ที่ค่อนข้างสูงเมื่อให้ร่วมกับฟลูดาราบีน (0.2–200 ไมโครกรัมต่อมิลลิลิตร) การศึกษาในระยะที่สองเป็นการประเมินผลตอบสนองขั้นต้นทางคลินิกและความปลอดภัยของการให้ ยิเทรียม ไอบริทูโมแมบ ไทยูซีแทน ตามด้วย CHOP ในมะเร็งต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์ จากผู้ป่วยที่เข้าร่วมโครงการ 10 ราย พบว่ามีการตอบสนองต่อยาที่ดีเพิ่มขึ้นเป็นลำดับเมื่อพิจารณาจากร้อยละที่เพิ่มขึ้นของผู้ป่วยที่หายจากโรคเมื่อประเมินที่ 3 สัปดาห์หลังจากการได้รับเคมีบำบัด มีการกลับเป็นซ้ำ 4 รายเมื่อประเมินที่ 3 เดือนหลังการได้รับยาเคมีบำบัด อาการไม่พึงประสงค์ที่ไม่รุนแรงและพบบ่อย คือ เม็ดเลือดขาวและเกร็ดเลือดลดต่ำอย่างช้าๆ ระยะเวลาเฉลี่ยที่ลดลงต่ำสุด คือ 5.80 $\pm$ 1.69 และ 4.50 $\pm$ 0.71 สัปดาห์ตามลำดับ การตอบสนองที่ดีในกลุ่มผู้ป่วยที่มี Bcl-2$^+$ ค่อนข้างสูงกว่ากลุ่มที่มี Bcl-2$^-$ โดยสรุป แผนการรักษานี้น่าจะมีประโยชน์สำหรับผู้ป่วยต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์ ซึ่งการตอบสนองที่ดีนี้สอดคล้องกับการศึกษาในระดับเซลล์ อนึ่ง ควรมีการศึกษาต่อไปถึงปัญหาการกลับเป็นซ้ำที่เกิดขึ้นค่อนข้างเร็ว.

สาขาวิชา เภสัชวิทยา
ปีการศึกษา 2550

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4689653220 : MAJOR PHARMACOLOGY

KEY WORD: ^{90}YTTRIUM IBRITUMOMAB TIUXETAN / LYMPHOMA / DIFFUSE LARGE B-CELL LYMPHOMA / CHEMOTHERAPY / RADIOIMMUNOTHERAPY

KRITTIKA TANYASAENSOOK: PRIMARY RESPONSE AND SAFETY OF ^{90}YTTRIUM IBRITUMOMAB TIUXETAN FOLLOWED BY COMBINED CHEMOTHERAPY OF CYCLOPHOSPHAMIDE, DOXORUBICIN, VINCRISTINE, AND PREDNISOLONE FOR DIFFUSE LARGE B-CELL LYMPHOMA. THESIS ADVISOR : ASSOC. PROF. SUMANA CHOMPOOTAWEEP, M.D., M.P.H. THESIS COADVISOR : ASSIST. PROF. UDOMSAK BUNWORASATE, M.D., 105 pp.

This study was aimed to demonstrate the synergism between ^{90}Yttrium ibritumomab tiuxetan (Y2B8) and commonly used antilymphoma drugs. The study was separated into 2 parts. First, to demonstrate the *in vitro* synergy in terms of direct cytotoxicity and antiproliferation to Ramos CD20-bearing lymphoma cells, 4-8 treatment arms due to drug order, different doses of ^{90}Yttrium and 2 different incubation periods were designed. Synergy was justified by decreased IC_{50} and drug modification factors (DMF) greater than 1. Although ^{90}Yttrium at 2 and 4 μCi/ml had no cytotoxic or antiproliferative effect, synergistic cytotoxicity was found when treated with doxorubicin (DMF 3.00-4.18), etoposide (DMF 1.96-2.36), and in ^{90}Yttrium followed by fludarabine (DMF 2.99-3.52). Besides, combined ^{90}Yttrium with cyclophosphamide, cytarabine (< 0.2 μg/ml), and vincristine provided 60-100% antiproliferative effect. Moderately high degree of synergy was found with 0.2-200 μg/ml fludarabine assessed after 96 hours incubation period (DMF 91.24-91.69). In the second part exploring clinical response and safety of the regimen Y2B8 followed by CHOP (Y-CHOP) for diffuse large B-cell lymphoma, 10 patients (4 male, 6 female) were enrolled during April 2006 - February 2007. The increased percentage of patients with complete response or unconfirmed complete response to 80% at 3 weeks after completion of CHOP indicated the development of good response along the program. However, 4 progressive/relapse cases were noticed at 3 months-visit. Factors related to progression were disease bulkiness and the presence of extranodal sites. Non-serious drop in neutrophil and platelet counts (nadir at 5.80 ± 1.69 and 4.50 ± 0.71 weeks, respectively) were common adverse events due to Y2B8. Higher percentage of good response was demonstrated among patients with Bcl-2$^+$ than those with Bcl-2$^-$. As summary, the regimen Y-CHOP provided more benefit in treating diffuse large B-cell lymphoma. This is related to the *in vitro* synergism with these drugs. In-depth investigation of rapid relapse should be performed further.

Field of Study : Pharmacology

Academic year 2007

Student's Signature : Krittika Tanyasaensook

Advisor's Signature : Sumana Chompootaweep

Co-advisor's Signature : Udomsak Bunworasate

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to Associate Professor Sumana Chompiitaweep and Assistant Professor Udomsak Boonworasate, my advisors, for their guidance, invaluable advice, supervision, and encouragement throughout the study.

My great appreciation is extended to Professor Tanin Intragamtornchai, Associate Professor Somjai Nakornchai, Associate Professor Supeecha Wittayalertpanya, Assistant Professor Wacharee Limpanasithikul, and Assistant Professor Suree Jianmongkol for their meaningful comments criticism and also for being members of the examining committee.

I would like to thank Miss Nui Sol, and all nurses at Vachiravuth-medicine ward, King Chulalongkorn Memorial Hospital for their co-operation.

Finally, I am most grateful to thank my parents, my family, and my colleagues for their unlimited love, encouragement, understanding and never ending support.

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LIST OF ABBREVIATIONS

μl	Microlitre
ABC type	Activated B cell-like profile
Bcl-2, Bcl-6	B-cell lymphoma like protein type 2, and type 6
cDNA	complimentary DNA
CD20	Cluster of definition no. 20
CHOP	A combined chemotherapy of cyclophosphamide, doxorubiocin, vincristine, and prednisone/prednisolone
CHOP-R or R-CHOP	A regimen of combined cyclophosphamide, doxorubicin, vincristine, and prednisolone/prednisone followed by rituximab or vice versa
CR	Complete response
CRu	Unconfirmed complete response
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
EPOCH	Etoposide, Vincristine, Doxorubicin, Cyclophosphamide, Prednisolone
ESHAP	a combination of etoposide, methylprednisolone, doxorubicin, high dose cytarabine, and cisplatin
Exp. Date	Expiration date
GELA study	the study performed by Groupe d'Etude des Lymphomes de l'Adulte
HGF	Hepatocyte growth factor
ICE	Ifosphamide, Cisplatin, Etoposide
IL-6	Interleukin-6
IgG1	Immunoglobulin G type 1
IPI	International Prognostic Index
IT MTX	Intrathecal Methotrexate
LDH	Lactate dehydrogenase

LN	Lot number
mCi/Kg	millicurie per kilogram
NHL	Non-Hodgkin's lymphoma
no.	number
PR	Partial response
S.D.	Standard Deviation
VEGF	Vascular endothelial growth factor
Y2B8	^{90}Yttrium Ibritumomab tiuxetan
Y-CHOP	A sequential therapy of ^{90}Yttrium ibritumomab tiuxetan followed by cyclophosphamide, doxorubicin, vincristine, and prednisone/prednisolone

CHAPTER I

INTRODUCTION

Background and Rationale

Non-Hodgkin's lymphoma (NHL), a malignancy of lymphoid cells, is one of the leading causes of death among the world population. An aggressive type Diffuse Large B-cell lymphoma (DLBCL) is the most common type. Its nature of heterogeneity, revealed by novel molecular-biological technologies like gene expression profiling, differentiates DLBCL from other lymphoma subtype, and possibly answers the diversity in treatment outcome *(1)*. Most DLBCL patients have worse prognosis at the time of diagnosis, suggesting the urgency and aggressiveness of treatment initiation. The standard treatment known as CHOP regimen (including cyclophosphamide, doxorubicin, vincristine, and prednisone) has been prescribed across the world for more than 25 years. This regimen turns DLBCL to a curable disease, however, relapse is still problematic *(2)*.

The inconsistency in treatment outcome among DLBCL patients challenges many researchers to define the affecting factors. The International Prognostic Index (IPI) derived from 5 clinical independent parameters including old age, presence of extranodal sites, increased serum lactate dehydrogenase level, un-acceptable patient performance status, and higher disease stage, has been widely used *(2)*. However, unexpected treatment failure was notified even in the identical IPI risk level. Some molecular parameters such as oncogenic proteins and drug metabolism enzymes are therefore investigated. Significance of B-cell lymphoma-2 (Bcl-2) proteins is now widely investigated. In terms of Bcl-2, Bcl-6 and some other biomarkers, DLBCL can be categorized to 3 subtypes which relate to differential outcomes *(3)*. Unfortunately, their predictive value could not be elucidated in the prospective trials of overall lymphoma patients, but its value was suggestive in some

populations *(1, 4)*. This controversy possibly depends on study procedures, analytical methods, and selected population.

Immunotherapy and radioimmunotherapy are two strategies aimed at maximizing tumor destroying while minimizing adverse reactions to normal cells. The application of both novel technologies to standard treatment is such an attempt to improve treatment efficacy. Rituximab is a chimeric murine-human monoclonal antibody specific to CD20 antigen, a key protein in cell survival found in over 90% of B-lymphoma cells *(2)*. This drug promises more benefit in NHL either used alone or as combination with standard regimen. However, rapid resistance to rituximab was reported recently, being partly due to cell adaptation and development of host antibody to the chimeric protein. Besides, less benefit was evidenced in bulky tumor mass treated with riuximab and/or chemotherapy *(5)*.

Radioimmunotherapy, on the contrary, was introduced for the treatment of lymphoma due to the high radiosensitivity characteristics of lymphoma cells and drug specificity to target molecule on cancer cells *(6)*. ^{90}Yttrium ibritumomab tiuxetan (Y2B8) is a radioimmunoconjugate approved in 2002 for NHL by the United State Food and Drug Administration Board *(1)*. Its effectiveness in follicular NHL even in refractory or rituximab resistance cases is well-accepted. This drug composes of a beta-emitter (^{90}Yttrium) and a murine immunoglobulin specific to CD20 (ibritumomab); both are linked to a linker-tiuxetan. It is generally safe except the most common reported reversible but delayed dose-limiting cytopenia *(7)*. Due to its crossfire effect from β-ray emission and its specificity to $CD20^{+}$ cells, Y2B8 has more benefit on bulkier mass than rituximab and conventional chemotherapy. ^{90}Yttrium ibritumomab tiuxetan might have place in DLBCL as a concern of disease bulkiness and heterogeneity *(8)*. Theoretically, repeated monotherapy of Y2B8 may not provide more benefit, partly is due to human antimurine-antibody development, and tumor re-population during long recovery phase from cytopenia. An unexpected low overall response rate of 44% was reported in a study of 104 DLBCL patients treated with Y2B8 as a second-line therapy. The response rate inversely correlated with the number of prior therapy *(1)*. The more benefit due to early treatment of Y2B8 was revealed in later study *(5)*. Interestingly, patients treated with

Y2B8 could tolerate later chemotherapy, bone marrow transplantation, and even rituximab. Meanwhile, the more favorable outcome with infrequent non-serious adverse events was revealed on interim analysis of an ongoing trial involving a sequential of ^{131}Iodine tositumomab followed by CHOP regimen in another aggressive type Mantle cell lymphoma *(9)*. ^{90}Yttrium ibritumomab tiuxetan has better safety profile and more predictable biodistribution, comparing to its allied drug, ^{131}Iodine tositumomab *(5)*. The last study so suggests the feasibility of a similar regimen of Y2B8 and CHOP regimen in other aggressive type lymphoma like DLBCL.

This study was therefore carried out to determine the *in vitro* synergism between ^{90}Yttrium and some commonly used antilymphoma chemotherapy and to explore the primary outcome and safety profile of the sequential therapy of ^{90}Yttrium ibritumomab tiuxetan followed by CHOP regimen in DLBCL patients. Some clinical factors including the expression of Bcl-2 are also investigated whether or not relate to primary outcome to the regimen.

Research Questions

Primary research questions

1. Whether or not that ^{90}Yttrium ibritumomab tiuxetan has *in vitro* synergy with some commonly used antilymphoma drugs (cyclophosphamide cytarabine, etoposide, doxorubicin, fludarabine, and vincristine) in terms of cytotoxic effect and antiproliferative effect.

2. Does the sequential administration of ^{90}Yttrium ibritumomab tiuxetan followed by CHOP regimen (Y-CHOP) have potential in treating patients with diffuse large B-cell lymphoma.

Secondary research question:

Does positivity to Bcl-2 correlate with primary response to the Y-CHOP regimen in these selected patients.

Objectives

Primary objectives

1. To define the *in vitro* synergistic effect between ^{90}Yttrium and other 6 antilymphoma drugs in terms of cytotoxicity and antiproliferation.

2. To explore the efficacy of Y-CHOP as the first line treatment for patients with diffuse large B-cell lymphoma in terms of biochemical responses (changes in blood picture and biochemistry), and primary clinical response (improvement or progression).

Secondary objectives

1. To investigate the difference in response to the treatment among patients whom verified positive or negative to Bcl-2.

2. To observe the safety of the Y-CHOP regimen in these patients during trial.

Hypotheses

1. ^{90}Yttrium has synergy with selected antilymphoma drugs (cytarabine arabinoside, cyclophosphamide, doxorubicin, etoposide, fludarabine or vincristine)

2. The sequential treatment of ^{90}Yttrium ibritumomab tiuxetan followed by CHOP regimen provides overall response rate higher than 80% (estimated from 70-90% overall response to standard CHOP treatment alone reviewed from literatures).

Limitation of the study

Since of time-limitation, each participant was followed-up for a maximum of 12 weeks after completion of consolidation phase with CHOP; and final endpoint as survival analysis was not included.

Expected benefit from the study

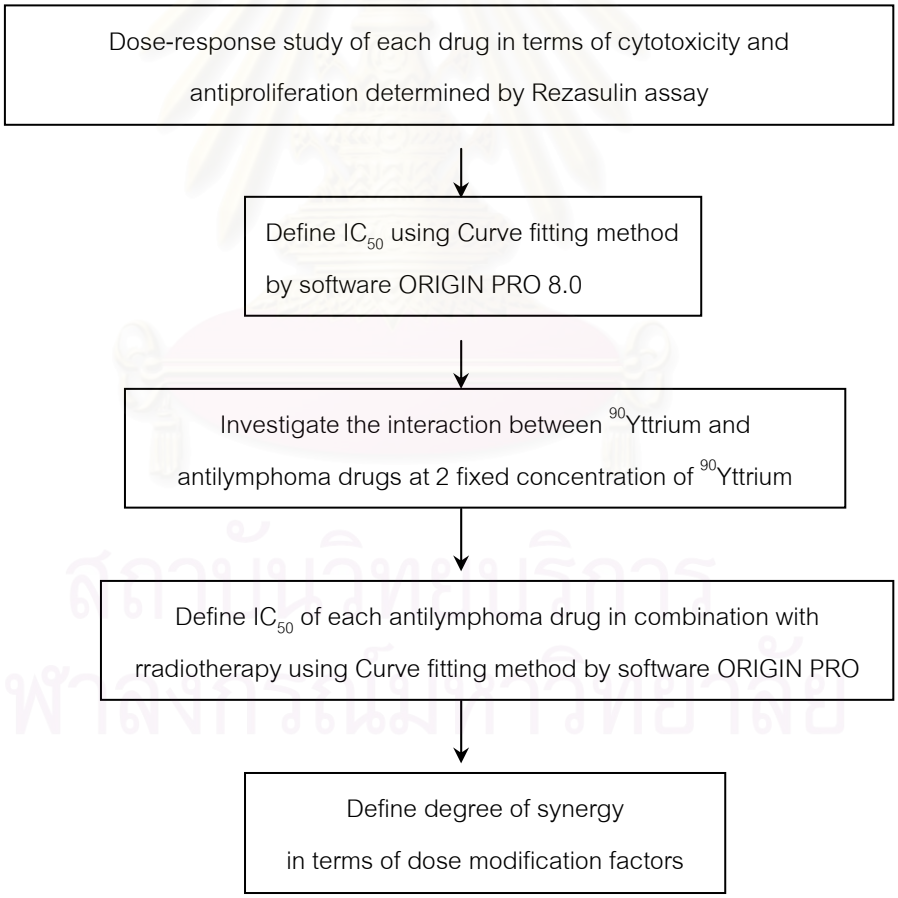
1. Since combination therapy is unavoidable in cancer treatment, the *in vitro* study of drug synergy should be a primary screening that used as guidance in developing treatment protocol. This *in vitro* model for drug-radiotherapy synergy therefore could be served as a model for other studies of similar drug pairs.

2. This exploratory study in human provides important information (potential benefit and safety) that may navigate a further multicentric phase III trial in larger population.

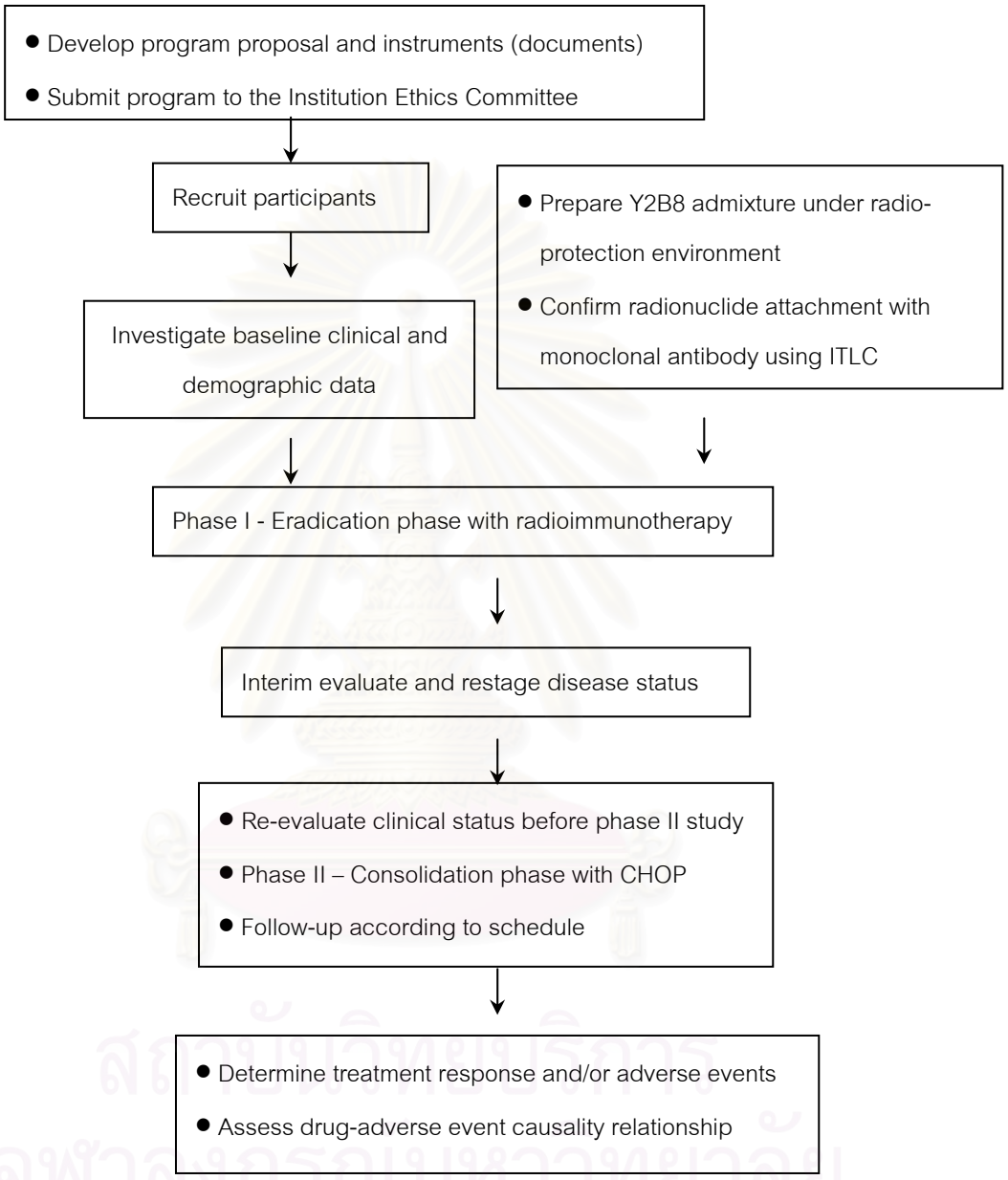
3. The preliminary investigation regarding the bcl-2 protein and treatment response can suggest further studies to prove their relationships particularly in DLBCL patients. If so, this might be used as a prognostic tool for differentiating the appropriate patient to the treatment protocol.

Conceptual Framework

1. To define the *in vitro* synergistic effect between ^{90}Yttrium ibritumomab tiuxetan and antilymphoma drugs



2. To explore the efficacy and safety of Y-CHOP regimen



CHAPTER II

REVIEW OF LITERATURES

Diffuse Large B-Cell Lymphoma

1. General background *(1, 2)*

Diffuse large B-cell lymphoma (DLBCL), characterized as an intermediate grade and aggressive type, is the most common type of non-Hodgkin's lymphoma (NHL). Approximately 75% of patients are highly moderate to high risk (justified as bulky stage II, or stage III-IV) associated with worse prognosis on diagnosis. The standard chemotherapy including combination of cyclophosphamide, doxorubicin, vincristine, and prednisone, known as CHOP regimen, is recommended for years since its low cost and comparable efficacy to other regimens. Prednisone is sometimes substituted with its derivative, prednisolone, in some countries due to local availability. Although DLBCL represents a curable disease since then, long-term survival is unpredictable with standard-dose treatment. One key explanation is that DLBCL is a non-uniform disease based on molecular pathology. Fortunately, the disease commonly remains chemosensitive even when relapse.

2. Prognostic factors of DLBCL patients

A high variation in outcome reflects heterogeneous pathology *(3)*. Management of DLBCL patients had been traditionally guided by the Ann Arbor staging based on tumor spreading to adjacent or remote lymph nodes. Later, the International Non-Hodgkin's lymphoma prognostic factor index (IPI) including 5 pretreatment clinical parameters- age, tumor stage, extranodal sites, performance status, and serum lactate dehydrogenase level- was developed in 1993. It has been used to define risk level which correlates with response and survival in each patient *(2)*. This model was tested in limited population most of whom treated with anthracycline based regimen due to existing standard guideline. The application to other regimens, therefore, maybe

inappropriate. Besides, the inconsistency in outcome is unexpectedly observed even in the identical IPI risk level. Other models concerning molecular parameters are currently investigated. The Leukemia-Lymphoma Molecular Profiling Project by means of complementary-DNA (cDNA) microarray technique demonstrated at least two distinct subpopulations of DLBCL. As distinct genotype, patients with a germinal center (GC) B cell-like signature have a more favorable course, and better response to chemotherapy than those with an activated B cell-like profile (ABC) *(1)*.

Cells in the emerging malignant clones accumulate genetic or epigenetic changes leading to an aberrant gene activity that finally affect cell behavior. Thus, tiny molecules taking part in cell cycle regulation (TP53, $p27^{KIP1}$, cyclin D, and Ki-67), apoptosis (Survivin, Bcl-2, caspases, Fas or CD95), B-cell differentiation (Bcl-6, HGAL, CD10, CD5, Foxp1, PKC-beta, CD21, CD44), inflammation (IL-6), and angiogenesis (VEGF, endostatin, matrix metalloproteinases), all have potential to be prognostic factors *(3)*. Elucidation of their relationship with outcome could lead to identification of patients who may or may not be candidates for any treatment approaches. Attempts to do so have been made recently. That sequential use of CHOP and rituximab (CHOP-R) appeared to be more effective than CHOP alone in Bcl-2 positive, but not in Bcl-2 negative patients, suggests prognostic value of Bcl-2 in the Groupe d'Etude des Lymphomes de l'Adulte (GELA) study. Confirmation of this association is ongoing in other similar trials *(1)*. Another phase III trial comparing CHOP and R-CHOP, on the contrary, demonstrated the predictive value of Bcl-6 protein (a marker of cells in germinal center origin), not Bcl-2. More favorable outcome of R-CHOP was observed in only Bcl-6 positive subset *(4)*. Next, the importance of the proliferation rate and the DNA-aneuplidy in NHL was demonstrated in patients especially in aggressive and advanced stage using molecular parameters. Higher fraction of cells in S-phase, indicating higher proliferation proficiency, correlated with unfavorable outcome to chemotherapy and radiotherapy *(10)*. Recently, plasma glutathione S-transferase P1-1(GSTp1-1), a phase II detoxifying enzyme known as a resistance factor to a number of chemotherapy, presented a stepwise increment behavior along with increasing

disease stages which associated with reduced complete response rate in CHOP treated patients *(11)*. Hepatocyte growth factor (HGF) and its tyrosine kinase type receptor (named c-MET), in addition, were found over-expressed in various aggressive cancers including lymphoma. Cases with HGF-positive and c-MET-positive, independent of IPI, accompanied with worse prognosis *(12).* Lastly, the expression of the Foxp1, a transcription factor, is strongly associated with less survival in DLBCL patients.

3. Treatment options

Many attempts have been made to improve the efficacy of standard chemotherapy regimen. Three approaches to do so are intensification of chemotherapy, addition of bone marrow transplantation, and potentiation with alternative or novel measures. The first two approaches frequently accompany with aggravated hematological toxicity that a salvage treatment is usually pre-prescribed in the protocol. On the contrary, novel modalities including immunotherapy and radioimmunotherapy are generally tolerable, less toxic and more specific to abnormal cells. Due to their novelty, only immunotherapy and radioimmunotherapy are further described.

Rituximab is an antibody-based immunological product designed to direct attack cancer cells as its specificity to CD20 antigen which is expected to involve in cell survival *(2*). It has benefit in treatment of hematologic $CD20^{+}$cancers such as DLBCL, however, rapid relapse is its drawback. Relapse within 2 years was noted in approximately 40% of subjects. The reasons for treatment failure are still unclear, and may be contributed by low levels or loss of receptor expression as cell adaptation, inaccessibility to target proteins or poor penetration of the antibody into the tumor, low serum concentrations of the antibody, Fc-gamma3 polymorphisms, and other unknown mechanisms. Several multicentric controlled phase III studies confirmed the more benefit of combined rituximab and CHOP over CHOP alone either in elderly or younger patients, resulting in recommendation of R-CHOP as the standard frontline therapy in DLBCL in the United State *(5)*. Aggressive toxicity and variation in response still exist, probably due to disease pathologic heterogeneity.

Radioimmunotherapy, a novel target therapy, is now considered as another alternative due to the high radio-sensitivity of most lymphoma cells. ^{90}Yttrium ibritumomab tiuxetan (Y2B8) is the first radioimmunoconjugate approved by the United State Food and Drug Administration Committee in 2002 for the treatment of NHL. Drug background is already described in the following section. Due to its crossfire effect and specificity to $CD20^{+}$ cancer cells, Y2B8 expresses more advantage over its allied antibody, rituximab, in treating bulkier tumors that are inaccessible by the latter antibody or that express insufficient antigens *(8)*. However, more benefit might not be achieved from repeated Y2B8 courses due to two theoretical reasons. One is the possibility of developing human antibody to foreign antigen in drug moiety similar to rituximab. This is considered risky even less than 2% of limited subjects from Phase I/II trials developed human anti-murine antibody. The other reason is the possibility of cancer cells re-growth during long recovery period from delayed cytopenia. An overall response rate of 44% was reported in a study of 104 DLBCL patients treated with Y2B8 as a second-line therapy. The response rate with Y2B8 inversely correlates with the extent of prior therapy. Another trial performed in relapsed or refractory cases demonstrated that early treatment of Y2B8 promised more durable and higher overall response rate *(5)*. All available retrospective data reveal 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. Taken together, these trials suggest the logical of developing a sequential treatment protocol of ^{90}Yttrium ibritumomab tiuxetan followed by chemotherapy as a first line treatment in DLBCL patients.

CD20 and B-Cell

CD20 is a cell surface protein containing four membrane-spanning regions, N- and C-terminal cytoplasmic domains, and a 50-amino acid loop that serves as the extracellular domain. The binding site for anti-CD20 presumably lies in this loop. Human CD20 shares 20% amino acid sequence identity with high-affinity IgE receptor β chain (FcεRIβ) *(13)*. Its expression is restricted to B-cells from the pre-B-cell stage until late in differentiation at plasmacytoid immunoblast stage. CD20 gene expression is first detected after cell surface expression of CD19 and intracellular expression of CD22 at the pre-B-cell stage of B-cell development *(14)*. Most plasma cells, for example, are CD20 negative.

Function of CD20 in B-cell remains mystery. It is believed that CD20 is functionally important for regulating cell cycle progression and signal transduction in B lymphocytes. Moreover, CD20 forms a homo- or perhaps heterotetrameric complex that regulates Ca^{2+} conductance by either forming or serving as a functional component of a Ca^{2+}-permeable cation channel *(13)*. Since B-cell influence the fate of other effectors in immune system such as T-cell, it can be assumed that CD20 has indirect role in immune cascade.

In addition to its restricted expression on B-cells, CD20 is served as an effective target for hematologic cancer because it is highly expressed about 100,000 molecules/cell; it does not shed or internalize in response to antibody binding; and it is not generally present in an appreciable amount in soluble form. In mice, both the native CD20 molecule and a human CD20 transgene have proven to be effective targets for B-cell depletion *(15)*.

Administration of some anti-CD20 monoclonal antibody like rituximab is followed by profound depletion of B-cell from the peripheral blood, providing its place in treating lymphoid malignancies due to abnormal B-cell. However, since CD20 has indirect influence on immune player cells, particularly T-cells and dendritic cells, accidental decrease in activated T-cells in the peripheral blood in some patients are found *(15)*. Molecular mechanism that antiCD20 induce B-cell depletion is still unclear.

The induction of lateral migration of CD20 into membrane micro-domains or rafts that promoting the activation of through Src-family PTK, phospholipase Cγ1 and PLCγ2 phosphorylation, increased cytoplasmic Ca^{2+}, and subsequent induction of caspase 3 promote cell apoptosis was reported previously *(16)*.

Bcl-2 Protein family

The BCL-2 gene was first cloned in 1985 from the t(14:18) chromosomal translocation found in nearly all follicular lymphoma. BCL-2 is understood to have a central role in inhibiting apoptosis that drive neoplastic accumulation. An entire family of Bcl-2 proteins, related by sequences homology and participation in the control of apoptosis was identified during the past decade. As the whole, three classes of Bcl-2 related protein were discovered. One share antiapoptotic activity of Bcl-2 includes BCL-X_L, BCL-w, MCL-1 and BFL-1. Second, including BAX and BAK, promote cell death. The rest including BID, BAD, BIK, PUMA, NOXA, BMF and HRK exhibit proapoptotic effect *(17)*.

Bcl-2 protein family is crucial regulators of mitochondria outer membrane permeabilization. Bcl-2 and related antiapoptotic proteins block the progression of a death signal by preventing BAX/BAK oligomerization. The imbalance in apoptotic/antiapoptitic Bcl-2 function indicate cell fate, resulting in their potential to be target of drug therapy. Inhibition of Bcl-2 by small hairpin RNAs can enhance radiation-induced apoptosis in A549 non small cells lung cancer suggesting the feasibility of drug synergism *(18)*. Many chemotherapy induce apoptosis in cancer cells through interaction with Bcl-2 protein, for example vincristine inhibits proapoptotic Bcl-2 resulting in cell death *(19, 20)*. Rituximab selectively modifies Bcl-XL resulting in cell apoptosis and sensitizes human B-lymphoma cell to paxlitaxel-induced apoptosis *(21)*.

The predictive value of bcl-2 in solid tumor like breast cancer is controversial *(22, 23)*. Bcl-2 is found correlated with treatment failure to CHOP regimen due to a retrospective study. Many studies concerning diffuse large B-cell lymphoma patients revealed the correlation between the inferior in disease free survival rate or overall survival rate in patients with high Bcl-2 expressiion in tumor site. Obtaining Bcl-2 included in prognostic marker for DLBCL patients *(3)*.

^{90}Yttrium Ibritumomab Tiuxetan

1. Basic structure

Radioimmunotherapy is a new therapeutic dimension that combines the benefit of exquisite targeting specificity of monoclonal antibody with the enhanced tumor killing power of cytotoxic radionuclide. The immunoconjugate permits sensitive discrimination between target and normal tissue, supposedly resulting in fewer side effects than most conventional chemotherapeutic agents *(6)*. ^{90}Yttrium ibritumomab tiuxetan (Y2B8) composes of ibritumomab (an murine IgG1 kappa producing in Chinese hamster ovary cells and reacts specifically with the CD20 antigen), the linker tiuxetan (MX-DTPA), and the toxic radioisotope ^{90}Yttrium, as shown in figure 1. Tiuxetan is stably bound to the antibody via a urea type covalent bond on one side, and to ^{90}Yttrium on the other side.

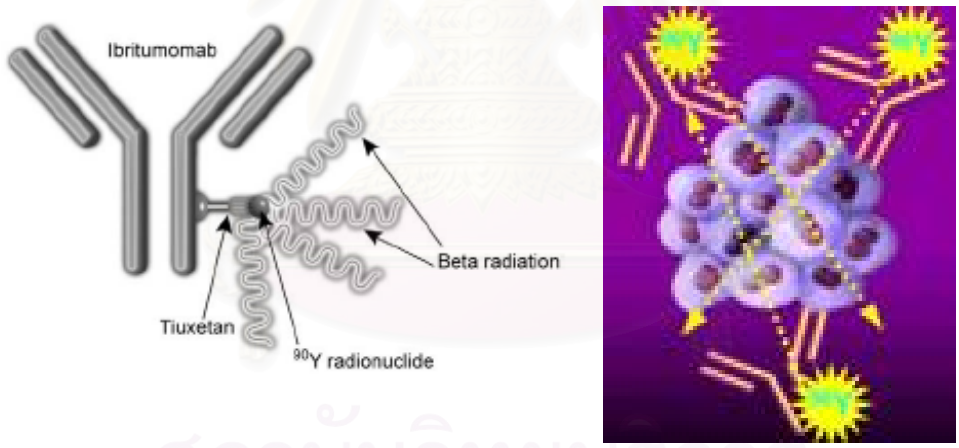


Figure 1 Molecular structure of ^{90}Yttrium ibritumomab tiuxetan (left), and its crossfire effect through bulky tumor mass (right) *(from 7)*

2. Pharmacological properties

^{90}Yttrium is a pure beta-emitter with a physical half-life of 2.7 days that finally decays to ^{90}Zirconium. Its main mechanisms of action are direct damage to DNA and indirect radiation-induced-oxidative damage that finally induces cell apoptosis. The variability in pharmacological effect so partly depends on tissue

antioxidant capacity *(24)* and cell proliferation proficiency *(25)*. Two radioresistance mechanisms-cancer cells repopulation and tissue hypoxia-occuring with external radiation, thus, may be applied to internal radiation like immunoradiotherapy *(26)*. Due to its high beta-energy and an effective long pathlength of 5.3 millimeters, it is provided that 90% of its energy is absorbed within a sphere with approximately 5 millimeters radius. This corresponds to 100-200 cell diameters, giving ^{90}Yttrium a broad crossfire effect for bulky tumor mass particularly when it is conjugated to a monoclonal antibody *(8)*. Since the murine based antibody counterpart has higher affinity but less activity to CD20 antigen, as well as more rapid clearance from human body, other mechanisms similar to its parent antibody, rituximab, including antibody-dependent cellular cytotoxicity, and complement-dependent cytotoxicity are insignificance.

In vitro stability studies of Y2B8 formulated in human serum albumin and stored at 37^{O}C indicated the averaging 1% loss of ^{90}Yttrium from the conjugate per day. Biodistribution studies in mice demonstrated its safety to normal organs and bone expressed as radiation absorbed dose while achieving high tumor uptake. These results are confirmed with those from Phase I/II clinical trials *(7)*.

3. Clinical benefits

The evidence that Y2B8 is effective as a frontline therapy for follicular lymphoma and in relapsed aggressive lymphoma led ^{90}Yttrium ibritumomab tiuxetan be approved by the United State Food and Drug Administration Committee in 2002 for the treatment of relapsed or refractory follicular, low-grade, or transformed B-cell NHL *(1)*. Its advantages over other radioimmuno-agents are negligible release of isotope from chelator, high beta energy and good tumor penetration providing the crossfire effect on bulky area, and negligible exposure risk to nearby persons *(5)*. ^{90}Yttrium ibritumomab tiuxetan can be easily dosed by patient weight since its tightly binding to antibody, short half-life, and rapid body clearance. The recommended dose is 0.4 mCi/kg with up to maximum dose of 32 mCi. The reduced dose of 0.3 mCi/kg is appropriate for whom with reduced platelet counts less than 100,000/μl. This dose is well-tolerated even in elderly patients *(28)*. Central nervous system infiltration may require a higher dose up to

57 mCi *(29)*. Since the presence of CD20 antigen in peripheral normal B-cells and some non-tumor cells, two sub-therapeutic doses of rituximab (250 mg/m^2 body surface area), one week apart, should be administered prior to Y2B8 administration. Rituximab, as a cold-antibody or unlabeled anti-CD20 antibody, will clear non-target CD20 antigen and improve biodistribution of the radionuclide to tumor sites in lymph nodes *(30)*. Various clinical prognostic factors of Y2B8 treatment in lymphoma are described in many clinical trials, but only tumor bulkiness is associated with a lower response rate to the treatment as demonstrated by a retrospectively multivariate analysis *(5)*. Even though predictive potential of some molecular prognostic factors is notified, it has not been proved statistically.

4. **Safety profile** *(Borghaei et al, 2004)*

Unlike chemotherapy, treatment with Y2B8 is not associated with severe mucositis, hair loss, or persistent or prolonged nausea/vomiting. The most common adverse event is transient but delayed myelosuppression, resulting in neutropenia, thrombocytopenia or anemia. The median duration of severe hematologic toxicities reported in clinical trials was 27 days for neutrophil, 23 days for platelet, and 15 days for hemoglobin level. Since that, treatment of Y2B8 should not introduce to patients with these following factors: extensive bone marrow infiltration ($\geq$ 25%), reduced bone marrow reserve, platelet count less than 100,000/μl, absolute neutrophil count less than 1,500 cells/μl, and prior stem-cell transplantation *(5)*. Other non-hematologic events observed in clinical trials include chills, fever, abdominal pain, hypotension, myalgia, dizziness, dyspnea, pruritus, and rash. According to the National Cancer Institute Safety Criteria, most of these adverse events are mild and transient.

Since no penetrating gamma-waves producing during treatment with Y2B8, there is minimal risk of radiation exposure to health care workers or to family members. The drug can be routinely administered on an outpatient basis. Patients can be released immediately after treatment, and patient separation is not required. The only suggestion to the family members is to avoid contamination with body fluids such

as saliva, blood, urine, and stool for several days. The family members usually have close contact to patients is suggested to wear a personal dosimeter as appropriate *(8)*.

Rezasulin assay (Alamar Blue assay) *(31)*

Rezasulin or Alamar Blue is a new rapid and simple non-radioactive assay to monitor and quantitatively determine the proliferation of various cells including cell lines, bacteria and fungi. This bioassay can be used to establish cytotoxicity and growth inhibitory effect due to various substances.

Principally, Alamar Blue contains an oxidation-reduction (REDOX) indicator which exhibits extinct characteristics between fluorescent, reddish reduced form and non-fluorescent, bluish oxidized form. Changes can be easily detected by either fluorometric or colourimetric instruments. Its advantages over other similar assays such as MTT assay are

- minimal toxicity to living cells.
- change in the property of redox indicator occurs in the appropriate oxidation-reduction range relating to cellular metabolic reduction.
- production of a clear distinct change which is easy to interpret
- being a water-soluble, thus eliminating the washing or fixing or extraction steps
- time saving
- no interference from the presence of protein equivalent to 10% fetal bovine serum or from the presence of phenol red in the growth medium

Viable cells with their innate metabolic activity results in a chemical reduction of the immediate surrounding environment. Continued growth maintains a reduced environment while inhibition of growth or dying cells maintains an oxidized environment. Reduction related to growth therefore causes the REDOX indicator to change from oxidized, non-fluorescent and bluish form to reduced, fluorescent and reddish form. Data may be collected using either fluorometric detector at 560 nm excitation wavelength and 590 nm emission wavelength, or colourimetric detector at 570 and 600 nm. The intensity of red color reflects the extent of cellular proliferation. The proliferation of cultures with Alamar Blue was determined under aseptic conditions at

specific time depending on type of cell lines. However, microbial contamination may produce mis-interpretation due to its ability to maintain reduction state in environment.

Generally, Alamar blue can be add directly to cell suspension in an amount equal to 10% of the culture volume. The absorbance is measured after incubation in incubator for additional of 3 or 4 hours. The incubation times may vary depending on the metabolic rates of the cell lines being tested. Trial for optimum condition is recommended before study. By measuring absorbance at 570 nm and 600 nm in a microELISA titer plate reader, absorbance of 600 nm and 570 nm wavelengths determines the absorbance of oxidized and reduced forms of Alamar Blue, respectively. Since there is some overlap of absorbance at 570 and 600 nm wavelength, it is recommended by the manufacturer to subtract the background absorbance at 600 nm from the absorbance at 570 nm, resulting in specific absorbance of reduced form which reflects specific level of viable cells or proliferation. Cells free blank with Alamar blue is suggested in each experiment.

CHAPTER III

MATERIALS AND METHODS

Research Methodology

1. **Study design**: An *in vitro* study for drug synergism and a prospective exploratory phase II clinical trial.

2. **Study site**: Hematology clinic, King Chulalongkorn Memorial Hospital

3. **Cell culture**: Ramos cell line (RA1) [ATCC Code CRL-1596, LN 3953138.] Cells were incubated in RPMI 1640 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^{O}C according to manufacturer' s product information sheet. Cells were replenished with fresh complete media every 2-3 days to maintain cell density between $2x10^{5}$ and $1x10^{6}$ cells/ml. Cells density above $2x10^{6}$ /ml was not recommended.

4. **Subjects**: Subjects recruited to phase II trial were new diagnosed patients with histological confirmed diffuse large B-cell lymphoma and comply to the following inclusion and exclusion criteria

4.1. Inclusion criteria

1. Aged between 18-75 years old
2. Had confirmed $CD20^{+}$ cancer cells
3. Justified as in advanced stage disease (bulky stage II, III or IV) accompanying with bidimensionally measurable disease
4. Had less than 20,000 lymphoid cells/μl on peripheral blood differential
5. Had no prior diagnosis of indolent lymphoma
6. Had no histologic transformation
7. Had performance status (Zubrod) less than 2
8. Had life expectancy not less than 3 months
9. Had no other malignant within the past 5 years

10. Had no prior chemotherapy, immunotherapy, and radiotherapy either for lymphoma, organ transplantation or any other diseases

11. Had written inform consent

4.2. Exclusion criteria

1. Had more than 25% lymphoma infiltration into bone marrow
2. Had clinical evidence of central nervous system involvement due to lymphoma
3. Had a drop in platelet counts less than 100,000/μl or neutrophil counts lower than 1,500 cells/μl
4. Had serum creatinine greater than 2.5 times of upper normal limit
5. Had suspected obstruction in hepato-biliary system evidenced by increased serum bilirubin greater than 2.5 times of upper normal limit
6. Had reduced cardiovascular function justified by echocardiogram or ejection fraction less than 45%
7. Were expected to have reduced pulmonary function due to requirement of oxygen therapy
8. Had positive serology to Human immunodeficiency virus
9. Had active uncontrolled infection
10. Had concurrent severe and/or uncontrolled medical problem that might interrupt patient's participation along the study
11. Unable to provide adequate sections from a paraffin-embedded block lymph node specimen
12. For female participant only: Suspected to be pregnant along the study period

4.3. Sample size determination: Since this is an exploratory phase II clinical study that compare to the standard treatment, sample size is estimated from the following equation *(32)*.

$$n = \frac{1}{d^2} \cdot [Z_{\alpha/2} \sqrt{\pi_1(1-\pi_1)} + Z_{\beta} \sqrt{\pi_0(1-\pi_0)}]^2$$

where $Z_{\alpha/2}$ = standard normal score at α level of 0.05, two-sided = 1.96

Z_{β} = standard normal score at 80% power = 0.84

π_1 = expected overall response rate in new treatment group equals to 0.95

π_0 = overall response rate in standard treatment group equals to 0.8 (estimated from 70-90% response rate reviewed from literatures) *(1)*

d = expected difference in overall response rate between two treatment equals to 0.15

$$n = \frac{1}{0.15^2} \cdot [1.96 \sqrt{0.95\ (1-0.95)} + 0.80 \sqrt{0.80\ (1-0.80)}\ ]^2$$

= approximate 25 subjects

However, the number of subjects in this study could be limited to a minimum of 10 subjects since this is an exploratory phase II clinical study which efficacy and safety as well as economic issue should be re-evaluated at interim.

4.4. Out of study protocol: Participants who meet these following criteria were allowed to terminate from the study.

1. Were reluctant to continue treatment
2. Were intolerant to adverse events
3. Had concomitant diseases or receive concurrent treatment that might interfere or could not compromise to the study treatment
4. Were considered as no improvement at any step of the treatment protocol or had disease progression
5. Had unexpected pregnancy during trial
6. Death from any causes other than study treatment protocol

The terminations according to no improvement, disease progression, intolerant to adverse events and death from disease itself were included for data evaluation.

5. Chemicals:

Rituximab injection 10 mg/ml [Roche, LN B2028, Exp.date Apr 2008]

Ibritumomab tiuxetan kit 1.6 mg/ml [Schering, LN 44094, Exp. Date Oct 2007]

Cyclophosphamide injection 20 mg/ml [Baxter, LN 7H547, Exp.date Aug 2010],

Cytarabine injection 20 mg/ml [Pfizer, LN 6P8021-C, Exp.date Nov 2011]

Doxorubicin injection 2 mg/ml [Pfizer, LN CN12D, Exp. Date Oct 2010]

Etoposide injection 20 mg/ml [Ebewe, LN 604404, Exp.date Apr 2009]

Fludarabine injection 50 mg [Schering, LN 71268E, Exp.date Mar 2010]

Vincristine injection 1 mg/ml [Pharmacia, LN CXZ 7F, Exp.date Mar 2009]

Prednisolone oral tablet 5 mg

^{90}Yttrium-EDTA

Rezasulin powder

RPMI 1640 [Gibco]

6. Instruments:

Documents : Program information for participants (Appendix B),

Participant consent form (Appendix C),

Case record form (Appendix D),

Adverse event-drug causality assessment form (Appendix E)

Culture well plates and culture flasks

Incubator controlled at standard environment

Plate centrifuge

Beta particle counting device

Protective devices for beta ray emission

Optical microscope

Microplate reader for absorbance

Computerized tomography (CT) scanner

5

7. Methods

7.1. Determination of Cell Growth *(33)*

Two experiments due to different starting cell density at $1x10^4$ and $1x10^5$ cells/ml were performed separately. Each experiment was repeated 3 times with duplicate. In each experiment, one milliliter of cells suspension was plated in 24 wells plates. Cells were incubated for over 6 days without fresh complete media replacement. Numbers of viable cells and dead cells were counted under optical microscope every day by means of Trypan blue staining. Changes in calculated cell viability and cell density over 6 days were plotted and doubling time was determined.

7.2. Determination of Optimum Rezasulin Concentration

Two experiments were done according to different conditions as mentioned in cytotoxic assay and antiproliferation assay. Primarily according to cytotoxic assay, 90 μl/well of cell suspension at density between $0.5\text{-}2.0x10^5$ cells/ml were incubated with 10 μl of Rezasulin (final concentration of 10-100 μg/ml) in 96 well plate in standard condition for cell culturing. The absorbance at wavelength 570/600 was measured for over 8 hours. Each plate was measured once every hour during 3-8 hours incubation period *(31)*.

Another experiment corresponding to antiproliferation assay was performed with 45 μl/well of cell suspension. The evaluation was performed similarly.

7.3. Radionuclide incorporation *(34)*

The radiolabelling process was performed on the day of administration at nuclear medicine laboratory according to manufacturer instruction. Ibritumomab tiuxetan was labeled with ^{90}Yttrium using ^{90}Yttrium chloride solution. Labeled product, ^{90}Yttrium ibritumomab tiuxetan (Y2B8), was then assayed for radionuclide incorporation using instant-thin layer chromatography (I-TLC), and beta particle counting device *(35)*. The minimum of 95% incorporation is required. Each radiolabelled dose specific for each patients was drawn just prior to administration time.

6

7.4. Treatment protocol for therapeutics:

Treatment protocol was composed of two drug regimens administered accordingly as described follows and in figure 2. The overall study time for each participant takes about 8-9 months.

1. ***Eradication phase with radioimmunotherapy*** (Y2B8): Each patients received two doses of 250 mg/m^2 rituximab as infusion over 10 minutes on day 1 and day 8. Then, immediately after the second dose on day 8, patients received Y2B8 injection 0.4 mCi/kg body weight (or 0.3 mCi/kg for whom with platelet counts less than 150,000/μl) up to the maximum total dose of 32 mCi. The radioimmunotherapeutic drug was administered as a slow intravenous push over approximately 10 minutes *(8)*. Due to no significant difference, the administration time may deviate within one day either sooner or later from scheduled date depending on patient's comfortability *(5)*. Patients achieving at least a stable disease response (according to *Criteria for primary response to tretment*) after radioimmunotherapy were further recruited for consolidation phase.

2. ***Consolidation phase with chemotherapy*** (CHOP regimen): After patients recovered from cytopenia, generally 10-12 weeks after radioimmunotherapy *(*modified from *9)*, patients were further treated with up to 6 courses of CHOP as ambulatory basis, each 21 days apart. The description of CHOP regimen is

Day 1 Cyclophosphamide 750 mg/m^2 intravenous infusion over 15-45 minutes
Doxorubicin 50 mg/m^2 intravenous over 5-20 minutes
Vincristine 1.4 mg/m^2 (up to a maximum of 2 mg) over 5-15 minutes,
administered accordingly in different syringes

Day 1-5 Prednisolone 40 mg/m^2 orally on day 1 through day 5.

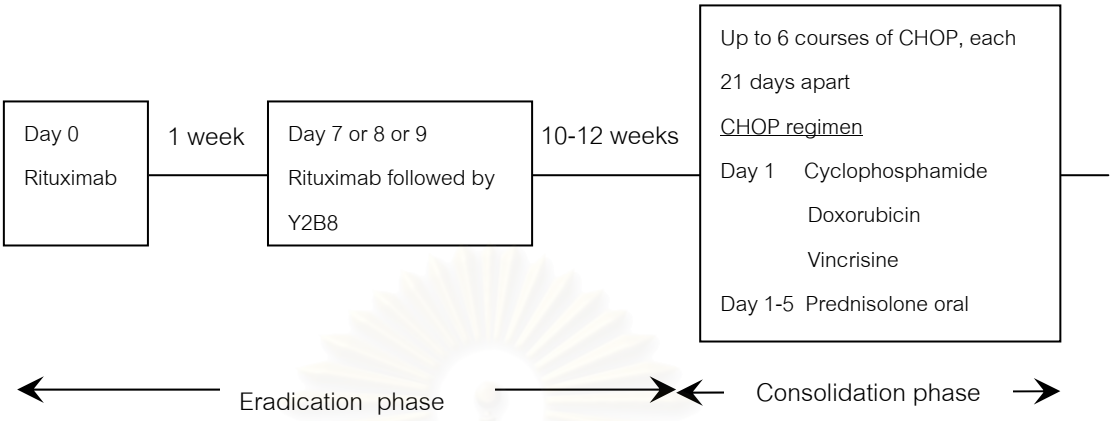


Figure 2 Diagram of treatment protocol

8. Research procedure: This study was divided into 2 parts: an *in vitro* study of drug synergism and a phase II clinical trial

8.1. The *in vitro* study of drug synergism:

- Lymphoma cells in the exponential growth phase, confirmed with over 90% survival by Trypan blue assay, are used in the experiments.

- All powdered drugs were reconstituted with sterile distilled water according to manufacturer's recommendation. 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.

- All data were generated from 3-6 repeated experiments which performed in triplicate. Cells were harvested and resuspended in 96 well culture plates as density of $1x10^6$ cells/ml. Cells were treated primarily with each drug alone (^{90}Yttrium-EDTA, Ibritumomab, rituximab, cytarabine arabinoside, cyclophosphamide, doxorubicin, etoposide, fludarabine, and vincristine) to create dose-growth inhibitory effect curve calculated from cell viability based on Rezasulin assay. Then, IC_{50} and IC_{90} values of each drug were determined using curve fitting method *(36, 37)* estimated by the program ORIGIN PRO 8.

8

- Cells were then treated with combination of ^{90}Yttrium-EDTA and each antilymphoma agent (cytarabine arabinoside, cyclophosphamide, doxorubicin, etoposide, fludarabine, and vincristine) as 2 different sequentials- ^{90}Yttrium-EDTA followed by antilymphoma drug, and vice versa. Two fixed dose of ^{90}Yttrium-EDTA at 2 and 4 μCi/ml were used in each sequential, resulting 4 arm treatments. In any 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 according to the first agent, cells were washed twice with incomplete media. Cell viability was determined by Rezasulin assay. The values of IC_{50} were determined similar to those treated with single drug alone. Cells treated with incomplete media were used as control.

- After completion of drug treatment either alone or in combination, cell viability was determined and data were further analyzed in two aspects as follows:

1. *Cytotoxic effect*: Each 90 μl of 1x10^6 cells/ml was plated into 96 well plate, and then 10 μl of each drug dilution were administered. After 24 hours incubation with drugs, cells were incubated with Rezasulin 40 μg/ml (final concentration) in standard condition for at least 5 hours. Absorbance was measured at the wavelength 570/600. Cytotoxicity was determined by % growth inhibition which calculated from this following equation

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

2. *Antiproliferative effect*: Each 45 μl of Ramos cells at density 1x10^6 cells/ml was plated into 96 well plate. Cells were then incubated with 5 μl of each drug for 24 hours according to the first agent administration. Finally, cells were washed twice with incomplete media and further incubated with 200 μl fresh complete media (providing 250 μl starting volume per well) for additional 72 and 96 hours. At the end of incubation period, cells were stained with rezasulin 20 μg/ml (final concentration) in standard condition for cell culture. The absorbance was determined at the end of incubation period at the wavelength 570/600. Cells treated with incomplete media

were used as control. The percentages of growth inhibition were calculated similar to those for cytotoxicity assay.

- Dose modification factors (DMF) are finally estimated from ratios of IC_{50} doses of drug when used alone and combined with ^{90}Yttrium as follows *(38)*.

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

Synergism was indicated by the value of DMF larger than 1.

8.2. The phase II clinical trial: (figure 3) The study was carried out as 3 steps as follows:

8.2.1. Program approval and Instruments development

1. Research proposal was developed based on previous clinical studies and available instruments at King Chulalongkorn Memorial Hospital. The program then was submitted to the Ethics Committee of Faculty of Medicine, Chulalongkorn University. This program was approved under its major project entitled "Phase II study of Yt^{90} Zevalin followed by CHOP chemotherapy (Z-CHOP) as the first line treatment for diffuse large B-cell lymphoma" as in Appendix A.

2. Four documents used as instruments of this study were Program information for participants (Appendix B), Participant consent form (Appendix C), Case record form (Appendix D), and Adverse event-drug causality assessment form (Appendix E) *(modified from 39)*

8.2.2. Patient recruitment and patient preparation The program started concurrently with the approval of the major project.

- New diagnosed CD20+ diffuse large B-cell non-Hodgkin's lymphoma cases that visit the Hematology clinic, King Chulalongkorn Memorial Hospital, and comply with the inclusion/exclusion criteria were recruited.

- The program description was discussed thoroughly to patients. Signed consent form was required at the beginning of the study to confirm their intention to co-operate.

- Demographic data including age, gender, and prior medical history were collected

- Pretreatment clinical data evaluated within one week prior to the study were collected including physical examination, hematologic parameters (blood counts and differentials), routine biochemical parameters (electrolytes, renal function tests, hepatic function tests, serum lactate dehydrogenase, and so on), performance status, chest radiography, echocardiography, and CT scan of lymphoma sites.

- Patients were justified for risk level using the International Prognostic Index (IPI) as no risk, low, low intermediate, high intermediate and high risk *(2)*.

8.2.3. Efficacy and safety study

- Treatment plan: Starting with radioimmunotherapy as described in *eradication phase with radioimmunotherapy section of "Treatment protocol"*, patients were then evaluated for primary response and re-evaluated at 2-3 weeks after completion of radioimmunotherapy. Patients achieving at least a stable disease response will be recruited for further study.

Participants were allowed to rest for 10-12 weeks after completion of Y2B8 while waiting for recovery from depressed blood cell counts. Afterwards, they were readmitted at hematology day-clinic and delivered CHOP regimen as described in *consolidation with chemotherapy section of "Treatment protocol"*. Acceptable peripheral blood picture (neutrophil counts greater than 1,500 cells/μl and platelet counts greater than 100,000/μl) as well as bone marrow status (less than 25% lymphoma infiltration) had to be confirmed before each administration.

Fertile participants were advised to receive effective contraception until the end of the treatment period.

- Follow-up plan: participants were scheduled to revisit with physician and investigator as follows- every other week for the first month and once a month afterwards until completion of radioimmunotherapy phase, before each CHOP administration, and 6-8 weeks after completion of consolidation with CHOP. Extra visits

were scheduled due to unexpected events. Investigations included physical examination, hematological and biochemical laboratory evaluation, interviewing and/or other specific investigations as appropriate.

- Data interpretation:

For efficacy:

1. Tendency of response or disease progression were evaluated along the study according to *"Criteria for response to treatment"* described below. Anyone who have tendency of no improvement or disease progression will be terminated from the study and considered to receive alternative salvage treatment according to attending physician and/or principal project investigator.

2. Individual primary response at 3 weeks and 3 months after completion of consolidation treatment based on physical examination, blood cell picture, chest radiography, CT scan. Their response was classified according to *"Criteria for response to treatment"*.

Criteria for primary response to treatment: Based on anatomic and biological change after treatment, responses were justified according to the International Workshop criteria as follows *(5)*:

- ***Complete response (CR):*** the disappearance of all lesions and of radiologic or biologic abnormalities observed at diagnosis and the absence of new lesions.

- ***Unconfirmed confirmed response (CR_u):*** persistence for 4 months of palpable node or mass on computed tomography that had regressed in size by at least 75% but not disappeared, normal bone marrow, normal performance status, no symptoms, and disappearance of initial biologic abnormalities.

- ***Partial response (PR):*** the regression of all measurable lesions by more than 50%, the disappearance of non-measurable lesions, and the absence of new lesions.

- ***Stable disease (SD):*** the regression of measurable lesions by 50% or less, or no change for the non-measurable lesions, and no growth of existing lesions or appearance of new lesions.

- ***Progressive disease (PD):*** the appearance of new lesions, growth of the initial lesions by more than 25%, or growth of measurable lesions that had regressed during treatment by more than 50% of their smallest dimensions.

Safety profile:

1. The type and severity of adverse events were primarily justified according to the National Cancer Institute Criteria, and result of adverse event causality assessment.

2. Suspected adverse event, suspected agent(s), and abnormal sign and symptom retrieved from medical record or interviewing.

Evaluation of adverse events: The drug-event relationship due to Y2B8 was tested using the RUCAM's algorithm (Appendix E). The involving information including type, severity, time-course, prior and concurrent drug administration, pre-existing and concurrent medical problems, were put into the algorithm of possible adverse drug events. The drug-event relationship was finally reported.

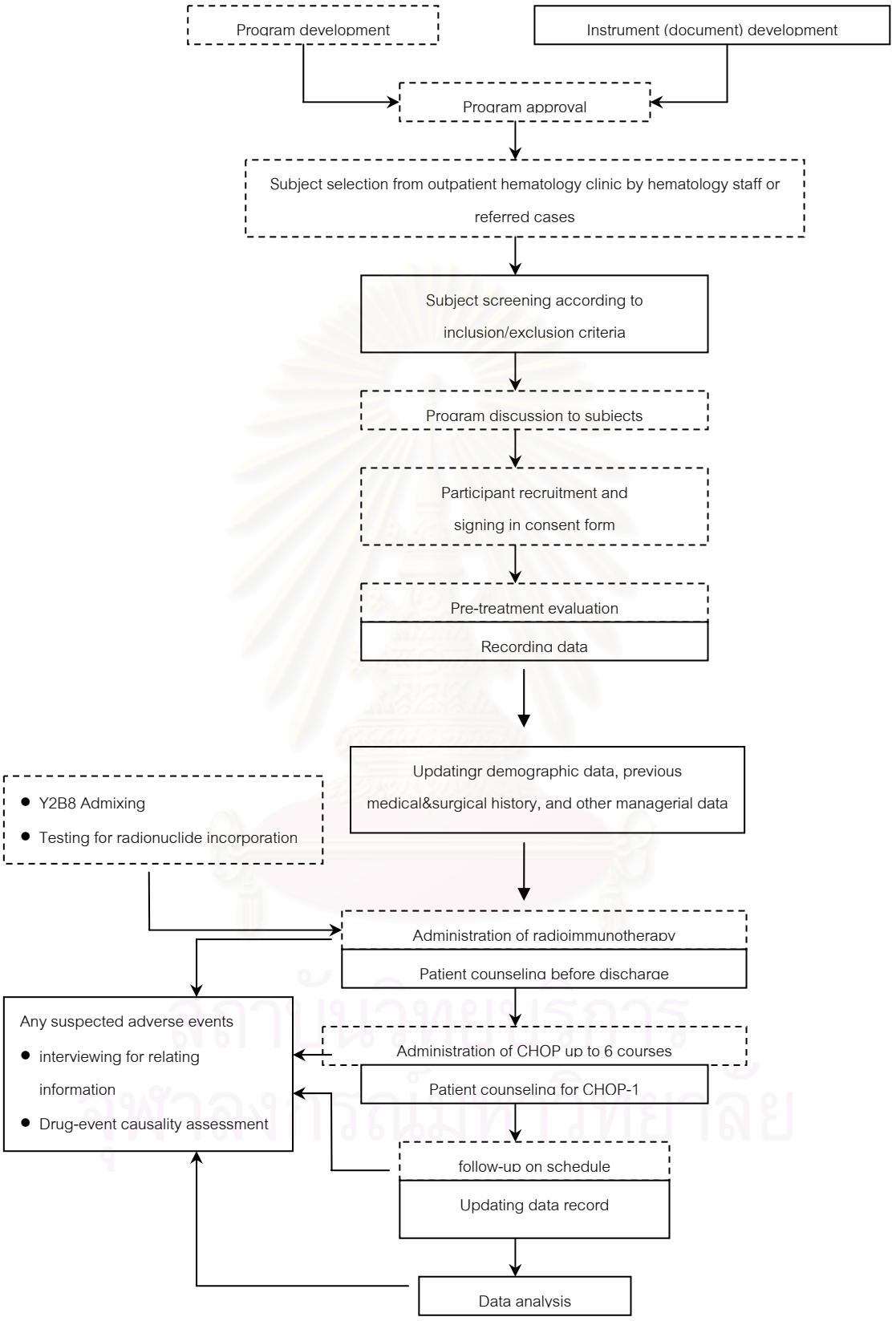


Figure 3 Workflow for phase II clinical trial to investigate efficacy and safety of Y-CHOP

jobs done by the investigator jobs done by physician and others

Statistical analysis:

Reliable correlation coefficient due to regression analysis in cell culture was set at > 0.9. In vitro study parameters including IC_{50}, dose modification factors, all are presented as range, mean $\pm$ standard deviation or other appropriate statistics according to the distribution of raw data. One way or two way ANOVA, where appropriate, is used to confirmed statistical difference at 95% significance level.

All analyses in phase II clinical trial were performed on an intention-to-treat basis. Patient characteristics, response to treatment, and adverse events were presented as mean and standard deviation or median, as appropriate, for continuous data, or number and ratio for discrete data. Difference in responses among different clinical parameters and biological parameters were compared using chi-square and Fisher exact tests at the 95% significance level.

Ethical consideration on clinical study:

- All performance was designed in accordance with the standard practices in *"Good Clinical Practices (GCP) principles and practices*". The clinical part of this study involving lymphoma patients is a part of the major project entitled *"Phase II study of Yt^{90} Zevalin followed by CHOP chemotherapy (Z-CHOP) as first line treatment in diffuse large B-cell lymphoma"* which was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University since March 10, 2006 (as document in appendix A).

- Participants who suffered from adverse events were handled appropriately and re-justified whether or not to further co-operate. A salvage treatment was provided as appropriate by attending physician.

- Participants who had tendency of disease progression according to attending physician investigation were considered to terminate from the program, and delivered the alternative treatment as appropriate.

- Participants being non-comfortable to co-operate were allowed to withdraw from the study immediately.

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- Participants were permitted to contact with the investigator whenever there was any problem.

CHAPTER IV

RESULTS

Growth Curve of Ramos Cell Line

To determine the doubling time of Ramos cell incubated in the culture condition in this study, cells at the starting density of $1x10^4$ and $1x10^5$ cells/ml were incubated in complete media supplemented with 10% fetal calf serum at 37^OC over 6 days without media replacement. As shown in table 1 and figure 4, cells with the density at start $1x10^4$ cells/ml still had over 95% viability on the sixth day. The highest density measured on the last day was $1.2x10^6$ cells/ml. Another experiment dealing with cells at higher density ($1x10^5$ cells/ml) revealed that cells viability started to decrease on the fifth day with the maximum cell density of $3.92x10^6$ cells/ml (Table 1 and Figure 5).

The doubling time estimated from both experiments were comparable at about 20 hours. This data implied that fresh complete media should be replaced every 2-3 days for normal growing cells in log phase.

Table 1 Cell density and viability of Ramos cells incubated over 6 days

Incubation period (Days)	Cell density (x 10^5 Cells/ml)	Cell viability (%)
Starting cell density 1x10^4 cells/ml		
0	0.10	97.56 ± 2.60
1	0.21 ± 0.07	100.00 ± 0.00
2	0.45 ± 0.18	100.00 ± 0.00
3	1.23 ± 0.35	98.29 ± 1.88
4	2.73 ± 1.02	99.49 ± 1.24
5	5.36 ± 1.23	98.78 ± 0.75
6	12.0 ± 0.19	98.52 ± 0.41
Starting cell density 1x10^5 cells/ml		
0	1.00	95.2 ± 1.89
1	2.88 ± 0.46	96.38 ± 5.12
2	4.93 ± 0.46	98.0 ± 0.18
3	11.8 ± 0.39	98.95 ± 0.26
5	39.2 ± 0.83	92.5 ± 1.78
6	37.2 ± 0.81	82.09 ± 0.07

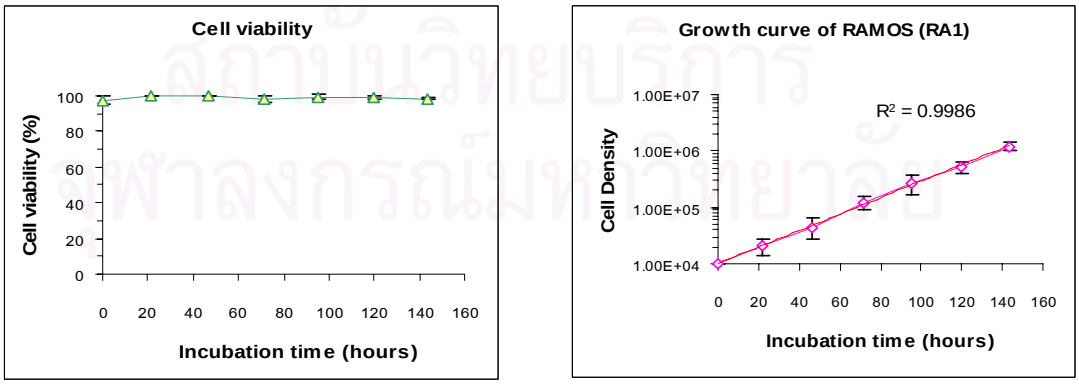


Figure 4 Viabilily and growth curve of Ramos cells (starting density 1x10^4 cells/ml) incubated in standard complete media over 6 days

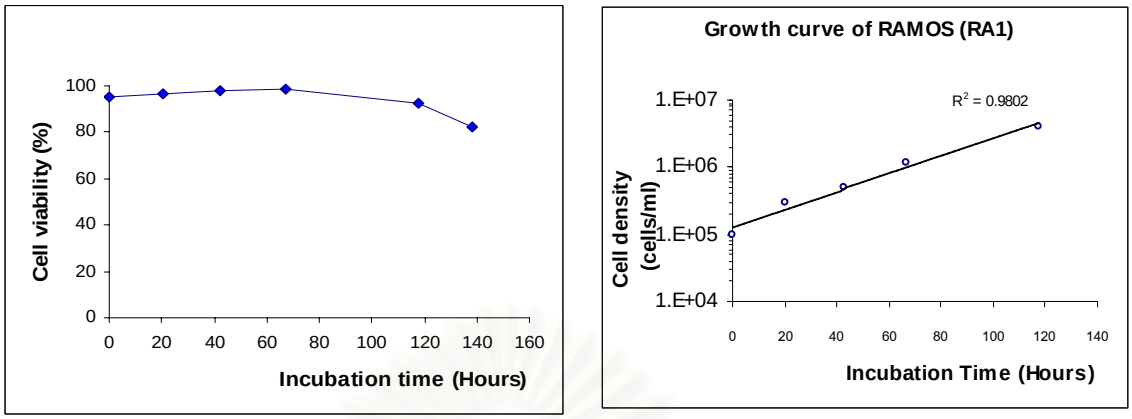


Figure 5 Viabilily and growth curve of Ramos cells (starting density $1x10^5$ cells/ml) incubated in standard complete media over 6 days

<u>The Optimum Condition for Rezasulin Staining</u>

Due to literatures search up to the time of this study, there was no previous report talking about Rezasulin staining in Ramos cell line. Since that, finding the optimum condition for Rezasulin assay was performed as two experiments according to different protocol for cytotoxic and antiproliferation assay.

Regarding cytotoxic assay, 90μl/well of cells at $1x10^6$ cells/ml were incubated with or without drug for 24 hours. Correspond to this experiment, Rezasulin at final concentration of 40-60 μg/ml gave an acceptable range of absorbance for cells at density $0.5\text{-}2x10^6$ cells/ml at the 5-hours incubation time (Figure 6). Similarly, the acceptable range of absorbance for 200 μl/well of Ramos cells as in antiproliferation assay was observed after incubating test cells with 20-30 μg/ml Rezasulin for 6 hours (Figure 7). A plateau due to the maximum capacity of the detector was detected after the extended incubation time with Rezasulin to 24 hours, suggesting that the determination of number of viable cells could not be performed correctly.

Therefore, the optimum condition of Rezasulin staining for cytotoxic assay was incubating cells with 40 μg/ml Rezasulin for 5 hours, and for antiproliferation assay was incubating cells with 20 μg/ml Rezasulin for 6 hours.

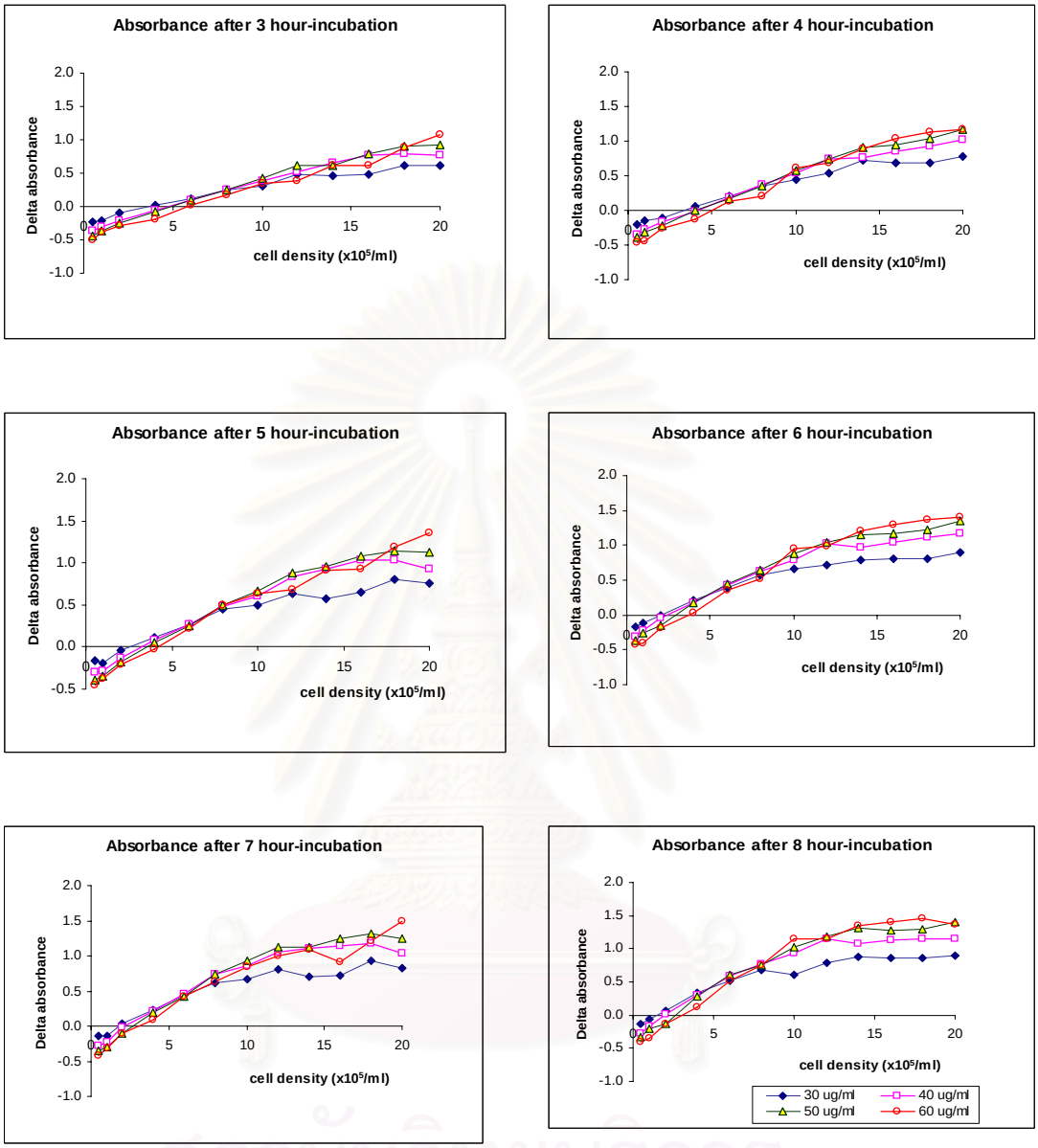


Figure 6 Absorbance of Rezasulin (final concentration 30-60 μg/ml) measured after incubating with Ramos cells at varied cell density (0.5 – 20x10^5 cells/ml) in 90 μl/well for over 8 hours

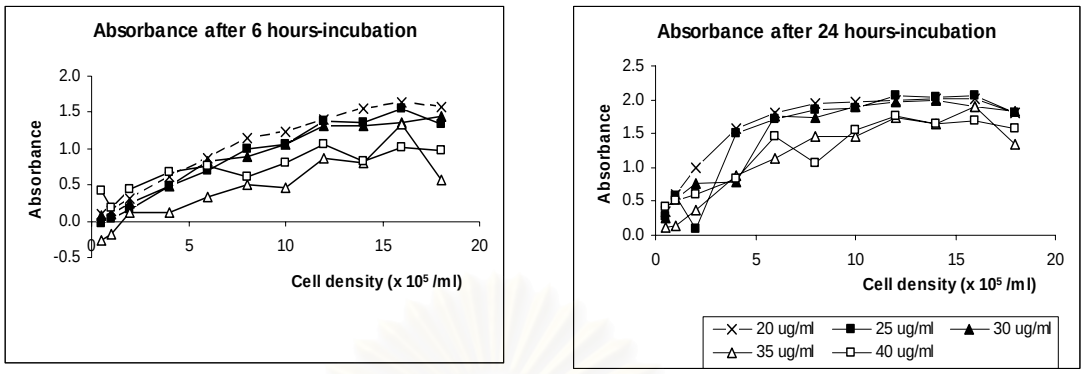


Figure 7 Absorbance of Rezasulin (final concentration 20-40 μg/ml) measured after incubating with Ramos cells at varied cell density (0.5 – 18x10^5 cells/ml) in 200 μl/well for 6 and 24 hours

Cytotoxicity of ^{90}Yttrium Alone or Combined with Antilymphoma drugs

Ramos cells were primarily treated with ^{90}Yttrium or each antilymphoma drug. ^{90}Yttrium alone at 10-100 μCi/ml had less cytotoxic effect on Ramos cells as seen under microscope (Figure 8). Slightly change in morphology was found only at high concentration of 90 and 100 μCi/ml of which were far high from therapeutic range. Rezasulin staining also confirmed the cytotoxic effect of ^{90}Yttrium to Ramos cells at high concentration over 90 μCi/ml (Figure 9). The maximum inhibitory effect at 100 μCi/ml ^{90}Yttrium was equaled to 31.91%. Therefore, it could be extrapolated that ^{90}Yttrium at 2 and 4 μCi/ml that used in this study had no cytotoxic effect on Ramos cells.

Rituximab, a chimeric antiCD20 used for lymphoma and leukemia, and ibritumomab, a murine antiCD20 used as carrier for ^{90}Yttrium in Y2B8 molecule, were also evaluated for their cytotoxicity to Ramos cells. The cytotoxic effect of both drugs could not be demonstrated statistically (Figure 9).

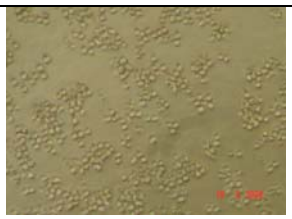
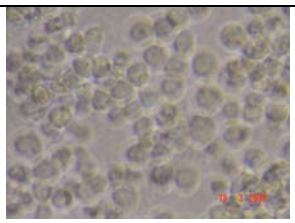
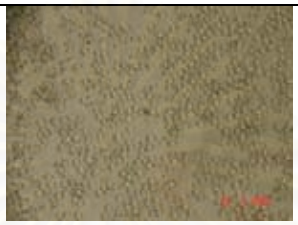
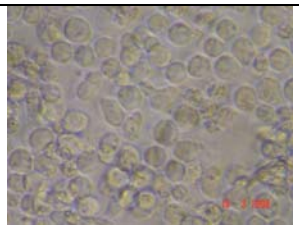
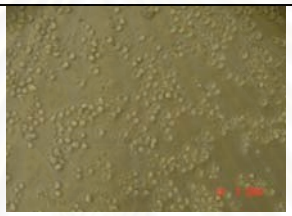
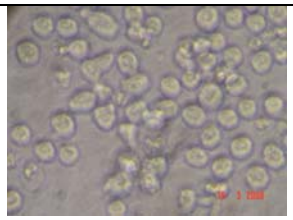
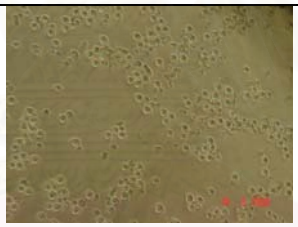
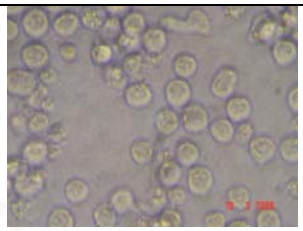
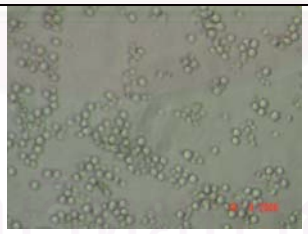
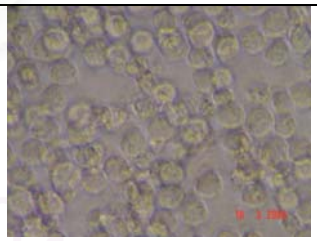
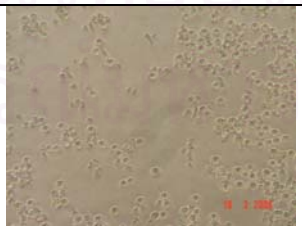
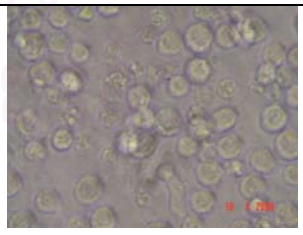
Concentration of ^{90}Yttrium	10X	40X
Control		
10 μCi/ml		
20 μCi/ml		
30 μCi/ml		
40 μCi/ml		
50 μCi/ml		

Figure 8 Microscopic illustrations of Ramos cells treated with up to 100 μCi/ml ^{90}Yttrium for 24 hours.

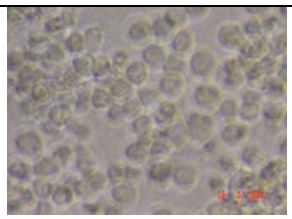
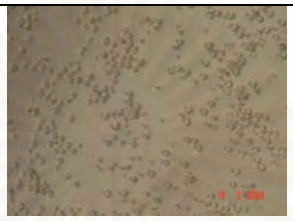
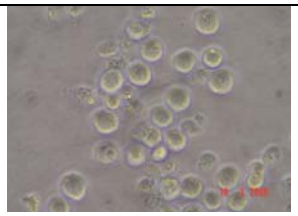
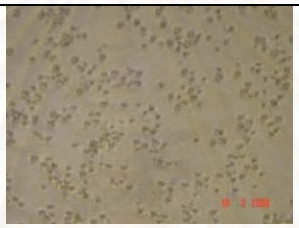
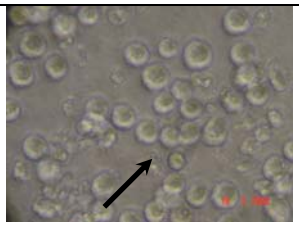
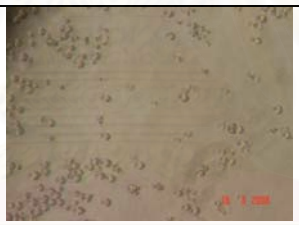
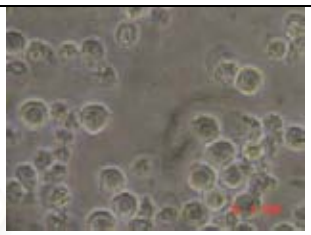
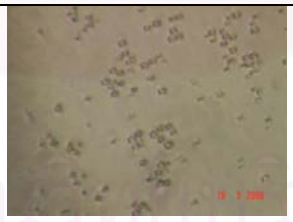
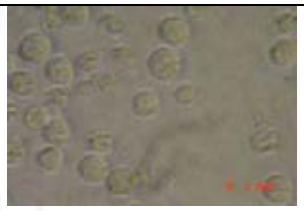
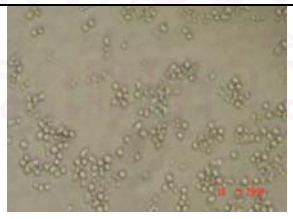
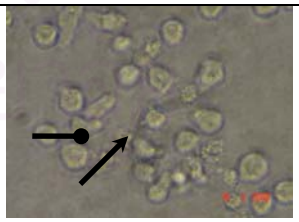
Concentration of ^{90}Yttrium	10X	40X
Control		
60 μCi/ml		
70 μCi/ml		
80 μCi/ml		
90 μCi/ml		
100 μCi/ml		

Figure 8 (cont.) Microscopic pictures of Ramos cells treated with up to 100 μCi/ml ^{90}Yttrium for 24 hours.

—● Cells with changed morphology → Indicates dead cells

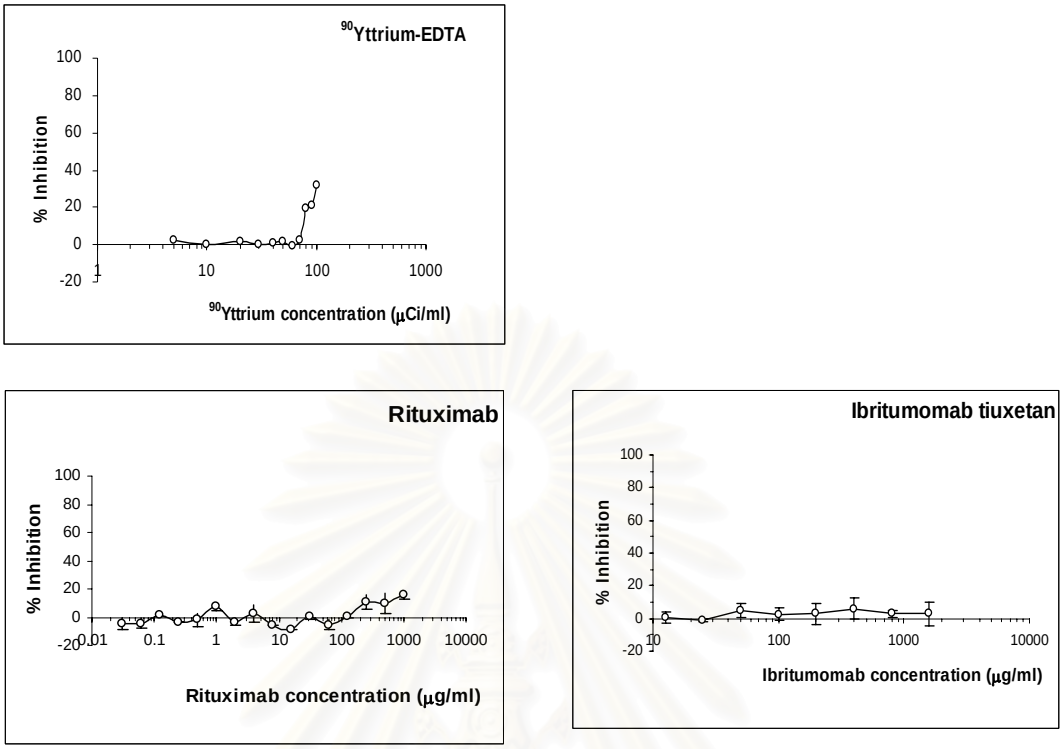
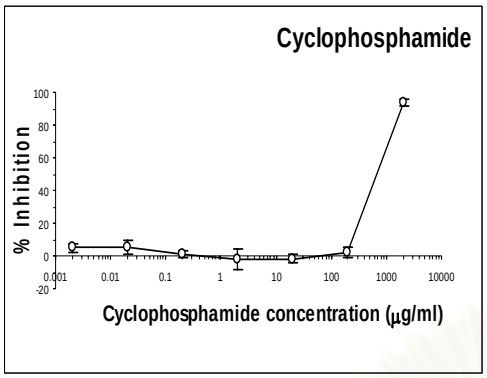
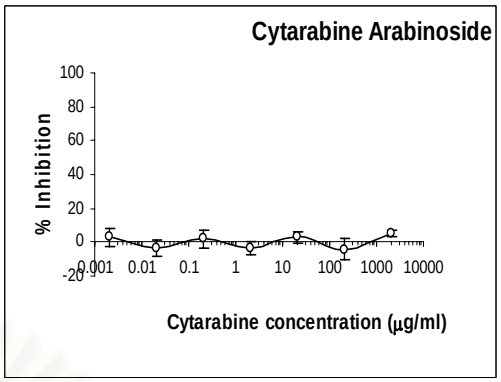


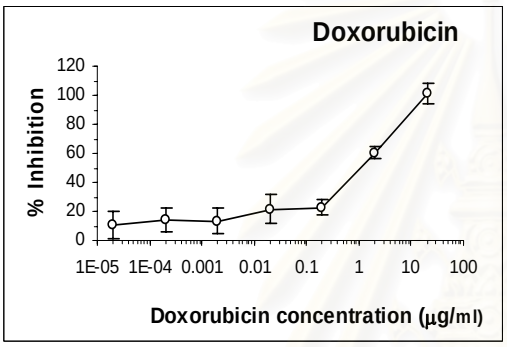
Figure 9 Cytotoxic effect of ^{90}Yttrium-EDTA, rituximab, and ibritumomab on Ramos cells, at density 1x10^6 cells/ml, measured after 24 hours incubation period

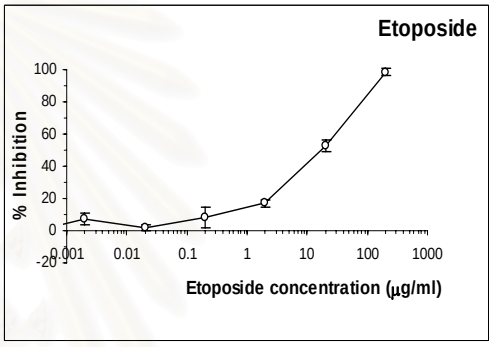
Concerning cytotoxicity of 6 antilymphoma drugs (figure 10), there were three different patterns of cytotoxic profile. Firstly, cytarabine up to its original product concentration (2 mg/ml as final concentration in test preparation) had no cytotoxicity to Ramos cells based on statistic analysis. Secondly, cyclophosphamide and fludarabine showed cytotoxic effect to the cells when treated with the highest concentration as their original product concentration (2 mg/ml). The IC_{50} and IC_{90} of cyclophosphamide were 1.07 and 1.93 mg/ml, while those of fludarabine were 1.11 and 1.96 mg/ml, respectively. And lastly, doxorubicin, etoposide, and vincristine were classified as potent antilymphoma drugs as in the order of vincristine > doxorubicin > etoposide. The IC_{50} of doxorubicin, etoposide and vincristine were 1.38 μg/ml, 17.82 μg/ml, and 3 ng/ml; while their IC_{90} were equaled to 5.16 μg/ml, 64.66 μg/ml, and 67 μg/ml, accordingly. The ratios of IC_{50} to their original product concentrations were approximately 1:145 for doxorubicin, 1:133 for etoposide, and 1:33,300 for vincristine, respectively.





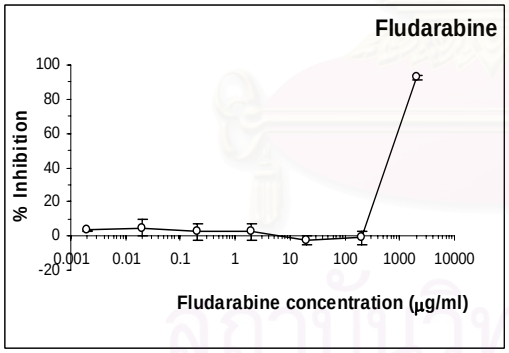
IC_{50} = 1.07 mg/ml, IC_{90} = 1.93 mg/ml

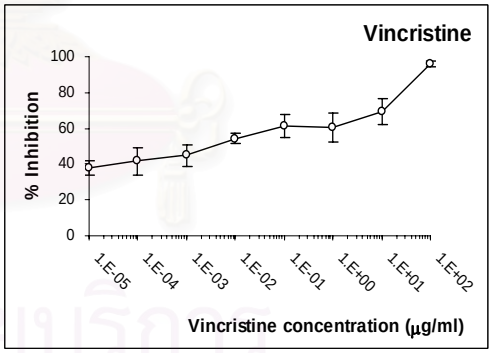




IC_{50} = 1.38 μg/ml, IC_{90} = 5.16 μg/ml

IC_{50} = 17.82 μg/ml, IC_{90} = 64.66 μg/ml





IC_{50} = 1.11 mg/ml, IC_{90} = 1.96 mg/ml

IC_{50} = 3 ng/ml, IC_{90} = 67 μg/ml

Figure 10 Cytotoxic effect to Ramos cells, at density $1x10^6$ cells/ml, treated with each antilymphoma drugs (cyclophosphamide, cytarabine arabinoside, doxorubicin, etoposide, fludarabine, and vincristine). Cytotoxic IC_{50} and IC_{90} were presented under each graph.

Cytotoxic effect of combined therapy was further tested with 2 fixed concentration of ^{90}Yttrium (2 and 4 μCi/ml), and as in 2 different sequential (^{90}Yttrium followed by antilymphoma drugs and vice versa). Results could not demonstrate the differential between 2 administration sequences, and between 2 fixed ^{90}Yttrium concentrations in all drugs except fludarabine (Figure 11 and 12). Cyclophosphamide and cytarabine when treated alone or in combination with ^{90}Yttrium could not demonstrate the difference in cytotoxic effect.. Regarding fludarabine, a significance difference was found only at 200 μg/ml point when ^{90}Yttrium was given prior to chemotherapy. Interestingly, combination of fludarabine at concentration lower than 20 μg/ml with ^{90}Yttrium seemed to induce proliferation in Ramos cells (figure 11).

Three patterns were demonstrated with the rest 3 antilymphoma drugs- doxorubicin, etoposide, and vincristine- that will be described further (figure 12). Doxorubicin at the concentration higher than 0.2 μg/ml showed synergism with ^{90}Yttrium. The largest degree of synergy was found at 2 μg/ml point which is slightly higher than IC_{50} of doxorubicin. Etoposide at the concentration range of 2-20 μg/ml, corresponding to approximately 0.1 to 1.2 times of its IC_{50} demonstrated synergistic effect with ^{90}Yttrium. On the contrary, synergistic effect due to vincristine and ^{90}Yttrium was found at the concentration of vincristine higher than 0.01 μg/ml, which is about 3 times of its IC_{50}.

Degree of synergy can be demonstrated by the value of drug modification factor (DMF) above 1. Corresponding to figure 11 and 12, calculated DMF of doxorubicin (3.00 – 4.18), etoposide (1.96 – 2.36), and fludarabine (1.80 – 3.57) confirmed their synergism with ^{90}Yttrium, while those of vincristine (0.27 – 0.62) suggested the absence of synergistic effect at 50%inhibition level (table 2). Concerning the doxorubicin-^{90}Yttrium pair, it seemed that pre-treatment with doxorubicin provided more benefit than prior treatment with ^{90}Ytrium. Conversely as in fludarabine-^{90}Yttrium pair, pre-treatment of fludarabine seemed to provide less benefit than prior treatment with ^{90}Yttrium.

Strong synergism in cytotoxic effect was demonstrated when treating Ramos cells with combination of ^{90}Yttrium and part of CHOP (including

cyclophosphamide, doxorubicin, and vincristine) using IC_{50} and IC_{90} dose levels. Viable cells disappeared in all wells except in control group.

As summary, among 6 commonly used antilymphoma drugs tested in this study, vincristine following by doxorubicin and etoposide were considered as potent antilymphoma drugs while cyclophosphamide, fludarabine, and cytarabine had less to no cytotoxic effect. Strong synergism was observed in the combination of doxorubicin or etoposide with ^{90}Yttrium, while moderate synergistic effect was found when pre-treated cells with fludarabine.

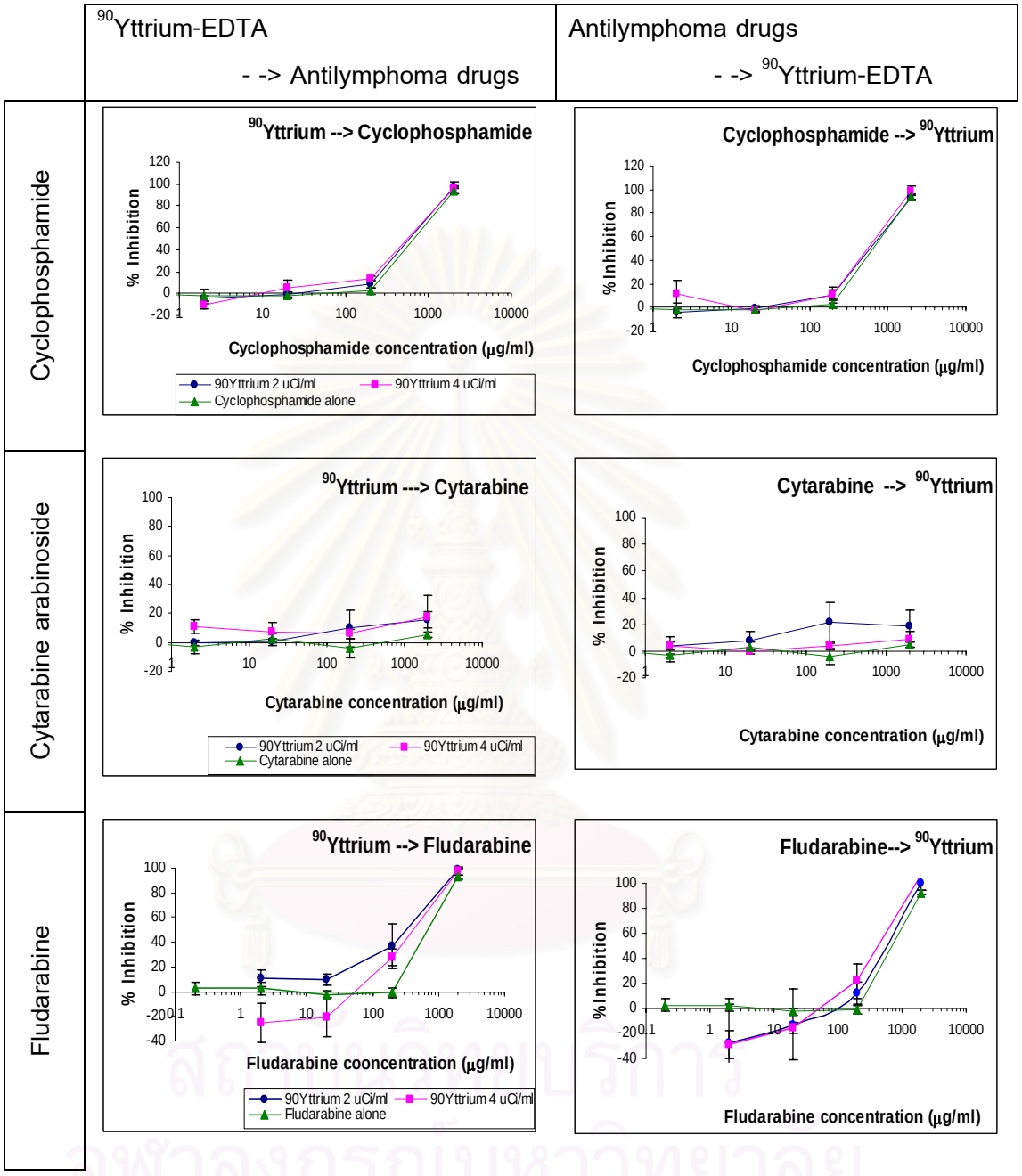


Figure 11 Cytotoxic effect on Ramos cell, at density $1x10^6$ cells/ml, of combined treatment of ^{90}Yttrium-EDTA and cyclophosphamide or cytarabine arabinoside or fludarabine comparing with each drug alone.

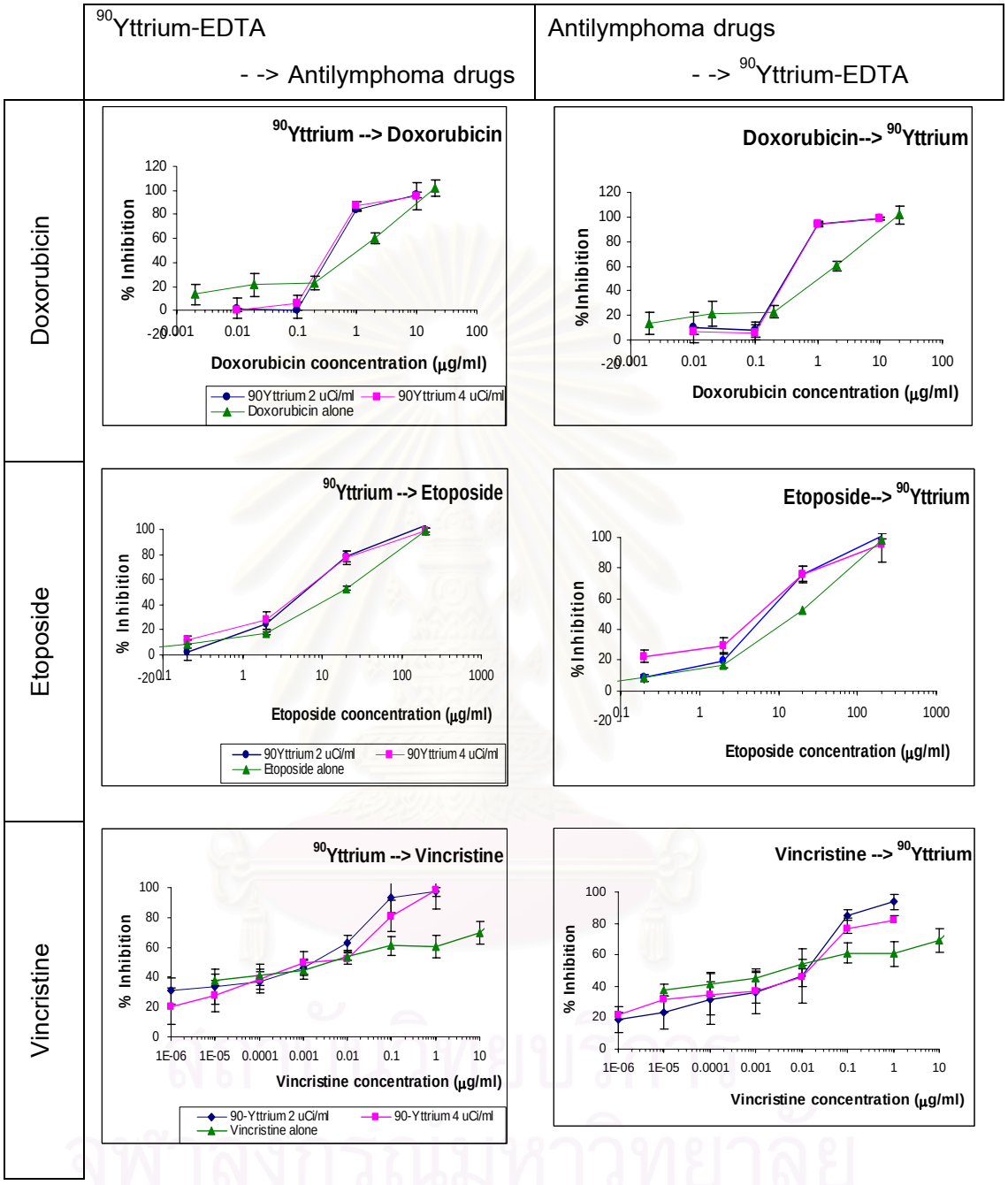


Figure 12 Cytotoxic effect on Ramos cell, at density $1x10^6$ cells/ml, of combined treatment of ^{90}Yttrium-EDTA and doxorubicin or etoposide or vincristine, comparing with each drug alone.

Table 2 Cytotoxic IC_{50} and Drug modification factor (DMF) of Combined therapy. Data was represented as IC_{50} [DMF].

Drug alone	^{90}Yttrium -> Antilymphoma drugs		Antilymphoma drugs -> ^{90}Yttrium	
	2 μCi/ml ^{90}Yttrium	4 μCi/ml ^{90}Yttrium	2 μCi/ml ^{90}Yttrium	4 μCi/ml ^{90}Yttrium
Cyclophosphamide IC_{50} = 1.07 mg/ml	1.01 mg/ml [1.06]	764.32 μg/ml [1.40]	904.21 μg/ml [1.18]	987.93 μg/ml [1.08]
Doxorubicin IC_{50} = 1.38 μg/ml	0.46 μg/ml [3.00]	0.39 μg/ml [3.54]	0.33 μg/ml [4.18]	0.35 μg/ml [3.94]
Etoposide IC_{50} = 17.83 μg/ml	8.40 μg/ml [2.12]	9.09 μg/ml [1.96]	9.09 μg/ml [1.96]	7.57 μg/ml [2.36]
Fludarabine IC_{50} = 1.11 mg/ml	311.18 μg/ml [3.57]	371.23 μg/ml [2.99]	615.70 μg/ml [1.80]	424.37 μg/ml [2.62]
Vincristine IC_{50} = 3 ng/ml	4.79 ng/ml [0.62]	7.54 ng/ml [0.40]	11.04 ng/ml [0.27]	11.32 ng/ml [0.27]

Antiproliferation of ^{90}Yttrium Alone or Combined with Antilymphoma drugs

Much difference from cytotoxic effect, ^{90}Yttrium showed antiproliferation to Ramos cells at the concentration higher than 10 μCi/ml (figure 13). Slightly difference was found when evaluated at 72 and 96 hours incubation period after washing out of drugs. Those cells that were 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. The antiproliferative IC_{50} were 40.61 and 49.69 μCi/ml assessed at 72 and 96 hours incubation time, accordingly. However, this result suggested that ^{90}Yttrium treated alone at 2 and 4 μCi/ml would not affect proliferation in normal Ramos cells. Another experiment also confirmed that ^{90}Yttrium at 2 and 4 μCi/ml did not inhibit cells proliferation.

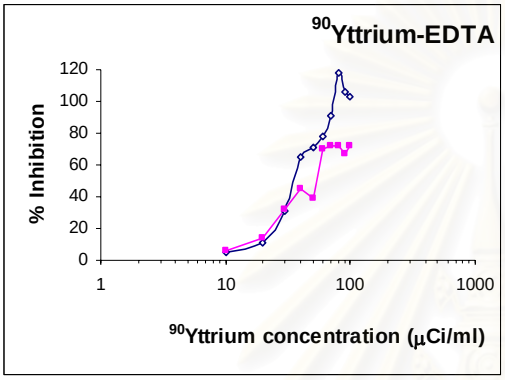
As shown in figure 13, rituximab and ibritumomab had no statistical significance difference in antiproliferative effect to Ramos cells when treated with each drug alone evaluated either after 72 or 96 hours incubation period. Rituximab, independent upon concentration, show about 10 % inhibition of cell proliferation, while ibritumomab did not demonstrate any inhibition.

Concerning of other 6 antilymphoma drugs (figure 14 and 15), only cytarabine demonstrated different degree of inhibition comparing between different incubation times. It seemed that incubation for 96 hours provided more benefit than incubation for 72 hours. Interestingly, cytarabine exhibited bell-shaped like profile as shown in figure 14. The effect started to increase from 0.02 μg/ml to the peak effect at 0.2 μg/ml, providing IC_{50}values at 0.2 and 1.5 μg/ml for 72 hours incubation group and 0.04 μg/ml for 96 hours incubation group, respectively.

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).

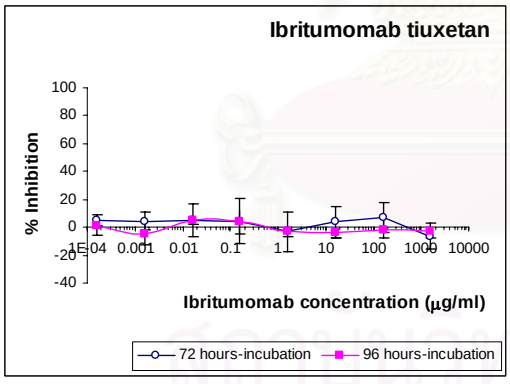
No significant difference in antiproliferation profile due to doxorubicin, etoposide, fludarabine, and vincristine when comparing between 72 hours and 96

hours-incubation period (figure 15). Their IC_{50} and IC_{90} for antiproliferative effect were demonstrated in figure 15. Generally, magnitude of these parameters were much lower than those for cytotoxic effect.



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

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



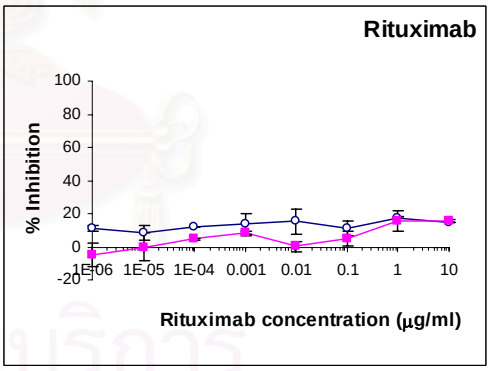
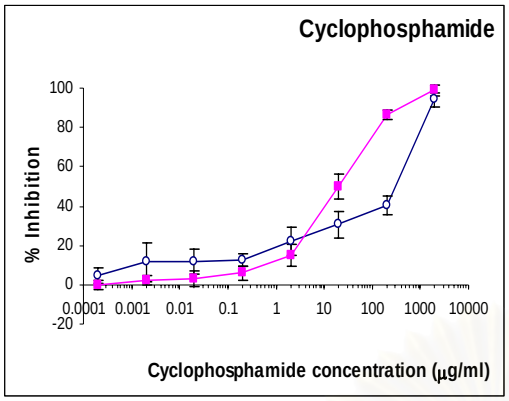


Figure 13 Inhibition of proliferation of Ramos cell, density $1x10^6$ cells/ml, evaluated at 72 and 96 hours after treating with each of ^{90}Yttrium, or ibritumomab, or Rituximab for 24 hours

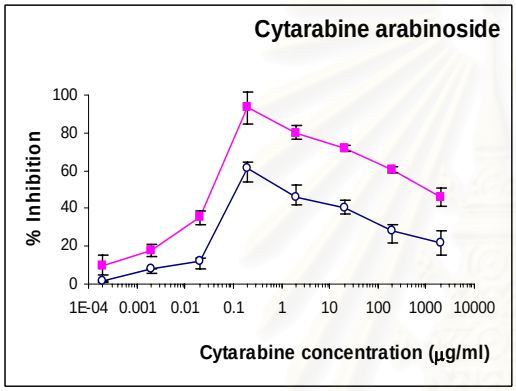


IC_{50} after 72 hours incubation = 268.19 µg/ml

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

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

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

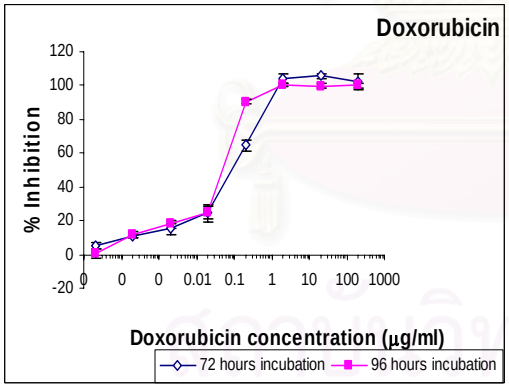


IC_{50} after 72 hours incubation = 0.2 , 1.5 µg/ml

IC_{50} after 96 hours incubation = 0.04 µg/ml

IC_{90} after 72 hours incubation = none

IC_{90} after 96 hours incubation = 0.25 µg/ml



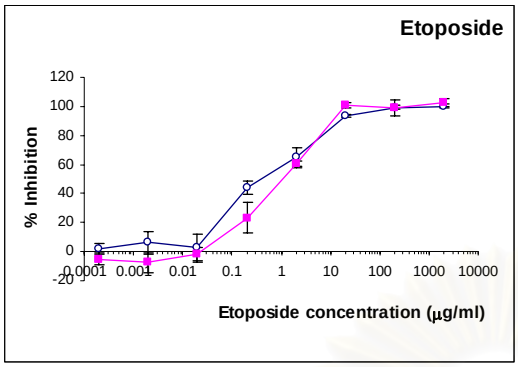
IC_{50} after 72 hours incubation = 0.12 µg/ml

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

Figure 14 Inhibition of proliferation of Ramos cell, density $1x10^6$ cells/ml, evaluated at 72 and 96 hours after treating with cyclophosphamide, cytarabine arabinoside, and doxorubicin for 24 hours. Their IC_{50} and IC_{90} were presented on the right to each graph.

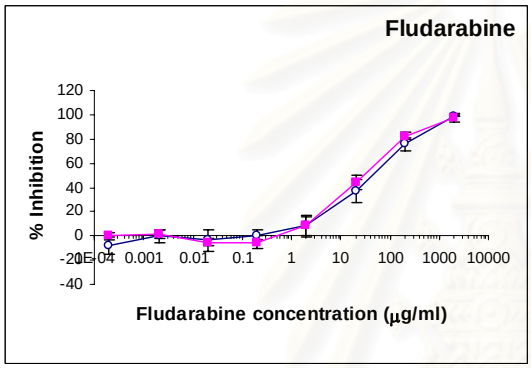


IC_{50} after 72 hours incubation = 0.95 µg/ml

IC_{50} after 96 hours incubation = 1.35 µg/ml

IC_{90} after 72 hours incubation = 4.15 µg/ml

IC_{90} after 96 hours incubation = 4.54 µg/ml

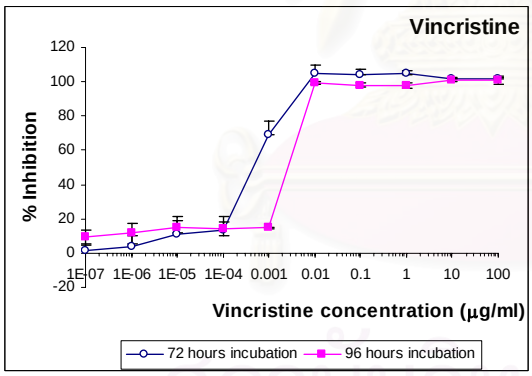


IC_{50} after 72 hours incubation = 3.07 µg/ml

IC_{50} after 96 hours incubation = 23.84 µg/ml

IC_{90} after 72 hours incubation = 12.71 µg/ml

IC_{90} after 96 hours incubation = 170.96 µg/ml



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

IC_{50} 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

Figure 15 Inhibition of proliferation of Ramos cell, density $1x10^6$ cells/ml, evaluated at 72 and 96 hours after treating with etoposide, fludarabine, and vincristine) for 24 hours. Their IC_{50} and IC_{90} were presented on the right to each graph.

Results of combined treatment of ^{90}Yttrium and antilymphoma drugs was demonstrated in figure 16 and 17.

Concerning cyclophosphamide , it seemed that combined treatment with ^{90}Yttrium provided higher degree of inhibition comparing with treatment with cyclophosphamide alone (figure 16, table 3 and 4). The larger difference was shown when cells were incubated for further 96 hours without any significance difference between two sequential or between 2 and 4 μCi/ml ^{90}Yttrium. On the contrary, in those incubated for 72 hours, treatment with ^{90}Yttrium -> 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 when comparing to cells treated with cyclophosphamide alone. This suggests the prolong effect of combination therapy.

In the aspect of cytarabine, there was no significant difference between combination with 2 and 4 μCi/ml ^{90}Yttrium (figure 16). Synergistic effect was demonstrated at the concentration lower than 0.2 μg/ml point in all conditions except treating with the sequential ^{90}Yttrium -> cytarabine and incubated for further 72 hours. The latter did not show any benefit comparing to treating with cytarabine alone. No benefit was delivered from combination with cytarabine at concentration higher than 0.2 μg/ml. However, DMF of combined cytarabine and ^{90}Yttrium could not be calculated since over 70% inhibition was observed (table 3 and 4).

Regarding fludarabine, synergism in antiproliferative effect was demonstrated in every treatment arm, both assessed after 72 and 96 hours incubation period (figure 16). The largest difference in antiproliferative effect was found at the 2 μg/ml point. Higher degree of synergism was found after 96 hours incubation time (DMF = 72.24 – 91.69) comparing with those incubated for 72 hours (DMF = 2.84 – 14.62) as shown in table 4 and 3, respectively.

Comparable effect was revealed among cells treated with doxorubicin alone or combined with ^{90}Yttrium (figure 17). Correspondingly, all calculated DMF could not demonstrate favorable synergy between doxorubicin and ^{90}Yttrium (table 3 and 4). This suggests no to little synergistic effect between doxorubicin and ^{90}Yttrium.

In terms of etoposide, there was no significant difference in antiproliferative effect between cells treated with etoposide alone or combined with ^{90}Yttrium and incubated for further 72 hours (figure 17). However, combined regimen provided slightly more benefit than treating with etoposide alone in those cells incubated for further 96 hours. In these groups, contrary to other previous results, the combination with 4 μCi/ml ^{90}Yttrium, independent upon administration sequential showed higher degree of inhibition than combination with 2 μCi/ml ^{90}Yttrium. This result corresponded to calculated DMF in table 3 and 4).

Lastly, the combination of vincristine, even at very low concentration, and ^{90}Yttrium in any condition provide strong antiproliferation to Ramos cells. Over 80% inhibition was found in every treatment arm, assessing either after 72 or 96 hours incubation time (figure 17).

The complete inhibition of cell proliferation was confirmed in another experiment of combined therapy between ^{90}Yttrium and part of CHOP regimen including cyclophosphamide, doxorubicin, and vincristine using IC_{50} and IC_{90} level of each drug. There was no viable cells found even after 96 hours incubation period.

To summarize, ^{90}Yttrium and all tested antilymphoma drugs exhibited antiproliferative effect to Ramos cell lines. Their IC_{50} for antiproliferation were much lower than IC_{50} for cytotoxic effect. Synergism was revealed when treated cells with ^{90}Yttrium and cyclophosphamide, lower than 0.2 μg/ml cytarabine arabinoside, 0.2 – 200 μg/ml fludarabine, and vincristine.

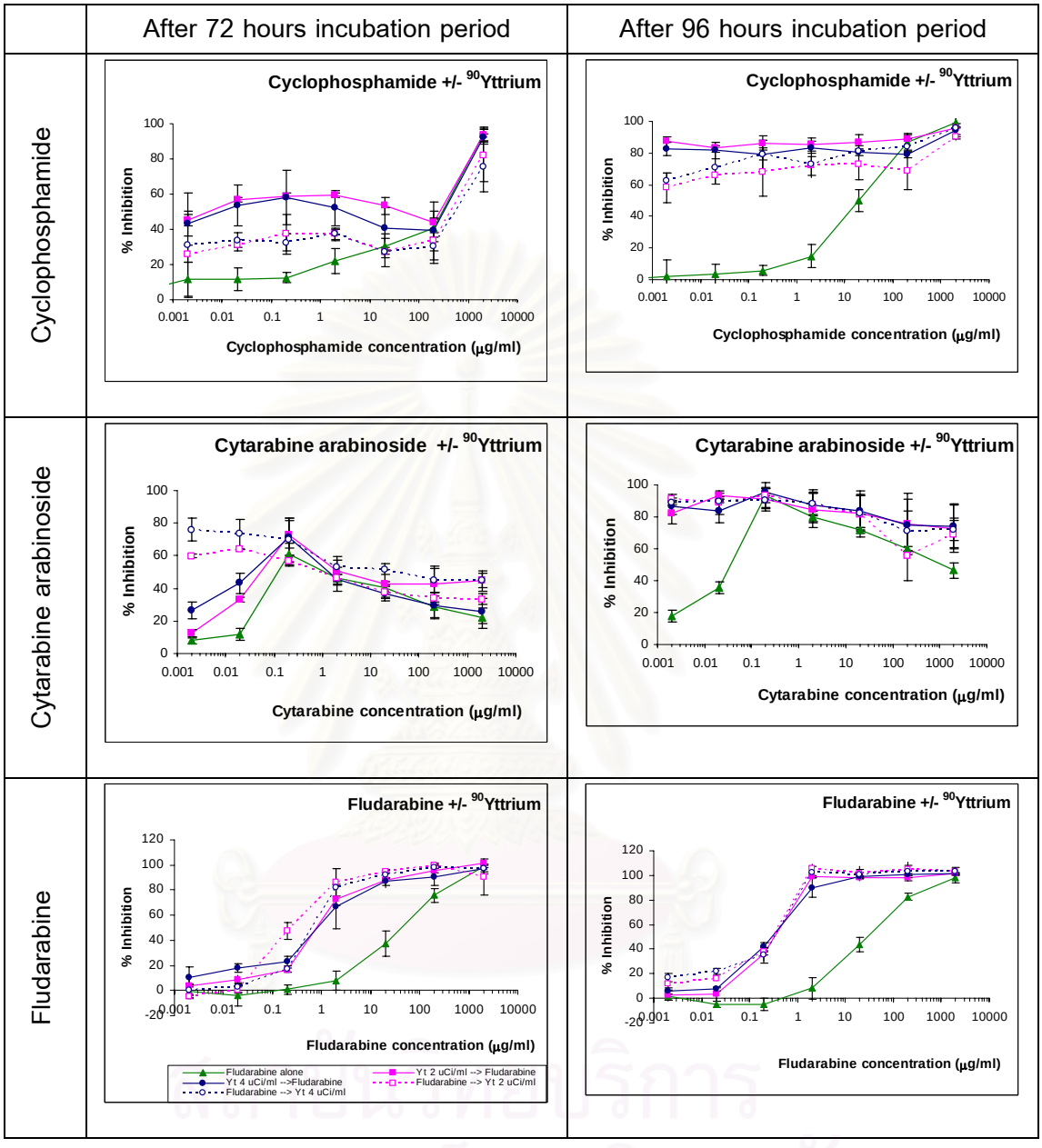


Figure 16 Inhibition of proliferation of Ramos cell, density $1x10^6$ cells/ml, evaluated at 72 and 96 hours after treating with ^{90}Yttrium combined with cyclophosphamide, cytarabine arabinoside, or fludarabine for 24 hours

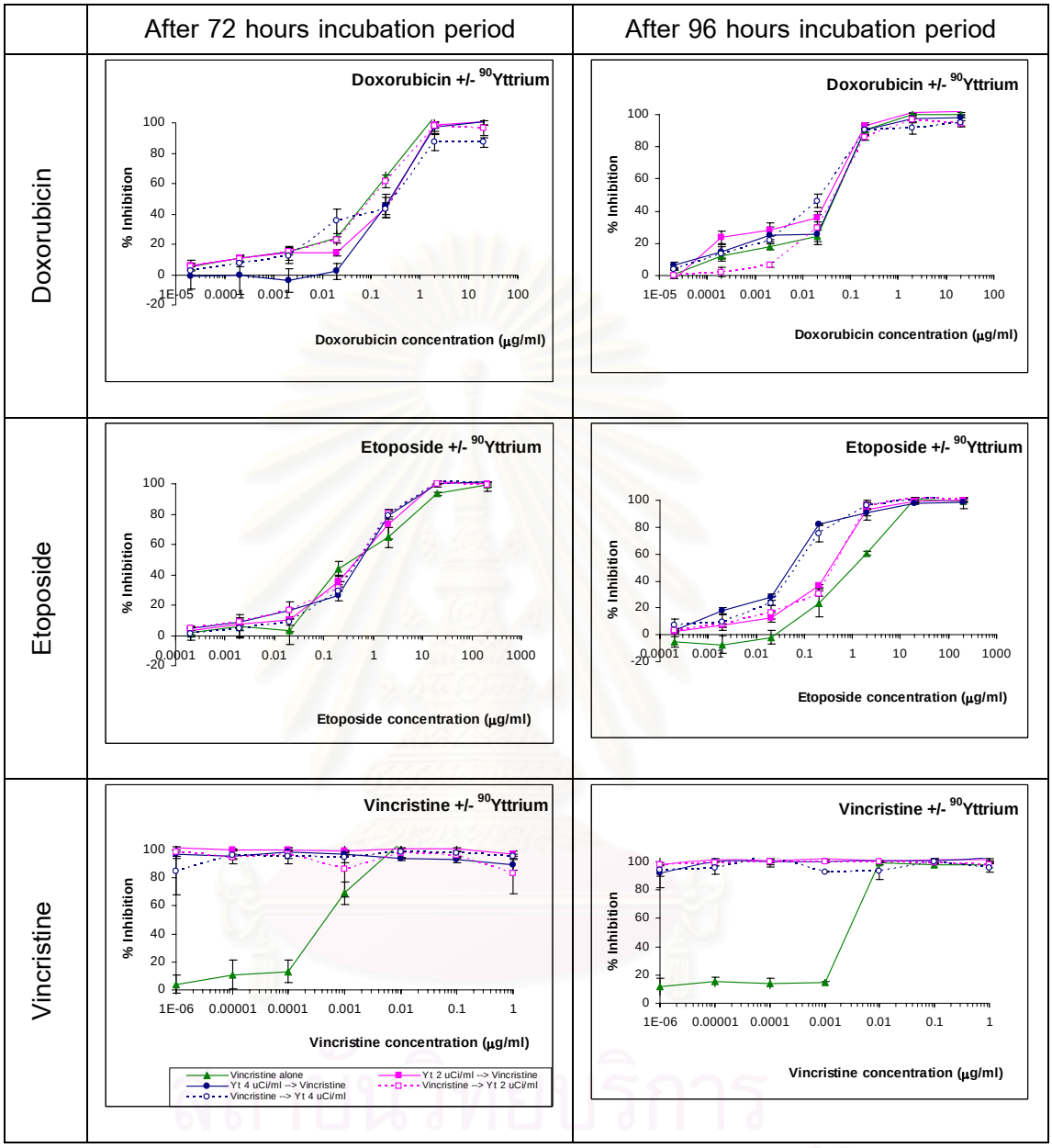


Figure 17 Inhibition of proliferation of Ramos cell, density $1x10^6$ cells/ml, evaluated at 72 and 96 hours after treating with ^{90}Yttrium combined with doxorubicin, etoposide, or vincristine for 24 hours

Table 3 Antiproliferative IC_{50} and Drug modification factor (DMF) of Combined therapy assessed after 72 hours incubation period. Data was presented as IC_{50} [DMF].

Drug alone	^{90}Yttrium -> Antilymphoma drugs		Antilymphoma drugs -> ^{90}Yttrium	
	2 μCi/ml ^{90}Yttrium	4 μCi/ml ^{90}Yttrium	2 μCi/ml ^{90}Yttrium	4 μCi/ml ^{90}Yttrium
Cyclophosphamide IC_{50}=268.19 μg/ml	219.48 μg/ml [1.22]	382.31 μg/ml [0.70]	688.52 μg/ml [0.39]	958.94 μg/ml [0.27]
Cytarabine IC_{50}=0.2,1.5 μg/ml	Unable to assess since over 70% inhibition of growth was found in all wells treated with combined therapy			
Doxorubicin IC_{50} = 0.12 μg/ml	0.24 μg/ml [0.50]	0.22 μg/ml [0.57]	0.13 μg/ml [0.93]	0.22 μg/ml [0.55]
Etoposide IC_{50} = 0.95 μg/ml	0.83 μg/ml [1.14]	0.76 μg/ml [1.24]	0.66 μg/ml [1.44]	0.73 μg/ml [1.29]
Fludarabine IC_{50}= 3.07 μg/ml	0.99 μg/ml [3.10]	1.08 μg/ml [2.84]	0.21 μg/ml [14.62]	0.76 μg/ml [4.04]
Vincristine IC_{50} = 0.59 ng/ml	Unable to assess since over 80% inhibition of growth was found in all wells treated with combined therapy			

Table 4 Antiproliferative IC_{50} and Drug modification factor (DMF) of Combined therapy assessed after 96 hours incubation period. Data was presented as IC_{50} [DMF].

Drug alone	^{90}Yttrium -> Antilymphoma drugs		Antilymphoma drugs -> ^{90}Yttrium	
	2 μCi/ml ^{90}Yttrium	4 μCi/ml ^{90}Yttrium	2 μCi/ml ^{90}Yttrium	4 μCi/ml ^{90}Yttrium
Cyclophosphamide IC_{50}=19.54 μg/ml	Unable to assess since over 60% inhibition of growth was found in all wells treated with combined therapy			
Cytarabine IC_{50}=0.04 μg/ml	Unable to assess since over 70% inhibition of growth was found in all wells treated with combined therapy			
Doxorubicin IC_{50} = 0.06 μg/ml	0.04 μg/ml [1.53]	0.06 μg/ml [1.00]	0.05 μg/ml [1.20]	0.02 μg/ml [2.62]
Etoposide IC_{50} = 1.35 μg/ml	0.33 μg/ml [4.17]	0.06 μg/ml [22.57]	0.40 μg/ml [3.39]	0.09 μg/ml [15.04]
Fludarabine IC_{50}= 23.84 μg/ml	0.26 μg/ml [91.69]	0.27 μg/ml [88.30]	0.29 μg/ml [82.21]	0.33 μg/ml [72.24]
Vincristine IC_{50} = 3.13 ng/ml	Unable to assess since over 90% inhibition of growth was found in all wells treated with combined therapy			

Efficacy and Safety of Combined ^{90}Yttrium Ibritumomab Tiuxetan and CHOP Regimen in Human Subjects

1. General information

There were 10 subjects recruited into the study during 10 months period (26 April 2006 – 23 February 2007) with the average of 1 case per month. All subjects complied with the inclusion and exclusion criteria mentioned in Materials and Methods. The overall study time including follow up time was 22 months (April 2006 – January 2008). The average follow up time for each of 9 subjects was 42.65 $\pm$ 4.16 weeks (range 36-52 weeks). A subject was followed up for only 9 weeks since her expiration.

As shown in table 5, 4 men and 6 women were recruited into the study. Their average age was 44.80 $\pm$ 12.69 (range 32-67) years old. Two from 10 subjects were considered as elderly. Concerning of primary sites of lymphoma, about 60% of subjects had an increase in size and number of lymph nodes in nasopharynx and shoulder area, while the other 30.0% presented with positive nodes in abdominal and pelvic area (Table 6). Approximately half of subjects presented with multiple bad prognostic factors including higher clinical stage III and IV (50.0%), presence of extranodal sites (60.0%), presence of B-symptom (20.0%), having bulky disease (40.0%), and splenomegaly (30.0%). As regarding to the risk level defined by the international prognostic index (IPI), 20.0% of subjects were classified as low risk level while 70.0% and 10.0% were classified as intermediate and high risk group, accordingly. About 70.0% and 30.0% of subjects were found positive to Bcl-2 and Hepatitis B test, respectively. Only one subject had lymphoma infiltration into less than 15% of bone marrow which was less than specified in the exclusion criteria.

Table 5 Demographic data of subjects

Demographic data	Distribution data
Gender	4 men, 6 women
Age (years)	Range 32-67
	Mean 44.80 $\pm$ 12.69,
No. of elderly	2 (20.0%)

Table 6 Pretreatment clinical data of participants

Clinical data	No. of patients (n = 10)
Primary sites of lymphoma	Nasopharynx and shoulder (6)
	Chest (1), Abdomen and Pelvic (3)
Clinical stage of lymphoma	
Stage II	5 (50.0%)
Stage III	4 (40.0%)
Stage IV	1 (10.0%)
Presence of extranodal sites	6 (60.0%)
Presence of B-symptom on admission	2 (20.0%)
Presence of bulky disease	4 (40.0%)
Risk level according to International	
Prognostic Index	
Low	2 (20.0%)
Low intermediate	5 (50.0%)
High intermediate	2 (20.0%)
High	1 (10.0%)
Positivity to Bcl-2	7 (70.0%)
Bone marrow involvement	1 (10.0%)
Presence of splenomegaly	3 (30.0%)
Having elevated LDH level > 500 u/l	6 (60.0%)
Positivity to hepatitis B test	3 (30.0%)

2. Clinical Response to the Combined Therapy

Since the attachment of ^{90}Yttrium to the carrier molecule, ibritumomab tiuxetan, over than 90% is the primary factor that support the efficacy of radioimmunotherapy such as ^{90}Yttrium ibritumomab tiuxetan (Y2B8). The attachment of ^{90}Yttrium in each preparation was found over 95% as shown in table 7 The mean time from diagnosis to Y2B8 administration was 27.5 $\pm$ 6.95 days (range 17-37 days). Since prolong waiting time for drug delivery, subject no. 1 received oral dexamethasone to control disease progression prior to Y2B8 treatment.

At the end of Y2B8 phase, 90.0% had good response to the treatment (8 with partial response, and 1 with unconfirmed complete response). Only subject no.9 had progression at 5 weeks after treatment that the urgent CHOP was considered. Moreover, progression responded to oral dexamethasone was observed in other two subjects (subject no. 1 and 2).

Due to cross comparing response with various factors as in table 8 , number of responder to Y2B8 was comparable among clinical stage II group (3 from 5 subjects) and stage III and IV group (4 from 5 subjects). Two subjects with low risk level had good response to Y2B8 while the one with high risk level had progression at the end of the course. Two (subject no. 1 and 2) from 5 subjects with low intermediate risk level experienced disease progression. However, both subjects, even though had clinical stage II, presented with bulky disease and extranodal sites since admission. Almost 75.0% (3 from 4 subjects) who had bulky disease and all of those with extranodal sites developed progression during therapy. About 67.0% (2 from 3 objects) who had spleenomegaly had good response to the treatment.

Concerning of Bcl-2, 5 of 7 subjects with positive Bcl-2 (representing 71.4%) and 2 of 3 subjects with Bcl-2 negative (representing 66.7%) showed good response to the treatment while about 30% of subjects either Bcl-2 positive or negative developed disease progression (table 9).

Table 7 Response at the end of ^{90}Yttrium ibritumomab tiuxetan phase

Subject no.	Time from diagnosis (days)	^{90}Yttrium attachment (%)	Response*	Management of problem -> response
1	36	97.74	Progression before and after 5 weeks -> PR	Oral dexamethasone -> improve
2	28	99.31	Progression after 5 weeks -> PR	Oral dexamethasone -> improve
3	36	99.04	PR	
4	37	98.68	PR	
5	21	97.09	PR	
6	24	98.39	PR	
7	23	96.89	PR	
8	24	98.09	PR	
9	29	97.18	Progression after 5 weeks	Urgent CHOP-1
10	17	98.89	CRu	
Range	17-37			
Mean	27.5			
$\pm$ SD	$\pm$ 6.95			

* CR = complete response, CRu = unconfirmed complete response, PR = partial response,

Table 8 Clinical response to ^{90}Yttrium ibritumomab tiuxetan related to various clinical factors on admission

Clinical factors	No. of subjects in each group			Total (n=10)
	Partial response (n=6)	Unconfirmed complete response (n=1)	Progression (n=3)	
Disease stage				
Stage II	2	1	2	5
Stage III	3	-	1	4
Stage IV	1	-	-	1
Risk level				
Low	1	1	-	2
Low intermediate	3	-	2	5
High intermediate	2	-	-	2
High	-	-	1	1
Presence of bulky disease (n=4)	1	-	3	4
Presence of extranodal sites (n=6)	3	-	3	6
Spleen involvement (n=3)	2	-	1	3

Table 9 Primary response to ^{90}Yttrium ibritumomab tiuxetan classified according to positivity to Bcl-2 protein

Bcl-2 protein	Response to ^{90}Yttrium ibritumomkab tiuxetan		
	Partial response	Unconfirmed complete response	Progression
Positive (n = 7)	4 (57.1%)	1 (14.3%)	2 (28.6%)
Negative (n = 3)	2 (66.7%)	-	1 (33.3%)

All 10 subjects were recruited into consolidation phase with 6 consecutive cycles of CHOP (a combination therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone) according to the protocol. Mean lag time between Y2B8 and CHOP-1 was 67.60 ± 12.95 days (range 48-92 days). Two subjects (subject no. 1 and 9) received CHOP much earlier than mentioned in the protocol since the disease progression. All subjects except subject no. 9, who died 1 week after CHOP-1, received 6 cycles of CHOP according to the protocol. The median lag time between each cycles was 3 weeks (range 3-7 weeks). Delay in CHOP administration, as usually found in subject no. 5, was due to the depression in blood counts (Table 10).

Table 10 Lag time between each cycle of CHOP and total follow up time

Subject no.	Time between each CHOP cycle (days)						Follow up time (weeks)
	Y2B8 to CHOP1	CHOP1-> CHOP2	CHOP2-> CHOP3	CHOP3-> CHOP4	CHOP4-> CHOP5	CHOP5-> CHOP6	
1	48	25	21	23	22	21	42
2	62	32	30	22	20	21	40
3	70	28	28	26	21	21	45
4	70	21	21	21	21	28	41
5	77	47	28	44	28	28	52
6	69	22	26	21	22	21	42
7	70	21	21	21	21	26	43
8	70	21	21	26	23	21	36
9*	48	-	-	-	-	-	9
10	92	21	28	21	21	21	43
Range	48-92	21-47	21-30	21-44	20-28	21-28	9-52
Mean ± SD	67.60 ± 12.95	26.56 ± 8.62	24.59 ± 3.82	25.00 ± 7.42	22.11 ± 2.37	23.11 ± 3.22	39.31 ± 11.25
		Range 20-47 days (3-7 weeks) Median 21 days (3 weeks)					

** Subject was expired 1 week after CHOP-1 administration.*

According to the study protocol, clinical response was evaluated four times for each subject:-at the completion of Y2B8 treatment or before CHOP-1, after the completion of CHOP-4, 3 weeks after CHOP-6, and 3 months after CHOP-6. As shown in figure 18, the number of subjects with partial response tended to decrease from 6 subjects after Y2B8 treatment to 5 and 1 subjects after CHOP-4 and 3 weeks after CHOP-6. On the contrary, the number of those receiving unconfirmed complete response increased from 1 subject after Y2B8 to 2 and 4 subjects after CHOP-4 and 3 weeks after CHOP-6, respectively. Similarly, the subjects with complete response increased from 2 subjects after CHOP-4 to 3 and 4 at 3 weeks and 3 months after CHOP-6, accordingly. This suggests the possibility of benefit from the combined therapy. Unfortunately, a progressive case was found at 3 weeks after CHOP-3 and the other 3 relapsing cases were found at the end of the study period. Changes in clinical response of each subject along the program was described in table 7. At least 50% of cases (subject no. 1, 3, 5, 6, and 7) seemed to gain benefit from the entire treatment.

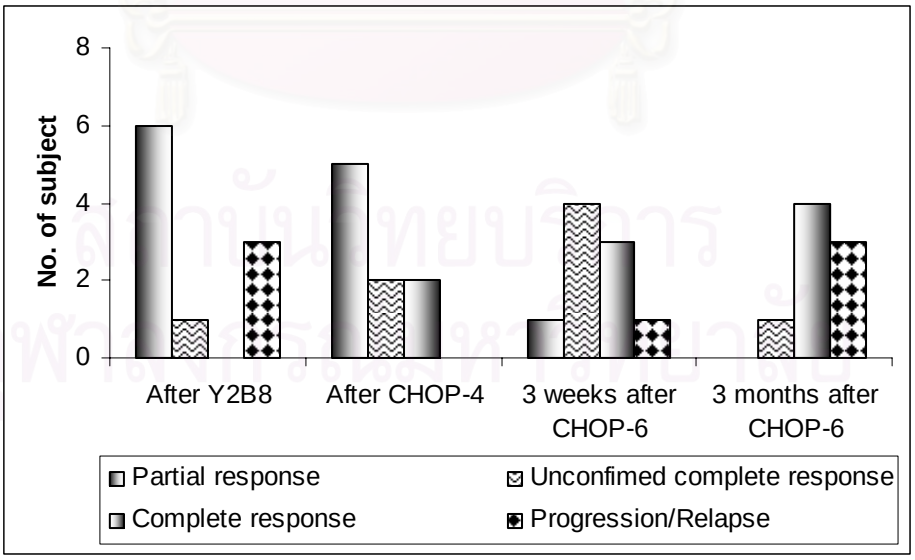


Figure 18 Clinical response evaluated along the program

Table 11 Changes in clinical response of each subject along the program

Subject no.	Clinical response*			
	After Y2B8	After CHOP-4	3 weeks after CHOP-6	3 months after CHOP-6
1	Progression	CRu	CRu	CR
2	Progression	PR	CRu	Relapse
3	PR	PR	PR	CRu
4	PR	CR	CR	Relapse
5	PR	CR	CR	CR
6	PR	PR	CRu	CR
7	PR	PR	CR	CR
8	PR	PR	Progression	Progression
9	Progression	NA	NA	NA
10	CRu	CRu	CRu	Relapse

* NA *Not access* — *Progression or Relapse*

PR *Partial response* — CRu *Unconfirmed complete response* — CR *Complete response*

3. Disadvantage of the Combined Therapy

Disadvantage of the program was investigated in two aspects- progression or relapse and adverse drug events.

Starting with progression and relapsing episodes, four cases with disease progression and 3 relapsing cases were revealed (Table 12). Among the progressive cases, 3 cases (subject no. 1, 2, and 9) were discovered at 5 weeks after Y2B8 treatment in spite of the good response (reduced number and size of affected lymph nodes) evaluated at 2 weeks after drug administration. The progressive disease in both cases could be controlled by oral dexamethasone, while the urgent CHOP-1 was introduced in another serious case. Subject no. 8 was found refractory to chemotherapy that her problem had to be handled by several aggressive treatments such as ESHAP (a

combination of etoposide, methylprednisolone, doxorubicin, high dose cytarabine, and cisplatin), autologous peripheral blood stem cell therapy, and finally, radiotherapy.

In the aspect of relapsing cases that were revealed on the 3 months-visit after the 6th CHOP, all three cases presented with lymphadenopathy in the same area as their primary lymphoma sites. Two of them were re-treated with alternative treatments for relapse lymphoma and one was still under observation at the end of the project. However, radiotherapy was required in subject no. 2.

Table 12 Progression and relapse occurring along the study period and their management

Subject no.	Time from treatment	Affected sites	Management* -> Response
Progression (n=4)			
1	5 weeks after Y2B8	Scapular mass	Oral dexamethasone ->improve
2	5 weeks after Y2B8	Neck lymph node	Oral dexamethasone -> improve
8	2 weeks after CHOP-6	Neck lymph node	1. ESHAP 5 cycles -> improve 2. Autologous peripheral blood stem cell therapy -> improve 3. Radiotherapy -> improve
9	5 weeks after Y2B8	Abdominal mass	Start CHOP earlier
Relapse (n=3)			
2	3 months after CHOP-6	Neck lymph node	1. ESHAP + IT- MTX 2 cycles -> ICE 2 cycles -> radiotherapy -> improve 2. EPOCH + IT-MTX 6 cycles -> radiotherapy -> improve
4	3 months after CHOP-6	Lymph node in abdomen and Thorax	Under observation till next visit
10	3 months after CHOP-6	Left breast	R-ESHAP + IT MTX 3 cycles

** ESHAP = Etoposide, Methylprednisolone, Doxorubicin, High dose Cytarabine, Cisplatin IT MTX = Intrathecal methotrexate*
EPOCH = Etoposide, Vincristine, Doxorubicin, Cyclophosphamide, Prednisolone ICE = Ifosphamide, Cisplatin, Etoposide

Adverse events due to the combined therapy were also evaluated along the study. According to laboratory and physical examination data, only hematologic adverse events were found. However, number of adverse event episodes might be less than expected since the prevention treatment such as blood cells stimulating factors were also prescribed in the treatment protocol as optional for the high risk patients.

Table 12 demonstrated the figures of adverse events to blood cells due to Y2B8 treatment. Eight subjects (80%) experienced depressed neutrophil counts with the mean time to nadir was 5.80 $\pm$ 1.69 weeks (range 2-8 weeks). Only 2 cases, representing 20.0% of subjects, suffered from severe grade 3 to 4 neutropenia. However, febrile neutropenia episode was not reported. Concerning the reduction in platelet counts, 9 subjects (90.0%) had decreased platelet counts to less than $150x10^3/\mu l$. The mean time to thrombocytopenia nadir was 4.50 $\pm$ 0.71 weeks (4-6 weeks). Among them, only 2 subjects, representing 20.0% of all subjects, were classified as severe grade 3 and 4 thrombocytopenia. One of which (subject no. 9) had many risk factors such as chronically ill, unstable clinical status, and complicated polypharmacy and treatments. There was only one mild anemic case found. As the whole, subject no. 5 and 9 were suffered from severe depression of neutrophil and platelet counts. Changes in neutrophil counts and platelet counts in each subject were also demonstrated in Figure 19 and 20, respectively.

The regimen CHOP tended to induce more serious hematologic disorders comparing to the Y2B8 treatment (Table 14). Nine subjects (90.0%) had decreased neutrophil counts, two-third of which had severe drop in neutrophil counts. Three (subject no. 3, 6 and 7) of 9 neutropenic patients were suffered from serious febrile neutropenic episodes that broad spectrum antibiotics were introduced immediately. A decrease in platelet counts was found in 4 subjects (representing 40.0% of all participants), two of which had moderate to severe thrombocytopenia requiring blood product treatment. Mild to moderate anemia was also found in 4 subjects. During CHOP phase, 4 febrile neutropenic episodes were found in 3 subjects with grade 4 neutropenia and in 1 subject with grade 2 neutropenia. Hematologic

disorders occurred after any cycle of CHOP depending on patient status. However, anyone who suffered from severe disorders will be considered to receive blood cells stimulating factors in the next chemotherapy course as declared in ethical consideration of protocol.

Table 13 Hematologic adverse events during ^{90}Yttrium ibritumomab tiuxetan treatment

Type and severity* of adverse events	No. of subjects (Subject no.)	Time to nadir (weeks)	
		Range	Mean ± SD
Decreased neutrophil counts (neutropenia)		2-8	5.80 ± 1.69
Grade 1	3 [2, 7, 8]		
Grade 2	3 [1, 4, 6]		
Grade 3	1 [9]		
Grade 4	1 [5]		
Decreased platelet counts (thrombocytopenia)		4-6	4.50 ± 0.71
Grade 1	6 [1-4, 7, 8]		
Grade 2	1 [10]		
Grade 3	1 [9]		
Grade 4	1 [5]		
Anemia		7	-
Grade 1	1 [5]		

** Based on the National Cancer Institute Classification on adverse events*

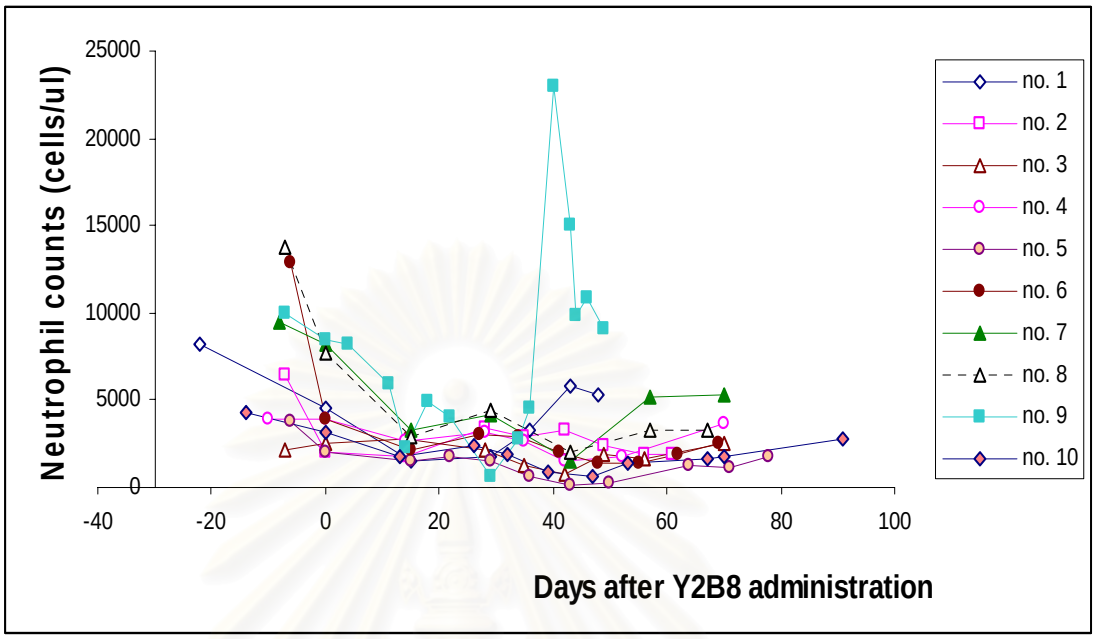


Figure 19 Changes in neutrophil counts during ^{90}Yttrium ibritumomab tiuxetan treatment

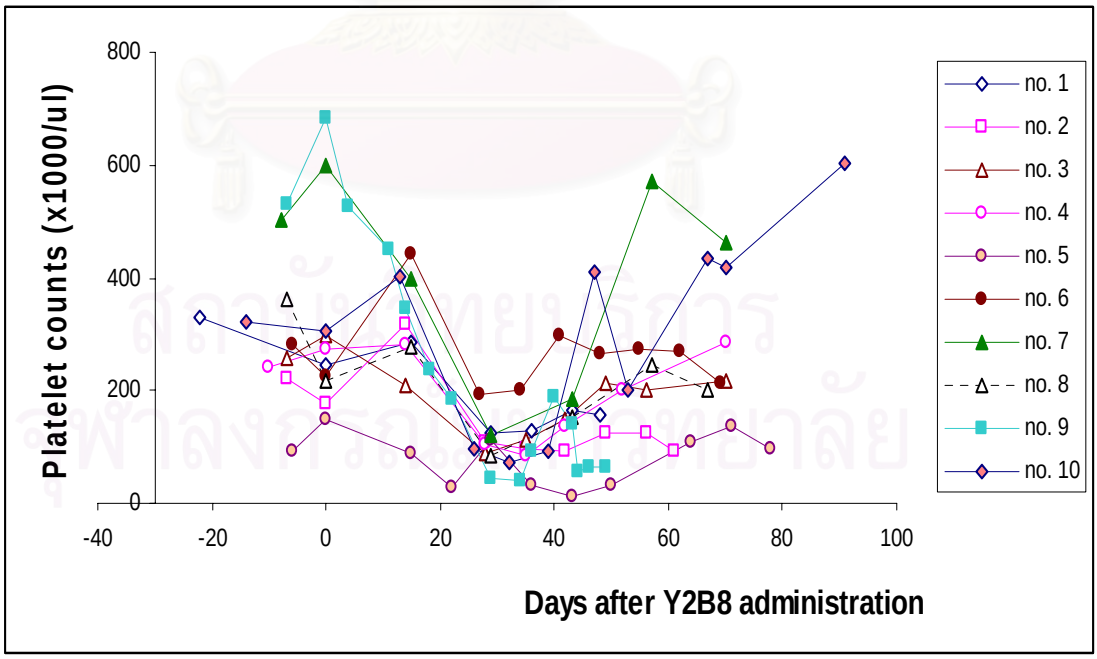


Figure 20 Changes in platelet counts during ^{90}Yttrium ibritumomab tiuxetan treatment

Table 14 Hematologic adverse events during CHOP treatment

Type and severity of adverse events*	No. of subjects (Subject no.)	Corresponding cycle of CHOP
Decreased neutrophil counts (neutropenia)		
Grade 2	3 [1, 2, 10]	1, 2, 6
Grade 4	6 [3-7, 9]	2, 1, 1, 2, 2, 1
Decreased platelet counts (thrombocytopenia)		
Grade 1	1 [1]	2
Grade 2	1 [6]	5
Grade 3	2 [2, 5]	4, 1
Decreased red blood cells		
Grade 1	1 [2]	4
Grade 2	3 [3, 4, 6]	2, 3, 5
Febrile neutropenia	4 [3, 6, 7, 10]	3, 5, 1, 3

** Based on the National Cancer Institute Classification on adverse events*

4. Overall Clinical Outcome

Overall clinical outcome at 3 months after the 6th CHOP was assessed as the final assessment of this study. Nine subjects (90.0%) were alive after the whole treatment. The cure rate, corresponding to 4 subjects who still alive with disease free, was approximately 40.0%. Only 10.0% of participants still had residual disease according to physical examination data. The other 4 subjects (representing 40.0%) may be considered as failure rate (Table 15). However, another expired subject was considered as questionable.

Regarding of Bcl-2 biomarker and primary outcome assessed at the end of study period, progression was found in both group of Bcl-2 positive and negative. According to the calculated percentage, 5 of 7 patients with Bcl-2 positive (71.4%) and 1 of 3 patients with Bcl-2 negative (14.3%) received good response. It seemed that Bcl-2 positive correlated with good response. However, other clinical factors, such as disease bulkiness, and extranodal sites, should be considered. Therefore, since a small sample size, correlation between Bcl-2 and clinical response to treatment could not be elucidated clearly.

Table 15 Clinical outcome at 3 months after 6th cycle of CHOP

Clinical status	No. of subjects (n=10)	Percentage of all subjects
Still alive	9	90.0 %
- with disease free	4	40.0 %
- with residual disease	1	10.0 %
- with progression / relapse	4	40.0 %
Dead	1	10.0%

Table 16 Clinical response at 3 months after 6th cycle of CHOP

Bcl-2 protein	Clinical response	
	Complete response / unconfirmed complete response	Progression / Relapse
Positive (n = 7)	5 (71.4%)	2 (28.6%)
Negative (n = 3)	1 (14.3%)	2 (66.7%)

CHAPTER V

CONCLUSION AND DISCUSSION

Ramos cell line is an CD20 expressing lymphoma cell derived from Burkitt's lymphoma cells, a serious type of lymphoma similar to diffuse large B-cell type (. Eventhough, it expresses less degree of CD20 than diffuse large B-lymphoma cells, it exhibits general property of B-lymphocyte as DLBCL cells.

Due to the availability of Yttrium ibritumomab tiuxetan (Y2B8), ^{90}Yttrium-EDTA was used instead in *in vitro* study part. The difference between these two molecules is the specificity to lymphoma cells containing CD20. However, since this study was performed with cell line containing CD20, the effect of ^{90}Yttrium apply directly to the cells can be applied to the effect of Y2B8 on in situ lymphoma cells.

Two fixed dosage of 2 and 4 μCi/ml ^{90}Yttrium used in this study were modified from its allied derivative, iodine tositumomab in another study . However, most experiments did not show the significant difference between the two dosages. This may due to too close dose level. Higher dose should not be considered since it might be comparable to toxic dose of Y2B8 in human. Therefore, further study should be done using lower concentration of ^{90}Yttrium.

Synergistic cytotoxic effect of ^{90}Yttrium and antilymphoma drugs

Generally, radioisotope particularly at low dose exhibits long term destroying effects on cells much more than short term effects like cytotoxicity. This support that ^{90}Yttrium at 10-100 μCi/ml used in this study did not show cytotoxic to the Ramos cell.(figure 8 and 9) but had antiproliferative effect after 72 and 96 hours incubation period (figure 13). This two graphs also imply that ^{90}Yttrium at 2 and 4 μCi/ml will not have effect on cytotoxicity or antiproliferation. Therefore, they are appropriate for further study dealing with drug combination.

Regarding cells treated with either drugs alone, 3 drug categories according to their potential to cause cytotoxic are classified (figure 10). The first group

with no cytotoxic effect is composed of rituximab, ibritumomab, and cytarabine. Rituximab acts on cells through cell signal transduction to induce ADCC, while ibritumomab works basically as a carrier of ^{90}Yttrium. This also implied that the efficacy of Y2B8 in the following clinical study is due to radio-destroying effect of attached ^{90}Yttrium. Furthermore, cytarabine is a nucleotide analogue that its effect always takes long time, maybe until to new cell generation. The second group which show cytotoxic effect just only at the highest concentration equivalent to product concentration include cyclophosphamide and fludarabine. Since metabolite of cyclophosphamide was not tested in this study due to its unavailability, cyclophosphamide is used in this study. Cyclophosphamide has less cytotoxicity may be due to its main action of immunosuppressant. The most potent group includes doxorubicin, etoposide and vincristine. The order of potency is vincristine > etoposide > doxorubicin.

Synergism of ^{90}Yttrium and antilymphoma drugs was found in the combination of ^{90}Yttrium and doxorubicin, etoposide, or vincristine (figure 11). On the molecular basis, this may result from their synergistic toxicity on DNA due to ^{90}Yttrium and doxorubicin. Both etoposide and vincristine are antimicrotubules that induce cell proliferation arrest, therefore this accompanying with DNA toxic effect of ^{90}Yttrium causes synergistic effect. It seems that antagonism occur when low dose fludarabine combined with ^{90}Yttrium.

Synergistic antiproliferative effect of ^{90}Yttrium and antilymphoma drugs

The cell proliferation was assessed after 72 and 96 incubation period. The reason for this separation is due to the very high cell density of $1x10^6$ /ml was used to imitate the dense property in lymphoma. However, easily cell dying since high density in culture, therefore, should be concerned. The problem of dying in control wells after 96 hours was not found may be due to most cells turned to lag phase after treatment with drugs.

All drugs tested, except ibrituximab, showed antiproliferative effect on Ramos cells with different degree (figure 14 and 15). Most drugs except rituximab and cytarabine exhibit cytotoxic dose-dependently with the IC_{50} lower than IC_{50} for cytotoxic

effect. Interestingly, cytarabine exhibits bell-shaped effect that peak effect was found at 0.2 μg/ml point. As mentioned previous, cytarabine acts as a nucleotide analog that produce long term effect on cell activity and finally cell proliferation. The effect after 72 and 96 incubation period was found significant difference only with cyclophosphamide.

Two sequentials of drug combination were chosen for each drug combination because the order of drug administration might produce different effect. Theoretically due to the crossfire effect of Y2B8 or the high penetration effect of ^{90}Yttrium pretreatment with isotope may induce eradication of lymphoma cells more than those vice versa treatment. However, the difference in two sequentials did not revealed in any drug pairs except ^{90}Yttrium-cytarabine assessed after 72 hours (figure 16). Treatment with cytarabine -> ^{90}Yttrium seems to produce high degree of antiproliferative effect. This might be due to less healthy cells are left after pre-treating with cytarabine in the cytarabine-> ^{90}Yttrium group than those pre-treating with ^{90}Yttrium in the ^{90}Yttrium -> cytarabine group.

Large difference was shown in the combination of ^{90}Yttrium and cyclophosphamide comparing between different incubation time. This corresponds with the experiment on cyclophohphamide alone. The higher degree in antiproliferation may due to the immunosuppressive effect of cyclophosphamide that suppress growth of new B-lympholyte cells. Therefore, less cells is left to interact with radioisotope when comparing to the effect of other drugs in other combinations. The high degree of synergism is also revealed with the combination with vincristine. Similar to cyclophosphamide but with different mechanism of action, vincristine reduces number of healthy cells in the well resulting in more effect of radioisotope on remaining cells.

<u>Efficacy and safety of the sequential therapy</u>

^{90}Yttrium ibritumomab tiuxetan (Y2B8) is a new target therapy composing of ^{90}Yttrium, a beta emitter isotope, attached to ibritumomab, a murine monoclonal specific to CD20 (mAb antiCD20) which found abundant in lymphoma cells. Theoretically, the reactor, ^{90}Yttrium, will be carried directly into lymphoma cells via the carrier mAb AntiCD20. Accordingly, benefit of Y2B8 over other mAb AntiCD20 alone

has been reported. As a whole of this study, almost 90% of patients had improvement at the end of the eradication phase with Y2B8. This is confirmed by reduction in number and size of positive lymph nodes, number of patient with good response evaluated periodically (figure 18) and gradual improvement demonstrated in each patient (table 11).

Many factors may affect the efficacy of Y2B8. They are the availability of Y2B8 at lymphoma sites, burden of disease, and patient characteristics. As general, it is recommended that determination of over 90% of ^{90}Yttrium attachment onto mAb Ibritumomab should be identified to confirm the maximum availability of radioisotope ^{90}Yttrium at the site of action. In this study, all preparations had over 95% attachment confirmed by thin layer chromatography. However, availability of Y2B8 is also dependent on blood supply around and inside lymph node. Since that, response in patients with bulky disease may unpredictable due to disease burden, less vascular supply inside lymphoma sites, the characteristic of cells re-growth. This may explain the 75% failure rate among subjects with bulky disease group in this study.

Concerning clinical factors affecting clinical response, the difference between those in stage II and those in higher stages cannot be clearly elucidated. This is probably due to other associated factors such as disease burden and presence of extranodal sites. Further analysis based on international prognostic index that includes 5 independent clinical factors (elderly, increased lactate dehydrogenase level, presence of exranodal sites, higher than level 2 performance status, and higher disease stage), are performed. It seems that all 2 subjects with IPI level 2 had good response to the treatment while the only one subject with high IPI level developed progression.

Since DLBCL is an easily progress type of lymphoma due to bulkiness, lag time of Y2B8 from diagnosis or active disease state is very important. In this study there are 2 progressive cases observed during waiting for drug delivery. However, these 2 cases still responded to conventional steroid therapy- dexamethasone as an immunosuppressant.

In terms of Bcl-2, no difference in clinical response found among those have Bcl-2 positive or negative. On the contrary from this study, 2 progressive case was

found among Bcl-2 negative group, while 2 from 7 subjects with Bcl-2 positive developed disease progression. Especially, subject no. 2 and 8 develop lymphoma considered as refractory to several salvage treatment that radiotherapy or stem cell therapy was introduced finally.

As summary for progression or relapsing cases, lag time between drug administration either from diagnosis to Y2B8 administration or during Y2B8 and CHOP administration; clinical factors such as presence of extranodal sites and disease bulkiness are factors that might indicate the possibility of progression.

In terms of adverse events, most subjects suffered from delayed type reduction in neutrophil and platelets counts (table 13, figure 19 and 20) with the mean nadir of 5.80 $\pm$ 1.69 and 4.5 $\pm$ 0.71 weeks, accordingly. This is similar to other reports. In this study group, decrease in platelets seems to be more serious than decrease in neutrophil counts. However, time of complete recovery could not be figured out since afterwards all subjects received CHOP. In this study, there was no subject suffered from serious side effects. On the contrary, about 90% of subjects experienced reduction in neutrophils counts during CHOP administration phase. Among them, four patients developed acute febrile neutropenia requiring broad spectrum antibiotics.

Concerning the overall benefit of the combination treatment, up to 90% of subject improve at the end of program. However, 40% developed disease progression assessed at 3 months later. This progression may be due to the characteristics of disease accompanying with the incomplete capability of treatment protocol for lymphoma cell eradication. The reason of this failure should be investigated on molecular basis.

Primary good response to the treatment protocol seems to correlate with *in vitro* study that there is synergism between ^{90}Yttrium and some antilymphoma drugs such as cytarabine, doxorubicin, etoposide, cyclophosphamide, and vincristine.

The result showing synergism between ^{90}Yttrium and doxorubicin, etoposide, or vincristine suggests the possibility of benefit from combined Y2B8 with many standard antilymphoma regimens such as CHOP (doxorubicin and vincristine are included), and ESHAP (etoposide, doxorubicin, and cytarabine)

CHAPTER VI

CONCLUSION AND SUGGESTION

According to the *in vitro* study, it strongly suggests that ^{90}Yttrium at 2 and 4 μCi/ml, which had no cytotoxicity or antiproliferation, potentiated the cytotoxic effect of doxorubicin, etoposide, fludarabine, and vincristine. The highest degree of potentiation was found close to IC_{50} of doxorubicin and etoposide, while the enhancement of vincristine effect was shown at the concentration above IC_{50}. Regarding synergism in antiproliferative effect on Ramos cell, ^{90}Yttrium combined with cyclophosphamide, cytarabine, fludarabine, and vincristine provided more benefit than treatment with each drug alone. There is no benefit observed from combination between radioisotope and doxorubicin. Slightly benefit was shown when use etoposide with high dose level ^{90}Yttrium and assessed after 96 hours incubation period. Differential in response due to order of drug administration was clearly demonstrated in the combined treatment with cyclophosphamide and cytarabine arabinoside after 72 hours incubation, and with fludarabine after 24 hours treatment. Taken together, these results suggest that the combination of ^{90}Yttrium and these drugs, which are usually included in standard treatment protocol and other salvage antilymphoma regimens, might provide more benefit than using those regimens alone.

As shown in the Clinical study of combined ^{90}Yttrium with CHOP regimen in diffuse large B-cell lymphoma patients, the combination treatment provided almost 90% improvement at the end of eradication phase with radioimmunotherapy. About 50% of patients were identified in complete remission state at 3 months after the completion of chemotherapy. Eventhough comparing to CHOP alone was not performed in this study due to ethical cosideration, this study suggests the comparable benefit of combined therapy to the standard treatment alone while there is less and non-serious adverse events to blood cells particularly neutrophil and platelets. However, uncontrolled disease, in terms of disease progression and relapsing cases were

observed at 3 months after completion of treatment protocol. Some primary clinical status including presence of extranodal sites, disease bulkiness, and easily adaptation characteristics of lymphoma cells might be the important factors.

The correlation of Bcl-2 and therapeutic response to chemotherapy is still controversial. Most studies suggested high ratio of response failure among Bcl-2 positive individuals. Contrarily, a higher response rate was found among Bcl-2 positive patients in this study. Since rather small sample size with unequalled number of patients in both Bcl-2 positive and negative groups as well as other clinical risk factors in this population study, the relation between Bcl-2 and response to treatment is inconclusive.

These results strongly suggest the benefit of combined ^{90}Yttrium and commonly used antilymphoma drugs. It is feasible to perform other clinical trial of ^{90}Yttrium ibritumomab tiuxetan and other antilymphoma drugs. Besides, further investigation such as clinical study in larger population of lymphoma patients or in other lymphoma types as well as the molecular insight of disease progression even during aggressive treatment should be performed.

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APPENDICES

APPENDIX A

No.117/2006
REC. No. 19/2549

Certificates of Approval

The Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol, the principal investigator and informed consent dated and/or amended as follows in compliance with the ICH/GCP.

Study Title : Phase II study of Yt^{90} Zevalin followed by CHOP chemotherapy (Z-CHOP) as first line treatment in diffuse large B-cell lymphoma

Study Code :

Centre : Chulalongkorn University

Principle Investigator : Dr. Udomsak Bunworasate

Document Reviewed :

1. Protocol , date 6 January, 2006
   Protocol Thai version date 6 March, 2006
2. Patient information sheet and consent form/Chulalongkorn hospital Thai Version date 6 March, 2006.

.....
(Professor Anek Aribarg, M.D.)
Chairman of Ethics Committee

.....
(Associate Professor. Vilai Chentanez, M.D.)
Associate Dean for Research Affairs

Date of Approval : March 10, 2006

Approval Expire Date : March 10, 2010

Approval is granted subject to the following conditions: (see back of this Certificates)

APPENDIX B

PROGRAM INFORMATION FOR PARTICIPANTS

โครงการวิจัย "ผลตอบสนองขั้นต้นและความปลอดภัยของการให้ยายิเทรียม ไอบริทูโมแมบ ไทยูซีแทน ตามด้วยแผนการรักษา CHOP ในผู้ป่วยมะเร็งต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์"

หัวหน้าโครงการวิจัย นพ. อุดมศักดิ์ บุญวรเศรษฐ์

สถานที่ทำวิจัย ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ความเป็นมาและความสำคัญของการวิจัย

มะเร็งต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์ เป็นมะเร็งต่อมน้ำเหลืองชนิดรุนแรง มักมีการกระจายของเซลล์มะเร็งไปยังต่อมน้ำเหลืองเกือบทั่วร่างกาย ผู้ป่วยส่วนใหญ่ที่มาพบแพทย์มักอยู่ในระยะที่มีการดำเนินโรคไม่ค่อยดี มีอัตราการรอดชีวิตต่ำ การรักษาที่ยอมรับกันในปัจจุบัน คือ การให้เคมีบำบัด การฉายรังสี และ การปลูกถ่ายไขกระดูก ซึ่งโดยส่วนใหญ่ผู้ป่วยมักเกิดอาการข้างเคียงขึ้นไม่มากก็น้อย โดยเฉพาะอาการข้างเคียงที่เกิดขึ้นต่อเนื้อเยื่อปกติอื่นเนื่องจากการออกฤทธิ์ที่ไม่จำเพาะของแผนการรักษาที่ใช้กันอยู่

ด้วยความก้าวหน้าในวิทยาการด้านภูมิคุ้มกันผสมผสานกับรังสีรักษา ได้มีการพัฒนายาขึ้นในลักษณะของผลิตภัณฑ์ชีวภาพติดสารกัมมันตรังสี ได้แก่ ยิเทรียม ไอบริทูโมแมบ ไทยูซีแทน (^{90}Yttrium ibritumomab tiuxetan) ยานี้มีคุณสมบัติบางประการเหนือกว่าการรักษาในลักษณะเดิม คือ มีความจำเพาะต่อเซลล์มะเร็งสูง สามารถนำพาอนุภาคสารกัมมันตรังสีไปทำลายเซลล์มะเร็งได้ค่อนข้างทั่วถึง ในขณะเดียวกันมีความเสี่ยงทำให้เกิดอันตรายต่อเซลล์ปกติในร่างกายน้อย ด้วยการออกฤทธิ์ที่แรง เร็วและมีความจำเพาะสูงทำให้ยาชนิดนี้สามารถใช้ได้กับเซลล์มะเร็งที่อยู่ลึกเข้าไปข้างในยากที่ยาเคมีบำบัดจะสัมผัสและฆ่าได้หมด เมื่อเทียบกับรังสีรักษาแล้ว ยาชนิดนี้เมื่ออยู่ในร่างกายจะถูกขับถ่ายออกจากร่างกายอย่างรวดเร็ว มีการแผ่รังสีออกมาน้อยมาก จึงค่อนข้างสะดวกที่ผู้ป่วยสามารถรับยาแล้วไม่ต้องนอนในโรงพยาบาลหรือแยกตัวออกจากผู้อื่น เนื่องจากผลกระทบของรังสีจากตัวผู้ป่วยต่อคนแวดล้อมต่ำมาก ยิเทรียม ไอบริทูโมแมบ ไทยูซีแทน ได้รับการยอมรับให้ใช้ในโรคมะเร็งต่อมน้ำเหลืองโดยองค์การอาหารและยาประเทศสหรัฐอเมริกา ตั้งแต่ ปี พ.ศ. 2545 จากรายงานการศึกษาหลายแห่งที่ทำการศึกษากับผู้ป่วยมะเร็งต่อมน้ำเหลืองชนิดไม่รุนแรงและรุนแรงหลายประเภท พบว่าการตอบสนองต่อยาดีในอัตราค่อนข้างสูง และใกล้เคียงกับแผนการรักษามาตรฐานด้วยยาเคมีบำบัดที่ใช้กันมานับ 20 ปี

ซึ่งประกอบด้วยยาไซโคลฟอสฟาไมด์ ดอกโซรูบิซิน วินคริสตีน และ เพรดนิโซโลน (CHOP) ในขณะที่ยานี้ค่อนข้างปลอดภัย มีผลข้างเคียงต่อระบบร่างกายอื่นน้อยกว่าเคมีบำบัด ยกเว้นการกดการสร้างเม็ดเลือดแบบไม่เฉียบพลัน

สำหรับผู้ป่วยมะเร็งต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์ แม้ว่าจะยังมีการตอบสนองต่อแผนการรักษา CHOP ค่อนข้างดี แต่ยังคงมีอัตราการกลับเป็นซ้ำค่อนข้างสูง ในขณะที่บางรายตอบสนองไม่ดีเท่าที่ควรต้องใช้การรักษาที่รุนแรงกว่าร่วมด้วย ซึ่งกลับทำให้ผู้ป่วยนอกจากจะเผชิญกับภาวะโรคที่ทรุดลงอย่างรวดเร็วแล้วยังต้องเสี่ยงต่อผลข้างเคียงที่เสริมกันขึ้นมาอีก การใช้เคมีบำบัด CHOP ร่วมกับผลิตภัณฑ์ชีวภาพชนิดใหม่นี้จึงน่าจะเป็นทางเลือกอีกทางหนึ่งที่สามารถกำจัดเซลล์มะเร็งได้อย่างมีประสิทธิภาพในเวลาอันรวดเร็ว ส่งผลพลอยได้ให้น่าจะช่วยยืดช่วงชีวิตของผู้ป่วยออกไปได้ยาวขึ้น การปรับลำดับการให้ยาที่เหมาะสมจะช่วยหลีกเลี่ยงอันตรายที่เกิดจากผลข้างเคียงต่อระบบเลือดที่อาจจะซ้ำซ้อนกันได้

โครงการวิจัยโดยสรุปและวัตถุประสงค์การวิจัย

ด้วยเหตุผลจากหลักฐานการศึกษาในรูปแบบต่างๆ ที่ผ่านมาในผู้ป่วยมะเร็งต่อมน้ำเหลืองนับ 1,000 คน การวิจัยนี้จึงจัดทำขึ้นโดยมีวัตถุประสงค์เพื่อประเมินผลตอบสนองขั้นต้นและความปลอดภัยของการให้ผลิตภัณฑ์ชีวภาพติดสารกัมมันตรังสี (ยิเทรียม ไอบริทูโมแมบ ไทยูซีแทน) ตามด้วยแผนการรักษามาตรฐานด้วยเคมีบำบัด (CHOP) เป็นการรักษาลำดับแรกในผู้ป่วยมะเร็งต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์

การศึกษาครั้งนี้กระทำในอาสาสมัครที่เป็นผู้ป่วยมะเร็งต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์ไม่ต่ำกว่า 15 คน ผู้ป่วยที่ผ่านการคัดกรองตามเกณฑ์การคัดเลือกที่ระบุไว้ในแผนการวิจัยจะได้รับการตรวจประเมินเบื้องต้นทั้งในด้านสถานะของโรคที่เป็น และ สภาพร่างกายทั่วไปพื้นฐาน โดยอาศัยการตรวจร่างกาย การตรวจทางห้องปฏิบัติการและการตรวจภาพรังสีตามความเหมาะสม พร้อมกับการสัมภาษณ์ประวัติความเจ็บป่วยอื่นที่เป็นอยู่ ทั้งนี้เพื่อใช้เป็นข้อมูลเปรียบเทียบผลการรักษา และ เฝ้าระวังผลกระทบต่อความเจ็บป่วยอื่นที่เป็นอยู่หรืออาจจะเกิดขึ้นในระหว่างเข้าร่วมงานวิจัย หลังจากได้รับการอธิบายถึงแผนการให้ยาและติดตามผลการรักษาเรียบร้อยแล้ว อาสาสมัครจะเข้ารับยาตามลำดับเริ่มจากผลิตภัณฑ์ชีวภาพติดสารกัมมันตรังสีแล้วตามด้วย CHOP 4-6 ครั้ง ตามกำหนดเวลา ในระหว่างการให้ยาแต่ละชนิดอาสาสมัครจะเข้ามารับการตรวจต่างๆ และการซักถามเป็นระยะตามกำหนดเวลาเพื่อติดตามผลการรักษาขั้นต้นและผลข้างเคียงที่อาจเกิดขึ้นโดยแพทย์และผู้วิจัย การประเมินการตอบสนองจะแบ่งเป็น 2 ช่วง คือหลังการได้รับผลิตภัณฑ์ชีวภาพติดสารกัมมันตรังสี และ หลังการได้รับเคมีบำบัดในแต่ละครั้ง ผู้ที่มีแนวโน้มของการตอบสนองที่ไม่ดีหรือมีแนวโน้มจะเกิดผลข้างเคียงจะได้รับการแก้ไขหรือการ

รักษาทดแทนที่เหมาะสมโดยแพทย์ตลอดระยะเวลาที่เข้าร่วมโครงการวิจัย อาสาสมัครแต่ละคนจะใช้เวลาเข้าร่วมโครงการโดยประมาณ 8-9 เดือน

การเข้าร่วมและการถอนตัวจากโครงการวิจัย

ผู้ป่วยมะเร็งต่อมน้ำเหลืองที่ได้รับการคัดกรองแล้วตรงตามเกณฑ์คัดเลือกจะได้รับการชี้แจงรายละเอียดเกี่ยวกับโครงการวิจัยตามเอกสารแนะนำนี้ พร้อมกับได้รับการตอบข้อซักถามอย่างเปิดเผยโดยละเอียดจากผู้วิจัยและ/หรือหัวหน้าโครงการวิจัย อาสาสมัครที่เข้าร่วมโครงการวิจัยจะต้องลงนามในใบยินยอมด้วยความสมัครใจ ซึ่งอาสาสมัครมีสิทธิ์จะถอนตัวออกจากการวิจัยได้ทุกเมื่อที่ต้องการโดยไม่มีผลใดๆต่อการรักษาพยาบาลที่จะเกิดขึ้นในภายหลัง

ขั้นตอนการปฏิบัติตัวของอาสาสมัคร

อาสาสมัครจะได้รับการประเมินขั้นต้นก่อนได้รับการรักษาใดๆ ได้แก่ การตรวจร่างกาย การตรวจเลือดทางห้องปฏิบัติการ การตรวจวิเคราะห์เนื้อเยื่อต่อมน้ำเหลืองและเซลล์ไขกระดูก การตรวจภาพรังสีตามความเหมาะสม และการสัมภาษณ์ประวัติความเจ็บป่วยและการรักษาพยาบาลก่อนหน้านี้

ในระหว่างการศึกษา อาสาสมัครจะต้องมาพบแพทย์ผู้วิจัยเพื่อรับการตรวจร่างกาย และการตรวจทางห้องปฏิบัติการต่างๆ ตลอดจนเข้ารับการรักษาพยาบาลตามแผนที่กำหนดไว้และได้นัดกับอาสาสมัครไว้ล่วงหน้า อาสาสมัครควรให้ข้อมูลต่างๆ แก่ผู้วิจัยอย่างเปิดเผย ในกรณีที่มีความผิดปกติหรืออาการไม่สบายเกิดขึ้นในระหว่างการศึกษา ควรแจ้งแก่ผู้วิจัยทันทีตามสถานที่ติดต่อท้ายคำชี้แจงนี้

อาสาสมัครที่เป็นสตรีวัยเจริญพันธุ์ควรปรึกษาแพทย์เพื่อทำการคุมกำเนิดอย่างเหมาะสม

ความเสี่ยงและ/หรือความไม่สบายที่อาจเกิดขึ้น

ผลข้างเคียงที่สำคัญที่อาจเกิดจากการได้รับผลิตภัณฑ์ชีวภาพติดสารกัมมันตรังสี คือ ภาวะเม็ดเลือดขาวชนิดนิวโทรฟิลด์ต่ำ เกร็ดเลือดต่ำ และ/หรือ โลหิตจาง ซึ่งมีโอกาสเกิดได้ร้อยละ 30, 10 และ 4 ตามลำดับ มีลักษณะไม่เฉียบพลันและไม่รุนแรง ผลข้างเคียงอื่นที่เคยมีรายงานจากการศึกษาในคนที่ผ่านมาซึ่งพบได้น้อยและไม่ค่อยรุนแรง ได้แก่ อ่อนเพลีย มีไข้ เบื่ออาหาร คัน ผื่น ลมพิษ ความดันต่ำ คลื่นไส้ อาเจียน ภาวะบวม ตามัว วิงเวียน เป็นต้น

สำหรับมาตรการเฝ้าระวังรักษา อาสาสมัครจะได้รับการดูแลอย่างใกล้ชิดในช่วง 24 ชั่วโมงหลังจากได้ผลิตภัณฑ์ชีวภาพติดสารกัมมันตรังสีและเคมีบำบัดครั้งแรก ได้รับคำแนะนำเกี่ยวกับยาทั้งสองที่ได้รับก่อนกลับบ้าน และได้รับการดูแลจากแพทย์และผู้วิจัยตลอดเวลาที่เข้าร่วมการศึกษา เมื่อมีอาการข้างเคียงเกิดขึ้นจะได้รับการแก้ไขอย่างเหมาะสมจากแพทย์ในโครงการ

ประโยชน์ที่อาสาสมัครจะได้รับ

การวิจัยนี้เป็นการศึกษานำร่องเพื่อนำไปสู่การศึกษาในลักษณะเดียวกันในระยะที่ 3 ซึ่งจะทำการวิจัยในกลุ่มผู้ป่วยที่กว้างขวางขึ้นร่วมกันจากหลายสถาบัน และ/หรืออาจมีความซับซ้อนมากขึ้น โดยทำการเปรียบเทียบกับกลุ่มควบคุม ในขณะเดียวกันอาสาสมัครจะได้รับแผนการรักษาที่คาดหวังว่าจะมีประสิทธิภาพและความปลอดภัยที่สูงกว่าแบบแผนการรักษาที่ใช้กันอยู่ในปัจจุบัน ผลการตอบสนองที่ดีประกอบกับความเสี่ยงต่อการอาการข้างเคียงที่ลดลงจะทำให้อาสาสมัครได้รับประโยชน์จากการรักษาอย่างเต็มที่ โอกาสการปลอดจากโรคมากขึ้นและน่าจะมีช่วงชีวิตที่ยาวขึ้นกว่าที่คาดไว้

ค่าใช้จ่ายในการวิจัย/ค่าชดเชยเดินทาง/ค่าเสียเวลา

อาสาสมัครทุกคนจะได้รับสนับสนุนค่าใช้จ่ายในการเข้ามาพบแพทย์ตามกำหนดเวลา การดำเนินการตรวจรักษาที่เกี่ยวข้องทั้งหมด รวมถึงค่าใช้จ่ายที่เกิดจากการแก้ไขปัญหาอาการข้างเคียงจากยา

การรักษาความลับ

ข้อมูลทุกอย่างของอาสาสมัคร จะถูกเก็บบันทึกไว้เป็นความลับในแฟ้มข้อมูลด้วยรหัสของอาสาสมัครแต่ละคน ข้อมูลเฉพาะที่จะนำสู่การเปิดเผยตัวอาสาสมัครจะถูกเก็บไว้เฉพาะผู้วิจัยและหัวหน้าโครงการวิจัย ผลการศึกษาจะถูกสรุปจากข้อมูลทางวิชาการและนำออกเผยแพร่แต่ในวงการวิชาการเท่านั้น ไม่มีการนำสู่สาธารณชนทั่วไป

การติดต่อกับผู้วิจัย

ไม่ว่าในกรณีที่อาสาสมัครมีความผิดปกติเกิดขึ้นในระหว่างการศึกษา หรือ ต้องการข้อมูลเพิ่มเติม สามารถติดต่อได้กับ

1. อ.นพ. อุดมศักดิ์ บุญวรเศรษฐ์ (ผู้รับผิดชอบโครงการวิจัย)หน่วยโลหิตวิทยา ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เขตปทุมวัน กรุงเทพฯ 10330 โทรศัพท์ 02-2564564
2. นส. กฤตติกา ตัญญะแสนสุข (ผู้วิจัยร่วม) นิสิตดุษฎีบัณฑิตสหสาขาเภสัชวิทยา ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เขตปทุมวัน กรุงเทพฯ 10330 โทรศัพท์ 087-0711404

APPENDIX C

PARTICIPANT CONSENT FORM

“ผลตอบสนองขั้นต้นและความปลอดภัยของการให้ยายิเทรียม ไอบริทูโมแมบ ไทยูซีแทน ตามด้วยแผนการรักษา CHOP ในผู้ป่วยมะเร็งต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์”

วันที่ให้การยินยอม .....

ข้าพเจ้า (นาย, นาง, นางสาว) ..... นามสกุล .....อายุ ..... ปี

ที่อยู่ .....

.....

ได้รับคำอธิบายเกี่ยวกับการเป็นอาสาสมัครในโครงการวิจัย “ผลตอบสนองขั้นต้นและความปลอดภัยของการให้ยายิเทรียม ไอบริทูโมแมบ ไทยูซีแทน ตามด้วยแผนการรักษา CHOP ในผู้ป่วยมะเร็งต่อมน้ำเหลืองชนิดดิฟฟิวส์ลาจบีเซลล์” และได้รับทราบถึงรายละเอียดของโครงการวิจัย เกี่ยวกับ วัตถุประสงค์ และ ระยะเวลาที่ทำการวิจัย ขั้นตอนและวิธีการปฏิบัติตัวที่อาสาสมัครต้องปฏิบัติ ผลประโยชน์ที่จะได้รับ ตลอดจนผลข้างเคียงหรืออันตรายที่อาจเกิดขึ้นจากการเข้าร่วมโครงการโดยละเอียดและเข้าใจดีแล้ว

ในการนี้ข้าพเจ้าได้เข้าร่วมการวิจัยด้วยความสมัครใจ และสามารถถอนตัวจากการศึกษาได้ตลอดเวลาตามปรารถนาโดยไม่เสียสิทธิ์ใดๆ ในการรับการรักษาพยาบาลที่จะเกิดขึ้นตามมาในภายหลัง

ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าเป็นความลับ และจะเปิดเผยเฉพาะข้อมูลในรูปผลสรุปการวิจัย การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆ ที่เกี่ยวข้องกระทำได้เฉพาะกรณีจำเป็นด้วยเหตุผลทางวิชาการและได้รับอนุญาตจากข้าพเจ้าเท่านั้น

ข้าพเจ้าได้อ่านและทำความเข้าใจในข้อความข้างต้นแล้ว จึงได้ลงนามยินยอมเป็นอาสาสมัครของโครงการวิจัยนี้

ลงชื่อ ..... อาสาสมัคร
(.....)

ลงชื่อ..... ผู้ให้ข้อมูล
(.....)

ลงชื่อ..... แพทย์ผู้เกี่ยวข้อง
(.....)

ลงชื่อ ..... พยาน
(.....)

หมายเหตุ ในกรณีที่อาสาสมัครไม่สามารถอ่านหนังสือ หรือ ลงลายมือชื่อได้ ให้ใช้การประทับลายมือแทนดังนี้

ข้าพเจ้าไม่สามารถอ่านหนังสือได้ แต่ผู้วิจัยได้อ่านข้อความในแบบยินยอมนี้ให้แก่ข้าพเจ้าฟังจนเข้าใจดี ข้าพเจ้าจึงประทับลายนิ้วมือขวาของข้าพเจ้าในแบบยินยอมนี้ด้วยความเต็มใจ

ลงชื่อ ..... ผู้อธิบาย

(.....)

ลงชื่อ .....พยาน

(.....)

ประทับลายนิ้วมือขวา

APPENDIX D

CASE RECORD FORM

Case record form	Primary response and safety of ^{90}Yttrium ibritumomab tiuxetan followed by combined chemotherapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone for Diffuse Large B-cell Lymphoma

	Date of recruitment	
Case ID	Age	Sex

Recruitment condition	Inclusion criteria	❑ all YES	❑ not all YES
	Exclusion criteria	❑ all NO	❑ not all NO

Disease status

	Before Zevalin	Before CHOP	After CHOP
Ann Arbor staging			
Presence of B symptom			
Extranodal sites			
Spleenomegaly			

Prognostic factors

International prognostic index (IPI)	❑ elderly (aged > 60) ❑ extranodal sites ❑ elevated LDH ❑ performance status > 2 ❑ bone marrow infiltration	Risk level ❑ no risk ❑ low risk ❑ low intermediate ❑ high intermediate ❑ high risk
Bcl-2 in lymph node		

Treatment outcome

	After Zevalin	3 weeks after CHOP-6	3 months after CHOP-6
Primary response (evidence)			
Adverse events			

Visit plan according to the protocol

wk 1 • screening • interviewing • writing consent form	**wk 2** Full scale evaluation	**wk 3** • *Rituximab*	**wk 4** • PE • Hematology • *Rituximab + Z*
wk 5 Hematology (optional)	**wk 6** Hematology	**wk 7** Hematology (optional)	**wk 8** Hematology
wk 9 Hematology (optional)	**wk 10** Hermatology	**wk 11** Hematology (optional)	**wk 12** Hematology (optional)
wk 13 Hematology (optional)	**wk 14** • *1^{O} response to Z* • Restaging • Full scale evaluation • *CHOP-1*	**wk 15**	**wk 16**
wk 17 • PE • Hematology • *CHOP-2*	**wk 18**	**wk 19**	**wk 20** • PE • Hematology • *CHOP-3*
wk 21	**wk 22**	**wk 23** • PE • Hematology • *CHOP-4*	**wk 24**
wk 25	**wk 26** • Full scale evaluation • *CHOP-5*	**wk 27**	**wk 28**
wk 29 • PE • Hematology • *CHOP-6*	**wk 30**	**wk 31**	**wk 32** • Full scale evaluation • *1^{O} response to CHOP*

Wk
1
2
3
4
5
6
7
8
9
10
11
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14
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32

Full scale evaluation: Performance status (PS), Physical examination (PE), Chest X-ray, CT scan, Lymph node and bone marrow biopsy (LNBx and BMBx), urinalysis (UA), hematology and blood chemistry

Previous medical & surgical history

Date	Medical & Surgical Problems	Management

Screening criteria

	Inclusion criteria	**Yes**	**No**
1.	Aged 18-75 years old.	❑	❑
2.	Have confirmed CD_{20}^{+} cancer cells	❑	❑
3.	Justified as in advanced stage disease (bulky stage II, III or IV) accompanying with bidimensionally measurable disease	❑	❑
4.	Have less than 20,000 lymphoid cells/μl on peripheral blood differential	❑	❑
5.	Has no prior diagnosis of indolent lymphoma	❑	❑
6.	Has no histologic transformation	❑	❑
7.	Have performance status Zubrod 0-2	❑	❑
8.	Have life expectancy ≥ 3 months	❑	❑
9.	Has no other malignant within the past 5 years		
10.	Has no prior chemotherapy, immunotherapy, and radiotherapy either for lymphoma, organ transplantation or any other diseases	❑	❑
11.	Have written inform consent	❑	❑
	Exclusion criteria	**Yes**	**No**
1.	Have more than 25% lymphoma infiltration in bone marrow	❑	❑
2.	Have clinical evidence of central nervous system involvement due to lymphoma	❑	❑
3.	Platelet counts < 100,000/μl or neutrophil counts < 1,500 cells/μl	❑	❑
4.	Serum creatinine > 2.5 times of upper normal limit	❑	❑
5.	Suspected obstruction in hepato-biliary system (serum bilirubin > 2.5 times of upper normal limit)	❑	❑
6.	Have reduced cardiovascular function justified on echocardiogram or ejection fraction < 45%	❑	❑
7.	Reduced pulmonary function due to requirement of oxygen therapy	❑	❑
8.	Positive serology to Human immunodeficiency virus	❑	❑
9.	Have active uncontrolled infection	❑	❑
10.	Have concurrent severe and/or uncontrolled medical problem that might interrupt patient's participation along the study	❑	❑
11.	Unable to provide adequate sections from a paraffin-embedded block lymph node specimen	❑	❑
12.	For female participant: Suspected to be pregnant along the study period	❑	❑

Drug administration according to protocol

Date	**Wt**	**Ht**	**BSA**	**Treatment phase**	**Dose**			
				Pre-Zevalin	Rituximab ……. mg			
				Zevalin	Rituximab …….. mg Zevalin …….. μCi/kg (……μCi)			
					C (mg)	H (mg)	O (mg)	P (date)
				CHOP-1				
				CHOP-2				
				CHOP-3				
				CHOP-4				
				CHOP-5				
				CHOP-6				

CHOP: Cyclophosphamide (C) 750 mg/m^2, Doxorubicin (H) 50 mg/m^2, Vincristine (O) 1.4 mg/m^2 with maximum of 2 mg/dose, and Prednisolone 100 mg/day for 5 days

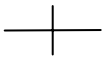
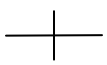
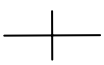
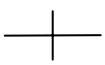
Physical examination

Visit/Date			
Performance Status (Zubrod score)			
Vital sign Blood pressure Heart rate Body temperature	/	/	/
General appearance			
Skin			
HEENT			
Lung			
Heart			
Renal			
Gastro-intestinal			
Genitourinary			
Musculoskeletal			
Neurological			
Others			

Hematology

Visit/Date				
Hb				
RBC				
Hct				
MCV				
MCH				
MCHC				
WBC				
N				
L				
Eo				
Mono				
Baso				
Platelet x 10^3				

Blood chemistry

Visit/Date				
Glucose				
BUN/Scr	/	/	/	/
Electrolyte Na | K Cl | HCO_3				
Ca/Phosphate	/	/	/	/
Total protein				
A/G	/	/	/	/
Alk phosphatase				
AST/ALT	/	/	/	/
GGT				
LDH				
TB/DB	/	/	/	/
Lipid profile TG | Chol HDL | LDL				

NA = did not assessed

Other examinations

Visit/Date			
Urinalysis			
Stool examination			
Immunohistochemistry CD_{20} lymphocyte			
BCl-2 in lymph node			

Biopsy/CT scan/ Imaging and Other investigations

Date	Investigation	Result

Concurrent medications

Date	Drug & Dosage	Indication	Drug problems

Adverse events

Date		
Suspected adverse events		
Description of reaction		
Suspected agent		
Seriousness*	❑ death ❑ life threatening ❑ disability ❑ new/prolonged hospitalization ❑ others …….	❑ death ❑ life threatening ❑ disability ❑ new/prolonged hospitalization ❑ others …….
Causality assessment result	❑ excluded ❑ unlikely ❑ possible ❑ probable ❑ highly probable	❑ excluded ❑ unlikely ❑ possible ❑ probable ❑ highly probable
Management		
Outcome	❑ completely recover ❑ relief with residual effect ❑ continuously	❑ completely recover ❑ relief with residual effect ❑ continuously

***** *Seriousness is categorized according to "The adverse event and problem report form", Faculty of Medicine, Chulalongkorn University*

APPENDIX E

ADVERSE EVENT-DRUG CAUSALITY ASSESSMENT FORM

Case ID	Total score
Adverse event Suspected agent	Assessment result ❑ excluded (≤ 0) ❑ unlikely (1 to 2) ❑ possible (3 to 5) ❑ probable (6 to 8) ❑ highly probable (> 8)

Criteria		Score
1. Time to onset of the reaction		
Highly suggestive	+3	
Suggestive	+2	
Compatible	+1	
Inconclusive	0	
If incompatible, then case "unrelated"		
If information not available, then case "insufficiently documented"		
2. Course of the reaction		
Highly suggestive	+3	
Suggestive	+2	
Compatible	+1	
Against the role of the drug	-2	
Inconclusive or not available	0	
3. Risk factor(s) for drug reaction		
Presence	+1[a]	
Absence	0	
4. Concomitant drug (s)		
Time to onset incompatible	0	
Time to onset compatible but unknown reaction	-1	
Time to onset compatible and known reaction	-2	
Role proved in this case	-3	
None or information not available	0	
5. Non-drug related causes		
Ruled out	+2	
Possible or not investigated[b]	+1 to -2	
Probable	-3	
6. Previous information on the drug		
Reaction unknown	0	
Reaction published but unlabelled	+1	
Reaction labeled in the product characteristics	+2	
7. Response to readministration		
Positive	+3	
Compatible	+1	
Negative	-2	
Not available or not interpretable	0	
or Plasma concentration of the drug known to be toxic	+3	
or Validated laboratory test with high specificity, sensitivity and predictive values	+3	
Positive	-3	
Negative	0	
Not interpretable or not available		

[a] on additional point for every validated risk factor [b] depending on the nature of the reaction

APPENDIX F

^{90}YTTRIUM RADIOACTIVE DECAY TABLE

Physical half-life 64.08 hours

Hours	Decay Factor	Hours	Decay Factor	Hours	Decay Factor
-54	1.793	-30	1.383	-6	1.067
-53	1.774	-29	1.368	-5	1.056
-52	1.755	-28	1.354	-4	1.044
-51	1.736	-27	1.339	-3	1.033
-50	1.717	-26	1.325	-2	1.022
-49	1.699	-25	1.311	-1	1.011
-48	1.681	-24	1.296	0	1.000
-47	1.683	-23	1.282	1	0.989
-46	1.645	-22	1.269	2	0.979
-45	1.627	-21	1.255	3	0.968
-44	1.610	-20	1.242	4	0.958
-43	1.592	-19	1.228	5	0.947
-42	1.575	-18	1.215	6	0.937
-41	1.558	-17	1.202	7	0.927
-40	1.541	-16	1.189	8	0.917
-39	1.525	-15	1.176	9	0.907
-38	1.508	-14	1.164	10	0897
-37	1.492	-13	1.151	11	0.888
-36	1.476	-12	1.139	12	0.878
-35	1.460	-11	1.126	13	0.869
-34	1.445	-10	1.114	14	0.859
-33	1.429	-9	1.102	15	0.850
-32	1.414	-8	1.090	16	0.841
-31	1.398	-7	1.079	17	0.832

Hours	Decay Factor
18	0.823
19	0.814
20	0.805
21	0.797
22	0.788
23	0.780
24	0.771
25	0.763
26	0.755
27	0.747
28	0.739

Hours	Decay Factor
29	0.731
30	0.723
31	0.715
32	0.707
33	0.700
34	0.692
35	0.685
36	0.677
37	0.670
38	0.663
39	0.656

Hours	Decay Factor
40	0.649
41	0.642
42	0.635
43	0.628
44	0.621
45	0.615
46	0.608
47	0.601
48	0.595
49	0.589
50	0.582

BIOGRAPHY

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INSTITUTIONS ATTENDED

1975 - 1979 Bachelor degree in Pharmaceutical Science, Chulalongkorn University

1983 - 1987 Master degree in Pharmaceutical Science (Hospital and Clinical Pharmacy), Chulalongkorn University

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