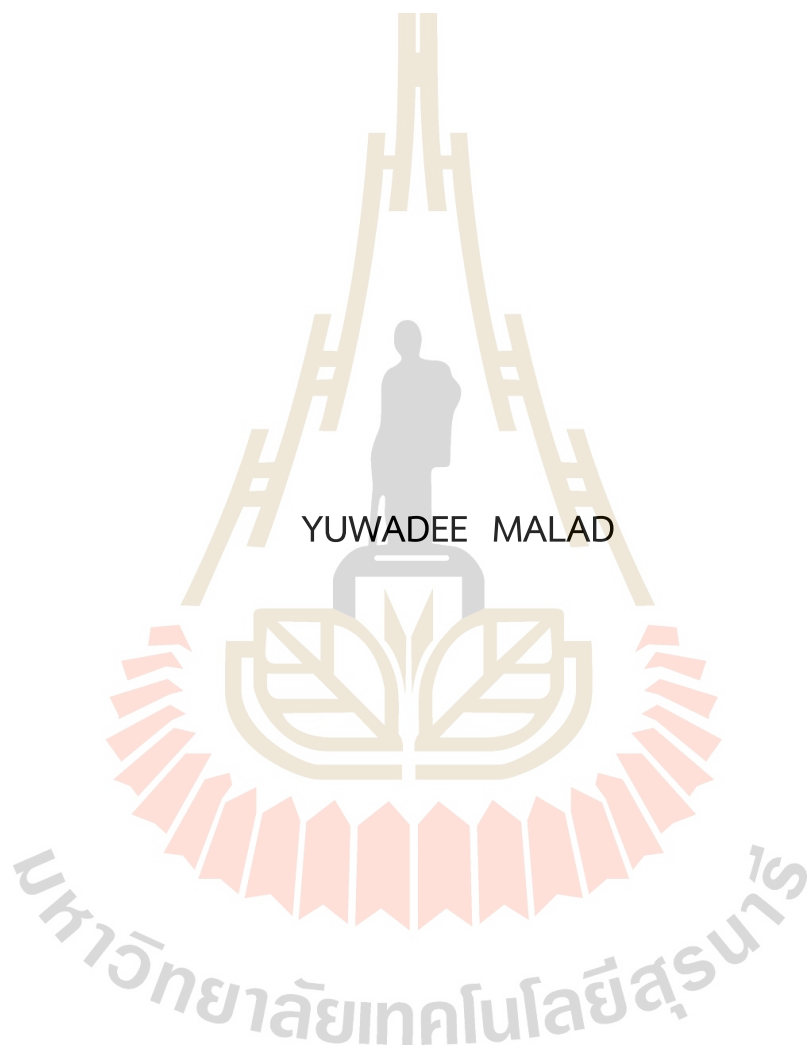
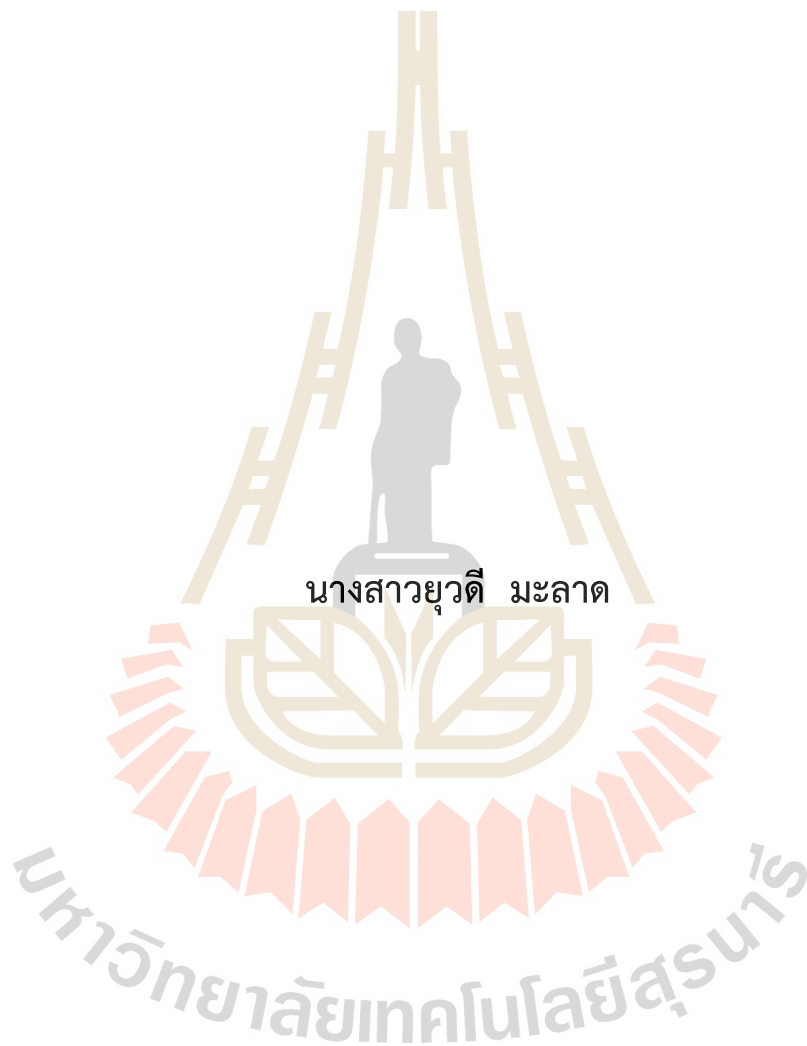


A FEASIBILITY STUDY OF USING BORON NEUTRON CAPTURE
THERAPY (BNCT) TO TREAT CHOLANGIOCARCINOMA (CCA)
CELL LINE



A Thesis Submitted in Partial Fulfillment of the Requirements for the
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การศึกษาความเป็นไปได้ในการใช้รังสีรักษาจากโบรอนจับยัदनิวตรอน
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A FEASIBILITY STUDY OF USING BORON NEUTRON CAPTURE THERAPY (BNCT) TO TREAT CHOLANGIOCARCINOMA (CCA) CELL LINE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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คำสำคัญ: รังสีรักษาจากโบรอนจับยัธนิวตรอน/ มะเร็งท่อน้ำดี/ ประสิทธิภาพทางชีวภาพสัมพัทธ์

รังสีรักษาจากโบรอนจับยัธนิวตรอน (Boron Neutron Capture Therapy: BNCT) เป็นรังสีรักษาแบบกำหนดเป้าหมายซึ่งอาศัยอันตรกิริยาระหว่างไอโซโทปโบรอน-10 (^{10}B) ที่มีความเสถียรกับนิวตรอนความร้อน เพื่อปลดปล่อยอนุภาคอัลฟาและลิเทียมพลังงานสูงซึ่งมีประสิทธิภาพในการทำลายสารพันธุกรรม (DNA) ภายในเซลล์มะเร็ง แม้ว่า BNCT จะถูกนำไปใช้รักษามะเร็งหลายชนิด แต่ยังไม่มีการใช้งานการประยุกต์ใช้เพื่อรักษามะเร็งท่อน้ำดี ซึ่งเป็นมะเร็งที่มีอุบัติการณ์สูงในภาคตะวันออกเฉียงเหนือของประเทศไทย การศึกษานี้จึงมีวัตถุประสงค์เพื่อประเมินผลของ BNCT ต่อเซลล์มะเร็งท่อน้ำดี โดยใช้สารแอล-โบโรโนฟีนิลอะลานีน (L-BPA) เป็นตัวนำส่ง ^{10}B จากนั้นจึงฉายรังสีนิวตรอนความร้อนจากเครื่องปฏิกรณ์ของสถาบันเทคโนโลยีนิวเคลียร์แห่งชาติ (สทน.) หรือจากมหาวิทยาลัย เกียวโต และเปรียบเทียบประสิทธิผลทางชีวภาพสัมพัทธ์ (Relative Biological Effectiveness: RBE) กับการฉายรังสีเอกซ์พลังงาน 6 เมกะโวลต์ ณ โรงพยาบาลจุฬาลงกรณ์ ผลการศึกษาพบว่า L-BPA แสดงความเป็นพิษต่อเซลล์มะเร็งท่อน้ำดีเพิ่มขึ้นตามความเข้มข้น แต่ที่ความเข้มข้น 1,000 ppm (ซึ่งมี ^{10}B ประมาณ 51 ppm) เซลล์ยังคงมีอัตราการรอดชีวิตสูงกว่าร้อยละ 85 นอกจากนี้ ปริมาณการสะสมของ ^{10}B ภายในเซลล์ยังเพิ่มขึ้นตามระยะเวลา โดยพบว่ามีการสะสมสูงสุดที่ 48 ชั่วโมง เมื่อนำเซลล์มะเร็ง ท่อน้ำดีที่สะสม ^{10}B ไปรับรังสีนิวตรอนความร้อนจากเครื่องปฏิกรณ์ทั้งสองแห่ง พบว่าความเสียหายของดีเอ็นเอเพิ่มขึ้นตามปริมาณรังสีและระยะเวลาที่ได้รับรังสีตามลำดับ ที่สำคัญคือ ค่า RBE ของ BNCT ในเซลล์มะเร็งท่อน้ำดีสูงถึง 3.466 เมื่อเทียบกับการฉายรังสีเอกซ์ ผลการทดลองนี้ชี้ให้เห็นถึงศักยภาพของ BNCT ในการรักษามะเร็งท่อน้ำดี เนื่องจากเซลล์สามารถนำเข้าสู่และสะสม ^{10}B ได้ในปริมาณที่เพียงพอต่อการทำลายดีเอ็นเอและนำไปสู่การตายของเซลล์เมื่อได้รับรังสีนิวตรอน

YUWADEE MALAD : A FEASIBILITY STUDY OF USING BORON NEUTRON CAPTURE THERAPY (BNCT) TO TREAT CHOLANGIOCARCINOMA (CCA) CELL LINE
THESIS ADVISOR : ASST. PROF. CHINORAT KOBDAJ, Ph.D. 67 PP.

Keyword: Boron neutron capture therapy (BNCT)/ Cholangiocarcinoma (CCA)/ Relative biological effectiveness (RBE)

Boron neutron capture therapy (BNCT) is a targeted radiotherapy in which the stable isotope Boron-10 (^{10}B) captures a thermal neutron, releasing highly energetic alpha and lithium particles that cause DNA damage in tumor cells. Although BNCT has been applied to various cancers, its application to cholangiocarcinoma (CCA), a cancer of high incidence in Northeast Thailand, remains unexplored. In this study, we aimed to evaluate the effects of BNCT on CCA cells. L-boronophenylalanine (L-BPA) was used as the ^{10}B delivery agent. The irradiation was performed using thermal neutrons from reactors at the Thai Institute of Nuclear Technology and Kyoto University. For comparison, the relative biological effectiveness (RBE) was calculated relative to CCA cells irradiated with 6 MV X-rays from Chulalongkorn Hospital. Our findings demonstrated that L-BPA exhibited dose-dependent cytotoxicity. However, the cell viability remained above 85% at an L-BPA concentration of 1000 ppm (containing 51 ppm of ^{10}B), indicating a suitable therapeutic window. Furthermore, intracellular ^{10}B accumulation in CCA cells increased in a time-dependent manner, peaking at 48 h of incubation. The subsequent irradiation of ^{10}B -loaded CCA cells with thermal neutrons at both facilities resulted in a dose-dependent increase in DNA damage. Moreover, BNCT demonstrated superior efficacy compared to conventional X-rays, with a calculated RBE value of 3.466. In conclusion, these findings demonstrate the potential of BNCT for CCA treatment, as CCA cells can accumulate sufficient ^{10}B to induce significant DNA damage and subsequent cell death upon neutron irradiation.

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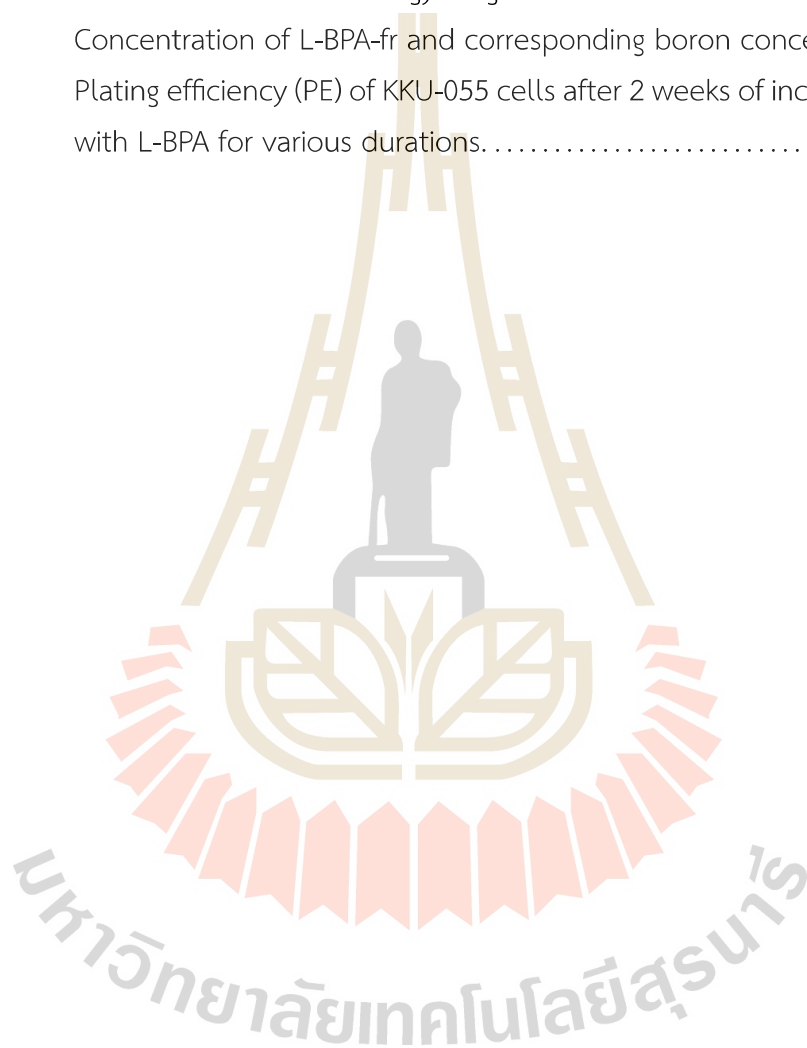
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LIST OF ABBREVIATIONS

CCA	Human cholangiocarcinoma
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
L-BPA	L-boronophenylalanine
L-BPA-fr	L-boronophenylalanine-fructose
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
AP/PA	Anterior-Posterior/Posterior-Anterior (radiation therapy)
KCMH	King Chulalongkorn Memorial Hospital
LINAC	Linear Accelerator
MU	Monitor Unit
TINT	Thailand Institute of Nuclear Technology
MCNP	Monte Carlo N-Particle Transport Code
HWNIF	Heavy Water Neutron Irradiation Facility
KUR	Kyoto University Research Reactor
TLD	Thermoluminescent Dosimeter
SSD	Source-to-Surface Distance
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
PE	Plating efficiency
HPGe	High Purity Germanium
DNA	Deoxyribonucleic Acid
RT-PCT	Reverse Transcription Polymerase Chain Reaction
BNCT	Boron Neutron Capture Therapy
SF	Survival fraction
ng	Nanogram
Gy	Gray (unit of absorbed radiation dose)
mL	Milliliter
μ l	Microliter
keV	Kiloelectron Volt
eV	Electron Volt

CHAPTER I

INTRODUCTION

Cancer is the leading second cause of one of the six deaths of people living on earth, accounting for over 10 million deaths in 2020 (World Health Organization, 2021). Cancer is an abnormal cell that causes unrestricted cell growth and transformation from normal cells to tumors, which then progresses to *malignant tumors* (World Health Organization, 2021). Cancer cells will proliferate rapidly and invade neighboring tissues, a process known as *metastasis*, which is the leading cause of death from cancer. Based on information from the World Health Organization (WHO), cancer is also the leading cause of death in Thailand. People in Thailand are diagnosed with cancer at a rate of 381 cases per day, or 139,206 cases per year. This disease kills 230 people every day, which adds up to 84,073 deaths every year (Record and Databased Cancer Unit, 2021). In Thailand, liver cancer, bile duct cancer, lung cancer, colorectal cancer, gallbladder cancer, and breast cancer are the top five types of cancer that are most likely to be fatal (Faculty of Medicine Siriraj Hospital, M. U., 2019).

Cholangiocarcinoma (CCA) or bile duct cancer is typically thought to be rare cancer (Ustundag and Bayraktar, 2008). These tumors cause harm to other vital organs, particularly the liver and pancreas. CCA mortality rates are particularly high in Thailand's northeast. Males were found to have a higher incidence than females, with 85.5% of CCA patients living in Nakhon Phanom (upper Northeast Thailand). Thailand now has the highest incidence of Cholangiocarcinoma in the world. The liver fluke *O. viverrini* infected by people who consume unsanitary food gives rise to CCA (Sripa and Pairojkul, 2008). Furthermore, patients with CCA were diagnosed too late because it is not easy to detect it at an early stage, making this disease being resistant to either chemotherapy or radical surgery (Gatto et al., 2010). Thai patients with diffuse-type CCA have a median survival time of only 65 days (Sripa and Pairojkul, 2008). Over the last 30 years, overall survival has been poor; five-year survival from CCA is less than 5% (Ustundag and Bayraktar, 2008). An attempt to cure CCA with radiotherapy after radical surgery is one of the few treatment options available to eliminate regional residual tumors and prevent metastasis progression. However, no effective patient treatment plan has been developed (Todoroki et al., 2001). Resection is the most effective treatment for CCA

in general (Gatto et al., 2010). Radiotherapy is frequently used as curative after resection and sometimes used to reduce the size of tumors. It can also be operated as the primary therapy for patients whose tumor has not spread to various organs. Despite the fact that it is only palliative care, it may help patients live longer lives. Recently, publication paper has been examined to show a significant advantage of radiotherapy for CCA, however the data is insufficient. As a consequence, a study of CCA with radiotherapy is required to find a defined summing to improve clinical treatment planning (Gatto et al., 2010).

Boron neutron capture therapy (BNCT) is a cancer treatment that uses non-invasive cellular target radiation. The first idea, developed in the mid-1930s and tried on a patient with Malignant Glioma in 1951, is that tumor cells can be eliminated if a sufficient amount of boron atoms are concentrated within tumor cells and then exposed to a neutron beam (Nedunchezian et al., 2016). As shown in Figure 1.1, BNCT utilizes the capture of a neutron with a stable boron ^{10}B isotope, which then fissions with a thermal neutron (kinetic energy of the order of 0.025 eV) to produce alpha particles with high linear energy transfer (LET) and recoiling ^7Li particles that destroy tumour cell DNA. Due to alpha's low penetrating properties, engagement is limited to cancer cells while normal tissues are preserved. Thus, BNCT can help with invasive cancers and has been demonstrated to be effective against glioblastoma (GBM) and head and neck cancer (Sato et al., 2018).

The first generation of BNCT was carried out in 40 patients using simple boron compounds, followed by clinical application of BNCT in Japan in 1968 utilizing Boro-captate Sodium (BSH) with outstanding results of reaching 58% of 5-year survival rate. Afterwards, the resurgence of BNCT occurred in the 1990s all over the world, including in the United States at Brookhaven and Cambridge, in Europe at Petten, in Finland, Sweden, and the Czech Republic, as well as in Argentina, Taiwan, and Japan (Nedunchezian et al., 2016).

Accelerator-Based Neutron Source (ABNS) and Nuclear Reactor are used to produce neutrons for BNCT. ABNS has recently been initiated, and it can be installed inside or adjacent to hospital areas, such as an electrostatic accelerator, cyclotron, and RFQ linac (Nishitani et al., 2022). Beside that, several nuclear reactors are available for research for BNCT clinical trials and other purposes such as neutron-activation analysis, gemstone coloration, and radioisotope production. The TRIGA Mark III reactor, operated by the Thailand Institute of Nuclear Technology (a Public Organization), is located in

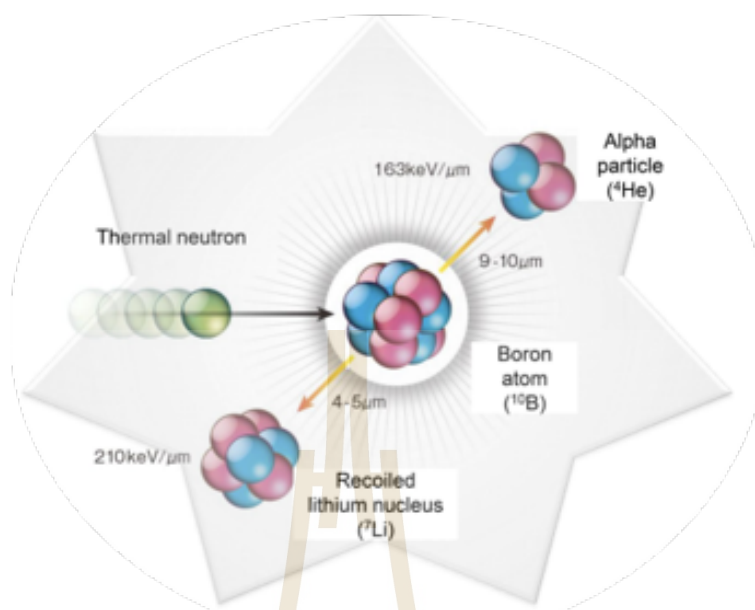


Figure 1.1 Schematic diagram of the interaction of Boron neutron capture reaction occurred with one cell (Suzuki, 2020).

Bangkok, Thailand. However, this reactor is not involved in BNCT research. Thailand is set to launch its new nuclear reactor, the SUT-RR, at Suranaree University of Technology. This reactor will support various fields of nuclear science, with a particular focus on the BNCT research center. Furthermore, the translation of recent BNCT clinical trial research in Thailand is not disclosed. Before moving on to clinical trials, we must envelope the treatment planning for BNCT, which is the most important stage space. Therefore, improvements in radiobiology are required for a more expansive clinical application of BNCT (Viegas et al., 2021).

This study aims to determine the boron uptake by CCA cells and investigate the expression of boron receptors. Additionally, it compares the biological response of CCA cell lines to photon and neutron irradiation to obtain radiobiological data relevant to CCA therapeutics utilizing BNCT. Moreover, the results may provide insights into how BNCT can be implemented in the treatment of Thai CCA patients.

CHAPTER II

BACKGROUND AND KNOWLEDGE

2.1 Cancer

Cancerous tumors can grow indefinitely because of cell mutations, which also cause normal cells to change into malignant tumors. Triggers for the development of cancer include hereditary, environmental, combined, and obfuscated defects. cancer cells are unable to develop into distinct cell types with unique functions like normal cells ,thus they continue to grow and spread to the nearby normal cells. They require some oxygen and nutrients to grow which is obtained the blood vessels of normal cells, and they can fend off the immune system in order to survive and develop on their own (Institute, 2021).

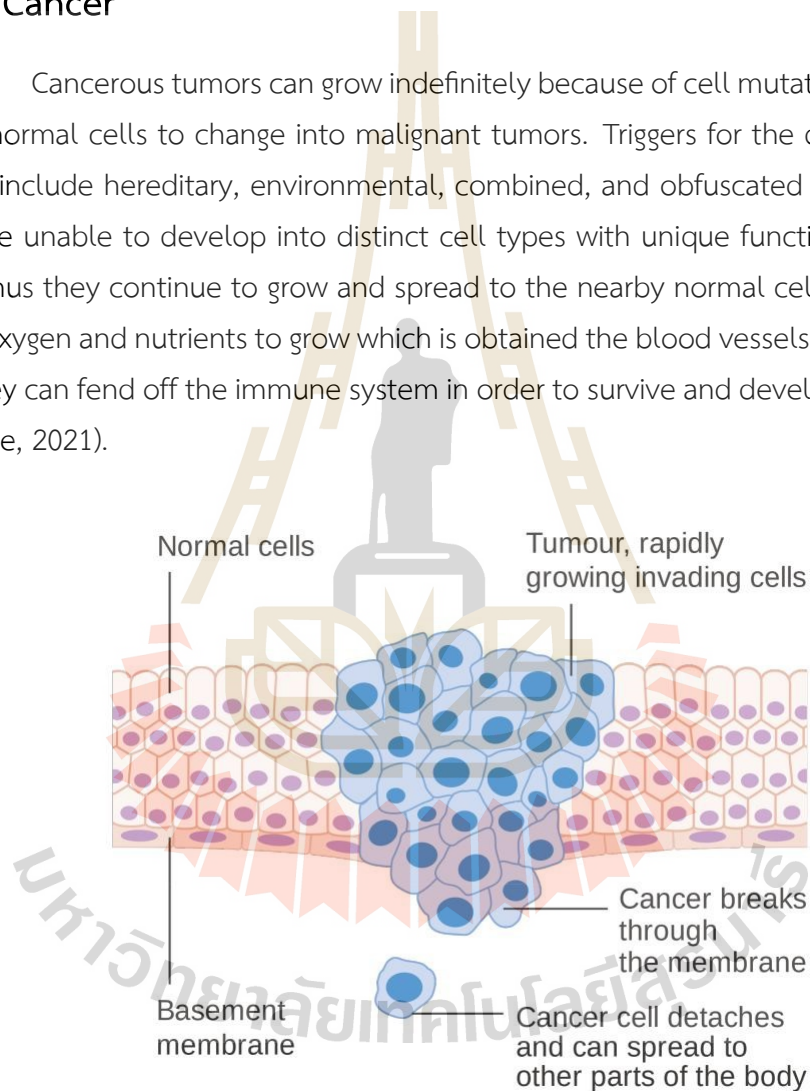


Figure 2.1 Several cancer cells in healthy cells proliferate and invade nearby cells (Cancer Research UK, 2020).

According to statistics in 2018, there were an estimated 18 million cases of cancer worldwide (International, 2018). In 2025, 20 million additional cancer patients are anticipated (Faculty of Medicine Siriraj Hospital, M. U., 2019). There are numerous

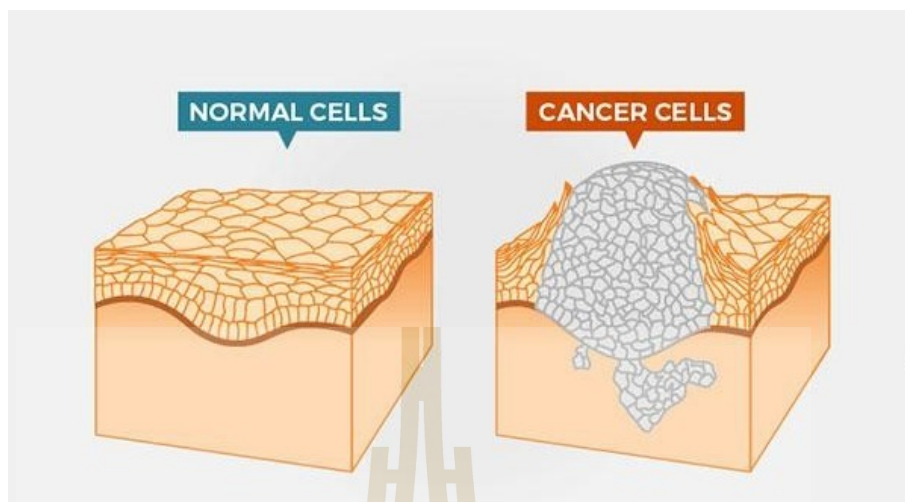


Figure 2.2 The distinction between cancerous and healthy cells (Institute, 2021).

types of cancer, and each type of cancers is treated using different methods. Cancer treatment plans have to take into account the type, location, severity, stage, and other aspects of the disease. Surgery, chemotherapy, radiation, immunotherapy, targeted therapy, and hormone therapy are all considered treatments for cancer. The medical community is currently searching for novel treatment options or combined strategies to increase upward patients' chances of surviving cancer. (Institute, 2021).

2.1.1 Cholangiocarcinoma (CCA)

Cholangiocarcinoma (CCA), also known as bile duct cancer caused by biliary epithelia, is a disease in which the bile duct grows abnormally and uncontrollably (Ustundag and Bayraktar, 2008). The bile duct's primary function is to transport bile from the liver and gallbladder to the small intestine. The small intestine is a vital organ that aids in the breakdown of dietary fats. Cholangiocarcinoma can be classified into three groups based on its location. This would include Intrahepatic, Perihilar (also known as hilar), and distal bile duct cancers. CCA can be spread to the liver or other organs (Society, 2018).

CCA is currently uncommon worldwide, however, its incidence rate is comparable to that of hepatocellular carcinoma (or liver cancer), at approximately 30% of the incident rate. (Gatto et al., 2010). In contrast, Thailand has the highest reported incidence of CCA in the world. The majority of cases are concentrated in the northeastern provinces of Thailand and predominantly affect males. According to Age-standardized incidence rates (ASR), Udon Thani has the highest incidence of CCA. The primary cause

is the regional population's lifestyle factors. Eggs of the parasite (*O. viverrini*) are frequently found in uncooked food and poor sanitation, which are frequently consumed by these individual persons. This leads to the risk of CCA incidents (Sripa and Pairojkul, 2008).

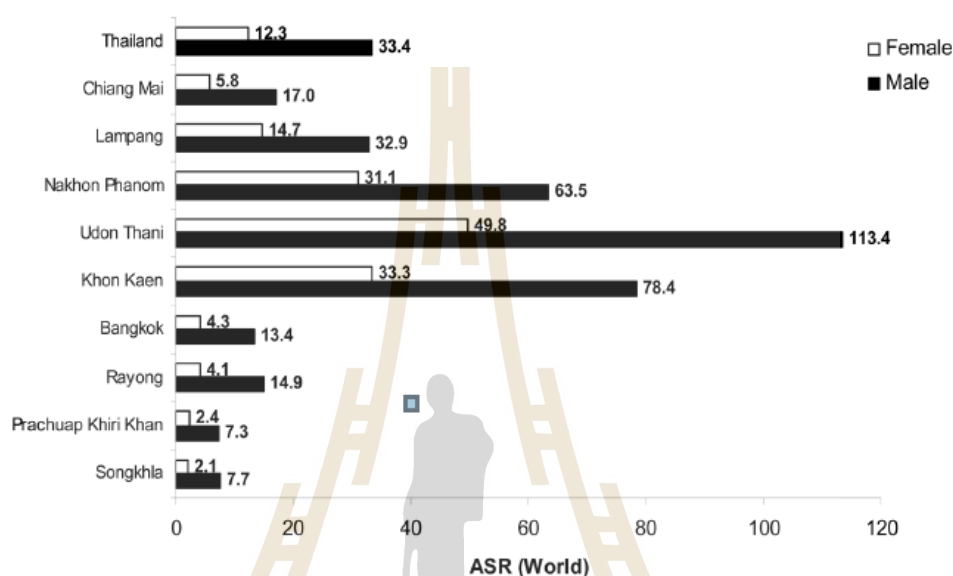


Figure 2.3 Age-standardized incidence rates (ASR) of liver and bile duct cancer in males and females in different regions of Thailand (1998–2000) (Sripa and Pairojkul, 2008).

Researchers are focusing on improving and finding new applications for advanced CCA treatment because of the disease's dismal prognosis and frequent late diagnosis (Gatto et al., 2010). Treatment options for CCA are radical resection, chemotherapy, palliative therapy, immunotherapy, targeted therapy, and radiation therapy (RT). Several factors need to be considered in order to determine the treatment for CCA patient such as the type of tumor present, its stage, the availability of a free surgical margin, the presence of distant metastases, and so on. Previous research on a patient treated with RT after surgery found that the additional treatment provided no benefit over surgical resection alone (Im et al., 2016). Chemoradiation is a combination of RT and chemotherapy; it employs a standard external beam of about 40 Gy and has shown promising results (Kim et al., 2002; Skowronek and Zwierzchowski, 2017). Radiofrequency ablation (RFA) and stereotactic body radiotherapy (SBRT) are two additional promising methods that have been shown to improve patient survival and tolerability (Bokemeyer et al., 2019). Toxic effects on nearby organs are still estimated

to be 5- 20% with SBRT (Mahadevan et al., 2015). There are many ways to treat this type of cancer depending on many factors. RT has been used for a long time but is not popular. Currently, there are not many cases reported about using BNCT which may be a good alternative for the treatment of CCA.

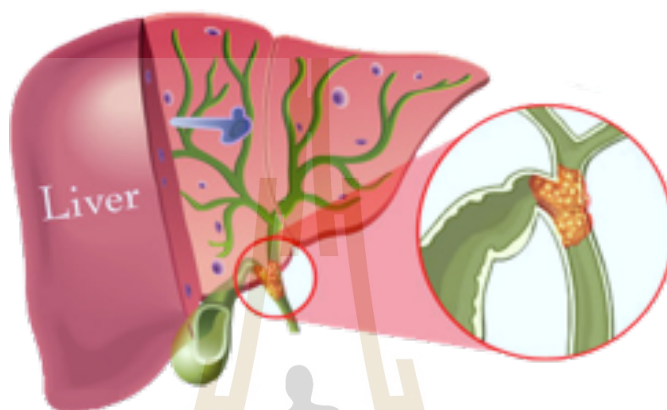


Figure 2.4 Anatomy of the intrahepatic bile ducts (News, 2019).

2.2 Radiotherapy

Radiotherapy, also known as Radiation Therapy (RT), is primarily used to treat cancer, which can cause DNA damage. In contrast to cancer cells, normal cells can repair DNA damage when exposed to radiation. The proliferation of cancer cells has ceased, and they have died out after irradiation. Because of this, RT could indeed kill cancer cells, and radiotherapy requires a treatment plan (Institute, 2021). External beam therapy is mostly used in many RT treatments for cancer. The most prevalent form of conventional (photon) therapy is a beam produced by a linear accelerator (LINACs). Typically, an electron beam generates energy between 6 and 20 MeV. Radiation dose is usually divided into several fractions. Most radiation therapy patients receive five daily doses over five to eight weeks. Some cancers need more than daily treatment. The new treatment option is ion beam therapy or charged particle therapy, such as proton therapy, carbon therapy, etc., with an inverted dose profile. The proton source for proton therapy is derived from a proton accelerator with an energy range of 70–250 MeV. When a charged particle passes through a material, if it has sufficient kinetic energy, it can deposit energy by ionizing or exciting atoms to a higher state or nonionized state. It can also be caused by collisions with an atom's electron (Das and Ferbel, 2003).

In addition, radiotherapy with thermal neutrons is used to treat brain cancer. This radiotherapy contains chemical agents that enhance cells' responses to radiation which is referred to as radiosensitizers and promotes both the direct and indirect effects of radiation.

2.2.1 Interactions of particles with matter

Particles and electromagnetic radiation can only be detected via their interactions with matter. There are distinct interactions between charged particles and neutral particles, such as photons, that differ from those of neutral particles. One could say that any interaction process can serve as the foundation for a detector concept. There are a large number of particle and radiation detection devices as a consequence of the diversity of these processes. In addition, different interaction processes at different energies may be relevant for the same particle. Ionization and excitation are the principal interactions of charged particles with matter. Bremsstrahlung energy losses for relativistic particles must also be considered. During an interaction, neutral particles must produce charged particles, which are then detected through their characteristic interaction processes. These processes are the photoelectric effect, Compton scattering, and electron pair production in the case of photons. The electrons produced by these photon interactions can be detected by their ionization in the detector's sensitive volume (Scharadt et al., 2010a).

2.2.2 Interactions of neutron

Neutrons have no charge and do not interact with orbital electrons; therefore, they can easily pass through the nucleus of a target to cause diverse reactions. However, not all potential reactions contribute equally to the dose distribution (Park and Kang, 2011). Neutrons are distinguished by their interactions with other forms of matter. The following conceptual categories can be used to categorize the interactions of neutrons with energies between 20 and 30 MeV, which are utilized in medical treatment.

- Elastic scattering (n,n) occurs when a neutron strikes a nucleus and bounces back elastically. The amount of kinetic energy transferred is determined by the impact angle and thus the direction of motion of the neutron and nucleus after the collision.
- Inelastic scattering (n,n'): This interaction dominates the interactions of fast neutrons in the therapeutic range, leading to the emission of photons, neutrons, and

charged particles. A nucleus is excited into an unstable state when a neutron collides with it and enters it. The excited nucleus then emits gamma rays to transition back to its stable state.

- Neutron capture (n, γ): Neutrons can be captured by the nucleus of absorbing matter, and gamma rays are only emitted by the absorbing atom. The interaction is identical to nonelastic scattering, but only occurs at low energies.
- Nuclear transmutation reactions (n, x): In a nuclear transmutation reaction, of which there are numerous types, a compound nucleus is formed by the collision of neutrons, and an isotope is split into a mixture of isotopes with a different state prior to the reaction.

2.2.3 The absorbed dose

The main focus of radiotherapy is dose deposition in tissue. When a photon passes through tissue, it loses energy and is eventually absorbed. The absorbed dose (D) is the mean energy deposited divided by the mass of ionizing radiation. The dose is measured in Gray units [$1 \text{ Gy} = 1 \text{ J/kg}$] and can be calculated using the equation below.

$$D = \frac{dE}{dm}, \quad (2.1)$$

where dE/dx defined as stopping power is energy loss particle per unit length of the path, and the relation dE/dx is a linear energy transfer (LET), energy deposited along a path segment up to bremsstrahlung loss (Weyrather W. K., 1999).

The absorbed dose from neutrons capture (thermal neutron) can be described as equation (Massachusetts Institute of Technology, 2004).

$$D = \frac{N\sigma E\phi}{\rho}, \quad (2.2)$$

ϕ = thermal neutron fluence (n/cm^2),

N = atom density (cm^{-3}),

σ = capture cross section (for each element),

E = energy from capture reaction,

ρ = tissue density.

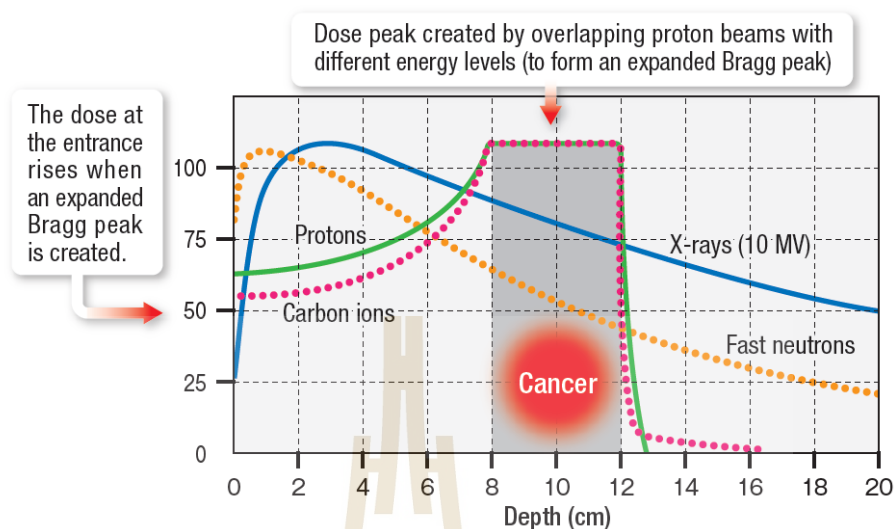


Figure 2.5 Depth-dose profiles of various types of radiation sources (Cente, 2013).

Figure 2.5 depicts a depth-dose profile as well as the Bethe-Bloch formula for calculating the maximum High energy deposited (Bragg peak) in particle beams (such as a proton or carbon ion). A charged particle beam has the advantage of being able to deliver the highest dose to cancer cells in the deepest layers of the body (Scharadt et al., 2010b). Bragg peaks do not appear for X-rays, electrons, gamma rays, or neutrons. In fact, because neutrons have a similar dose-depth curve to gamma rays, direct neutron irradiation without sensitizing the tumor has the distinct disadvantage of producing a high amount of biologically very effective damage in the healthy tissue surrounding the tumor.

2.2.4 Biological effective

Survival curve is a straightforward method for measuring the response of cell lines to different radiations. One or two weeks after irradiating the cells, the colony-forming assay is used to analyze the proliferation cells (Scharadt et al., 2010b). Colony consisting more than 50 cells can be counted as a surviving cell. This method refers to the proportion of inoculated single cells that produce expanding colonies. Each cell line has a unique value for plating efficiency (PE) which is the ability of cells to attach and proliferate into colonies. Therefore, we must consider the PE experiment both before and after irradiating the cell. It is defined as follows (Podgorsak, 2005):

$$PE = \frac{\text{Number of colonies counted}}{\text{Number of cell seeded}}. \quad (2.3)$$

Cell survival data are generally plotted in semi-logarithmic scale. The most common model used to describe the relationship between radiation dose and the cell survival is the linear-quadratic model (LQ) :

$$s(D) = e^{-\alpha D - \beta D^2}, \quad (2.4)$$

where S is the survival fraction of cell at dose D while α and β are constants.

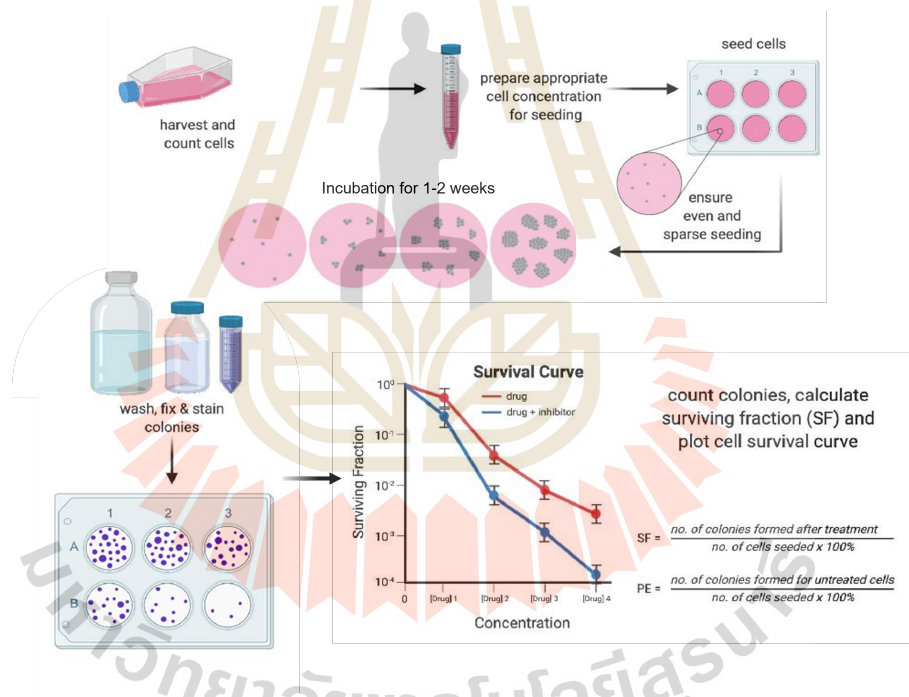


Figure 2.6 The clonogenic assay process, including cell harvesting, cell seeding, incubation, washing, fixing, staining, colony counting, and plotting the cell survival curve. (Axion BioSystems, 2023).

2.2.5 Relative biological effectiveness (RBE)

BNCT involves mixed field irradiation consisting of several different radiation dose components, with different physical properties and relative biological effective-

ness (RBE). The biological effective dose of BNCT depends on the RBE of different dose components that contribute the total irradiation dose. The RBE is a clear, unambiguous, and well-defined radiobiological concept. It is the ratio of absorbed doses of a reference radiation such as X-rays or gamma rays and the radiation under study, required to produce the same biological effect. RBE value depends on the type of radiation source, type of tissue (tumor or normal tissue), and LET of radiation source. As the LET of radiation increases, the ability of the radiation to produce biological damage also increases. RBE is defined by the following ratio (Podgorsak, 2005):

$$\text{RBE} = \frac{\text{Dose from standard radiation to produce a given biological effect}}{\text{Dose from test radiation to produce the same biological effect}}. \quad (2.5)$$

2.3 BNCT physical dose

2.3.1 BNCT

Boron neutron capture therapy (BNCT) allows for the selective destruction of malignant cells while sparing healthy cells. It is based on the high propensity of the non-radioactive nuclide boron-10 to capture low-energy thermal neutrons, resulting in the prompt nuclear reaction $^{10}\text{B}(n,\alpha)^7\text{Li}$ via two way channels: in 94% of cases, the Li nucleus is in an excited state that decays quickly to the ground state accompanied by a 0.48 MeV photon, and in 6% of cases, it enters the ground state directly (Yue et al., 2018). The fundamentals of nuclear reaction are shown in greater detail below:

Taylor and Goldhaber described the $^{10}\text{B}(n,\alpha)^7\text{Li}$ reaction in 1935 (Taylor and Goldhaber, 1935), after Chadwick discovered the neutron in 1932 (Chadwick, 1932). Locher published the fundamental idea of using neutron capture reactions to treat cancer in 1936 (Locher, 1936). Following this, Hatanaka started using sodium mercaptododecaborate (BSH) and a thermal neutron source in 1968, which improved some of his patient's chances of surviving surgery.

Most recently, clinical interest in BNCT has extended to head, neck and liver cancers for the treatment of unresectable tumors. Considering that BNCT is a biologically rather than physically targeted form of radiation therapy. Especially, the tumor (atoms or cells) must receive a sufficient dosage of ^{10}B and must be able to absorb an adequate number of thermal neutrons. Regarding that, the capture reaction shown in Figure 1.1 materialized, resulting in extensive linear energy transfer (LET) particle paths

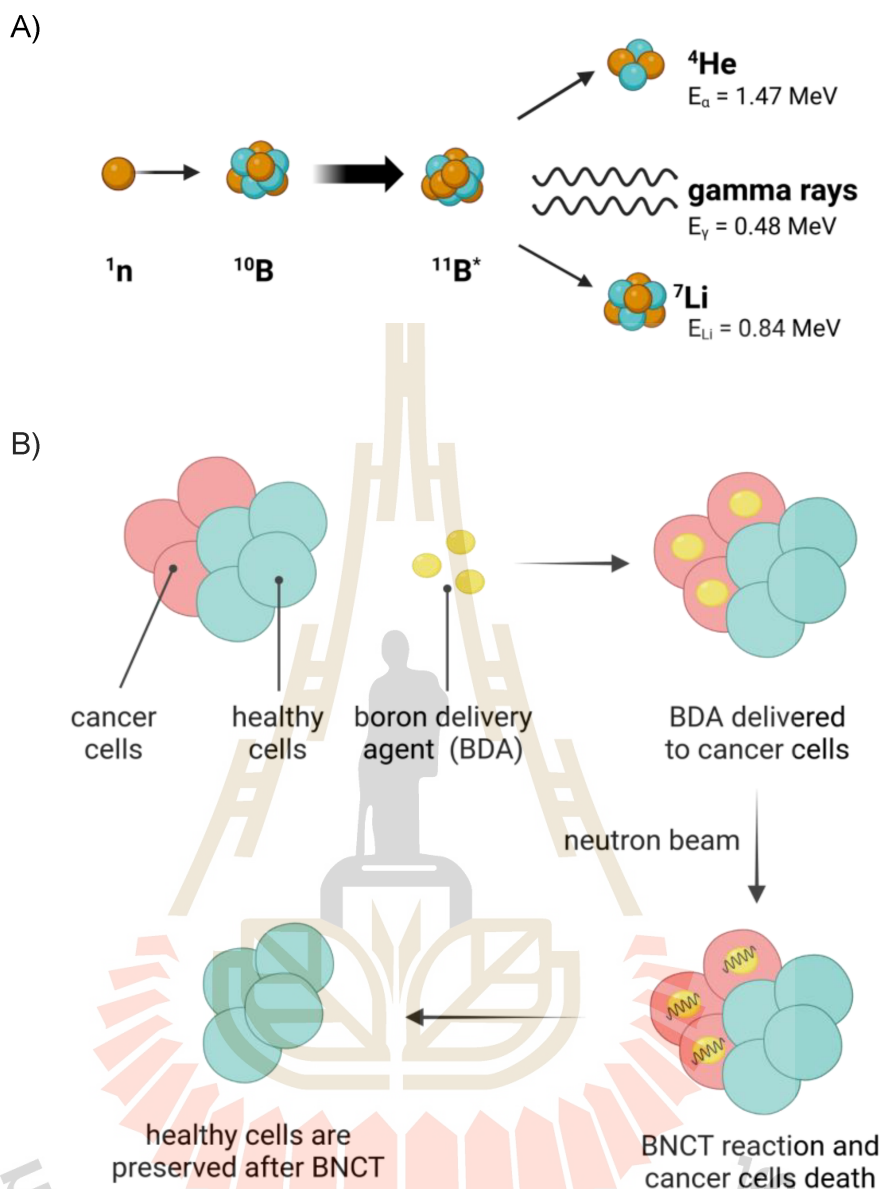


Figure 2.7 Schematic of Boron Neutron Capture Therapy (BNCT). (A) The nuclear reaction where a Boron-10 nucleus captures a neutron, releasing a destructive alpha particle and Lithium-7 nucleus. (B) The therapeutic strategy where a boron agent targets cancer cells, which are then destroyed by a neutron beam while sparing healthy cells (Marforio et al., 2023).

in tissue ($5\text{--}9 \mu\text{m}$). Thus, only boron-containing cells are subject to this destructive effect (Barth et al., 2005).

Despite its straightforward concept, BNCT requires the administration of a pharmaceutical in conjunction with the application of the neutron, a relatively uncom-

mon form of ionizing radiation. In order to achieve the most optimal energy range for treatment, related clinical designs and operations. The Figure 2.8 depicts the schematic diagram of the AB-BNCT system. The accelerator produces a proton beam and then, the proton beam is focused using the beam transportation system. Next, the beam moves into the neutron irradiation system also known as the beam shaper assembly (BSA), consisting moderator that slows fast neutron emission before the beam is delivered into the treatment room where a patient is precisely placed (Suzuki, 2020).

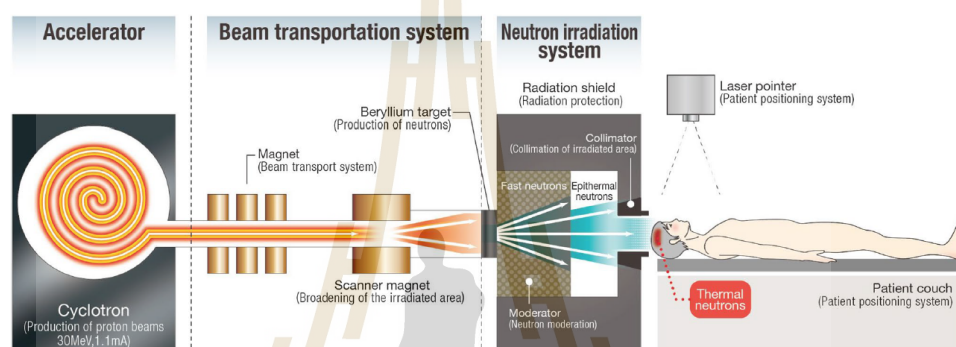


Figure 2.8 Schematic view of the accelerator-based BNCT system showing the principal components of the accelerator (Suzuki, 2020).

2.3.2 Neutron cross-section

The neutron has a mass of 1.675×10^{-27} kg and a half-life of about 14 minutes and 39 seconds. Neutron is a neutral particle and therefore leaves no track in the matter except when it undergoes a nuclear interaction. Neutrons are comparable to protons in most ways. They are the building blocks of nuclei and have the same mass, a number of nucleons, and spin as protons (Das and Ferbel, 2003). Neutrons are incapable of perceiving the nuclear Coulomb force; therefore, even slow neutrons can be scattered or captured by the strong nuclear force. When low-energy neutrons interact inelastically, they can leave nuclei in excited states, which can then decay to ground levels by emitting photons or other particles. Neutrons are produced with an energy proportional to the nucleus from which they are emitted and the energy of the interacting particle that triggers their emission.

In general, the range of neutron energies used in BNCT is summarized in Table 2.1, with neutron energies categorized using terms like *thermal*, *epithermal*, and *fast*. All neutron sources produce the vast majority of fast neutrons. As illustrated by the

neutron cross section for boron, it is desirable to distinguish and measure neutrons in each region due to their varying radiation properties. The thermal neutron flux required by the BNCT radiation field at the tumor site is approximately 10^9 n/cm²/s, which is computed using Monte Carlo simulations as well as other methods (Barth et al., 2005).

Table 2.1 Definition of neutron energy ranges used in BNCT.

Range name	Thermal	Epithermal	Fast
Neutron energy	<0.5 eV	0.5 eV - 10 keV	>10 keV

2.3.3 Radiation component

The dosimetry for BNCT is much more complex than the standard radiotherapy, where collimated photons mainly produce electrons, releasing all their kinetic energy by ionization. There are four types of direct ionizing radiation with LET characteristic differences (Barth et al., 2005):

(a) Photon dose or low LET γ rays caused by normal tissue hydrogen atoms capturing thermal neutrons [$^1\text{H}(n, \gamma)^2\text{H}$].

(b) Thermal neutron dose or nitrogen dose, which is made up of high-LET protons that come from fast neutrons scattering and thermal neutrons being taken in by nitrogen atoms [$^{14}\text{N}(n,p)^{14}\text{C}$].

(c) Boron dose, high LET, heavier-charged particles (stripped-down ^4He nuclei) and ^7Li ions, released as products of the thermal neutron capture and fission reactions with ^{10}B .

(d) Fast neutron or Hydrogen dose is the (n,n') moderation reaction mainly with ^1H in the body. The energy released by recoiling protons was produced by fast neutron.

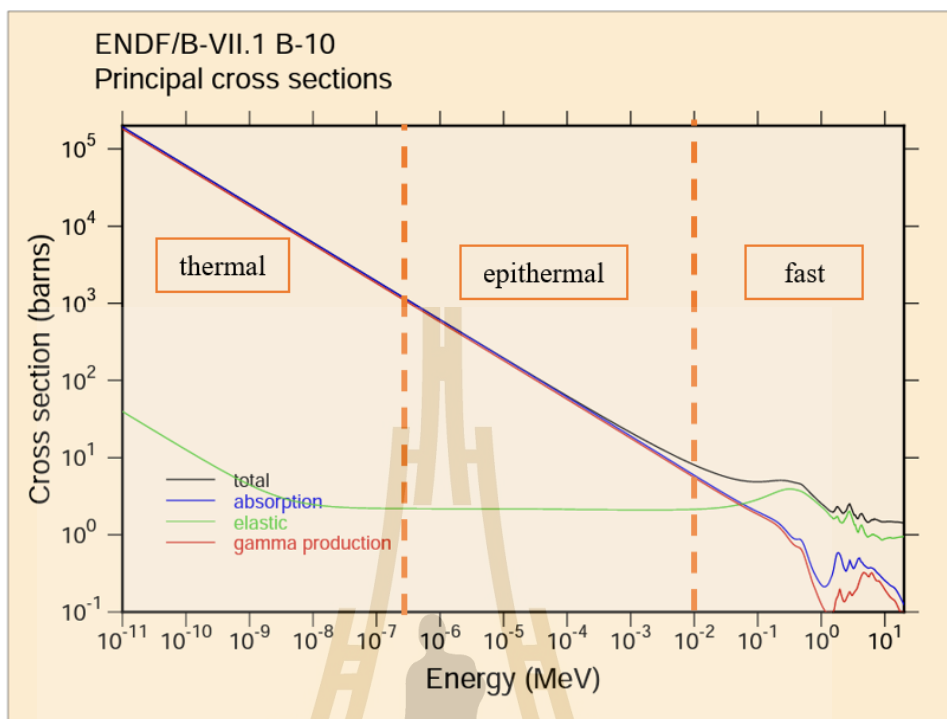


Figure 2.9 Neutron cross-section for boron-10 (IAEA, 2011).

2.4 Boron content or Boron agent

^{10}B is the sole isotope of boron that contributes to BNCT. The natural abundance of ^{10}B in boron is only 19.8 percent of the amount required to reduce the boron agent's pharmaceutical dose for patients. Thus, boron agents with a high level of ^{10}B enrichment are needed (Tanaka et al., 2014). Ideally, it would: (a) have low systemic toxicity sufficient to ensure normal tissue uptake with high tumor uptake and concomitantly high tumor/brain and tumor/blood concentration ratios (>3-4:1); (b) be able to maintain the ^{10}B concentration in the tumor tissue at a level where an antitumor effect can be anticipated during neutron irradiation; and (c) have rapid clearance from blood and normal tissues while remains in tumor during BNCT (Barth et al., 2005).

2.4.1 L-p-Boronophenylalanine (L-BPA)

The first boron agents used in the BNCT treatment of brain tumors with high antitumor effect were sodium mercaptododecaborate (^{10}B) and BSH in 1995. Four years later, 4-borono-L-phenylalanine (BPA), which is similar in structure to the essential amino acid phenylalanine and has a boric acid group on the benzene ring of L-phenylalanine

(see Figure 2.10), was used to treat malignant brain tumors. The boron compound L-p-Boronophenylalanine (L-BPA) is now widely used in clinical settings. BPA has three structural isomers that are different based on where the boric acid residue is on the aromatic ring in relation to the alanine group (Hatanaka, 1975).

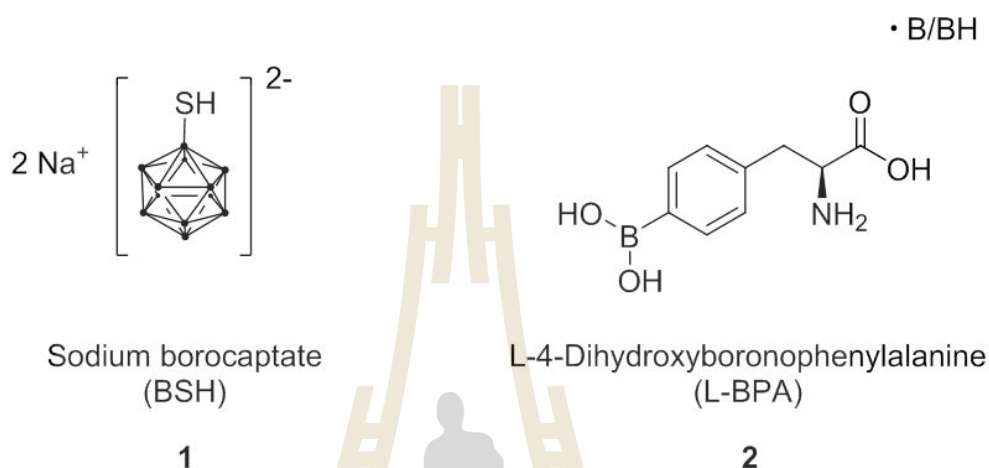


Figure 2.10 Boron compounds widely used in clinical studies (1) BSH, (2) L-BPA (Kellert and Hey-Hawkins, 2020).

BPA concentrates in tumors at levels 3.5 to 4 times higher than in blood or brain tissue (Coderre et al., 1992) and is barely affected by a pH of 7.4 and insoluble in water. By using D-fructose as a solubilizer, it was discovered that BPA had high water solubility (MORI et al., 1989). The BPA-fructose (BPA-fr) solution has a pH of 7.94, which is close to physiological pH. However, the BPA-fr solution is unstable; within two to three days of preparation, it turns brown due to the Maillard reaction, and the concentration of BPA decreases. Therefore, it is recommended that the solution be prepared immediately prior to irradiation.

2.4.2 Cellular transport

The transport mechanism of L-BPA-fr and BPA-fr in cells have been studied. They are transported via L-type neutral amino acid transporter (LAT), which operates across the blood-brain barrier, and two transporter components (LAT1 and LAT2) (Coderre et al., 1990).

LAT1 (L-type amino acid transporter 1) is a transmembrane protein belonging to the solute carrier (SLC) family of transporters. Its primary function is to transport large neutral amino acids, such as leucine and phenylalanine, across the plasma membrane

of cells, as shown in Figure 2.11. LAT1 is composed of a light chain subunit (LAT1, SLC7A5) and a heavy chain subunit (CD98 or 4F2hc, SLC3A2), which acts as a molecular chaperone to localize LAT1 at the plasma membrane (Puris et al., 2020). LAT1 plays a critical role in various physiological processes, including protein synthesis, cell growth, and energy metabolism. Furthermore, it has been implicated in the pathogenesis of several diseases, such as cancer and neurological disorders. Many cancer cells highly express LAT1, making it a potential target for cancer therapy. Selective inhibition of LAT1 could lead to the selective starvation of cancer cells, providing a novel strategy for cancer treatment.

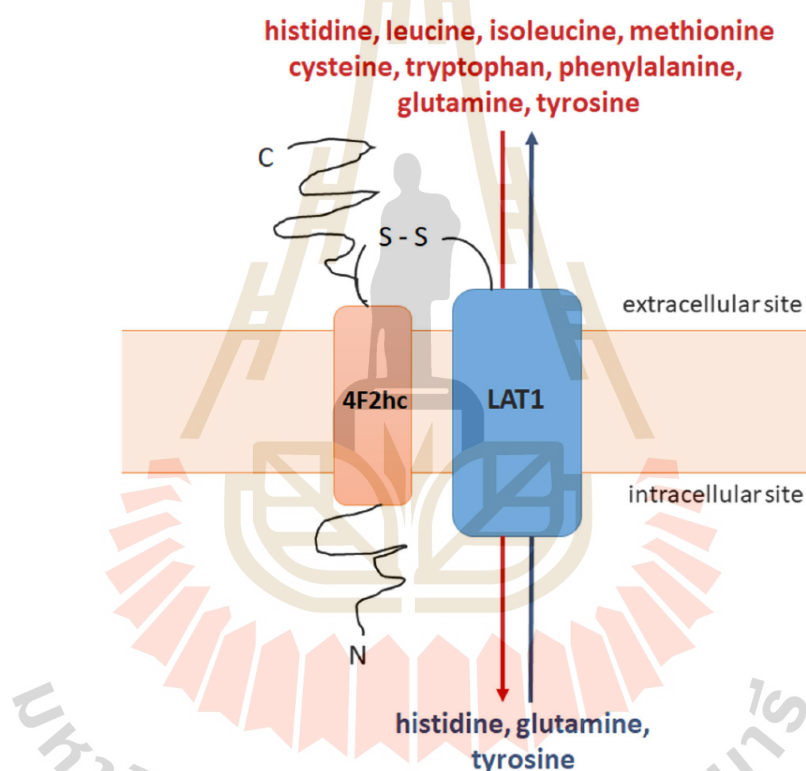


Figure 2.11 Heterodimeric transporter LAT1/4F2hc (CD98) mediated transport of essential and non-essential amino acids across the cell membrane (Puris et al., 2020).

2.4.3 Inductively coupled plasma (ICP)

Inductively coupled plasma (ICP) is produced by transferring the energy from a radio frequency generator into a suitable gas, usually ICP argon. This generates an alternating electromagnetic field (inductive coupling) for any fluid that moves through the coils. Adequate power is used to heat and ionize the sample so that it turns into plasma. Two ICP methods are mostly used in BNCT applications.

1. ICP Optical Emission Spectrometry (ICP-OES) is sensitive enough to measure boron levels in samples with low boron concentrations. Many research studies mentioned that the ICP-OSE can be used to measure the amount of boron in BNCT. It is less costly to purchase and operate than ICP-MS (Linko et al., 2008).

2. ICP mass spectrometry (ICP-MS) is an analytical method that can be used to measure the trace amounts of elements in biological fluids. It has detection limits that are significantly lower than ICP-OES (about three orders of magnitude better), and it is also sensitive to the isotopic composition of boron (Triatmoko and Sutjipto, 2017).

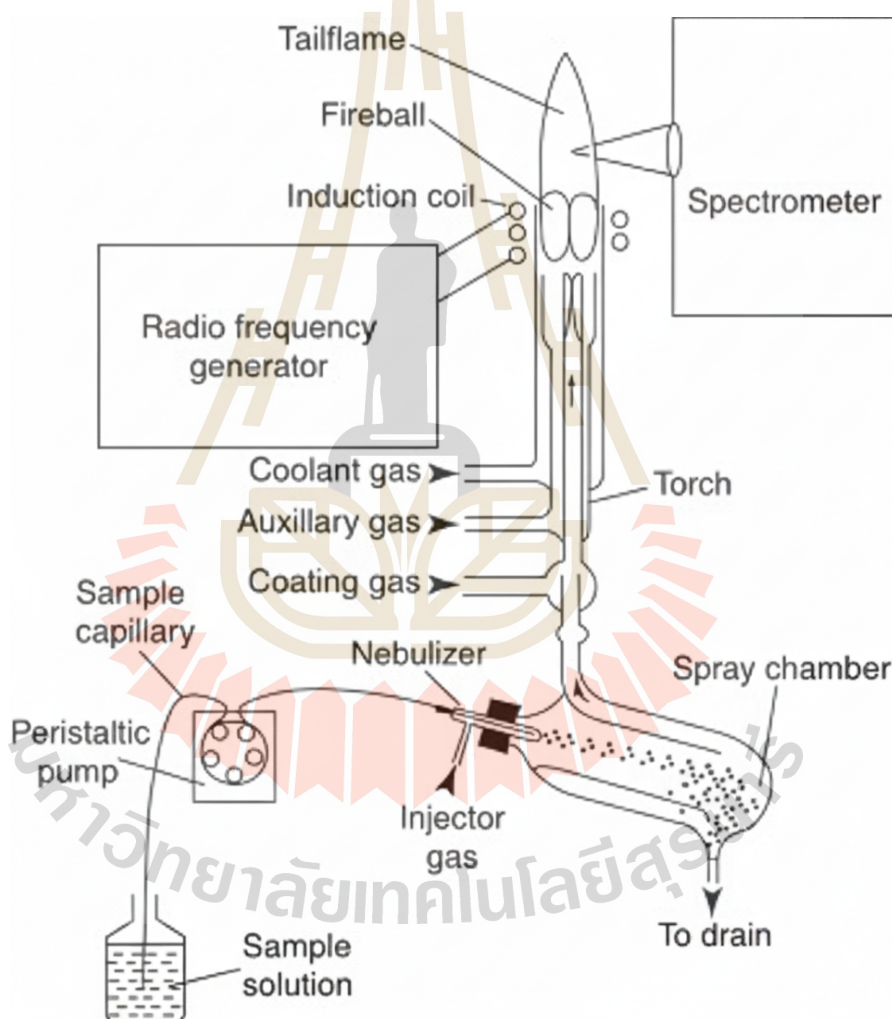


Figure 2.12 Major components and layout of a typical ICP instrument (Kumar and Mazumder, 2023).

CHAPTER III

MATERIAL AND METHODS

This chapter describes the materials and methods we used to study cholangiocarcinoma (CCA) cells in vitro for BNCT (boron neutron capture therapy). Primarily, we prepare an L-BPA-fructose solution and investigate its uptake, cytotoxic effects on CCA cells, and L-BPA receptor expression. After the in vitro setup was optimized, the cells were exposed to doses ranging from 0 to 5 Gy of X-ray and thermal neutron beam radiation to assess the efficacy of BNCT. We used assays for colony formation, DNA damage expression, and cell viability to evaluate the biological effects of radiation exposure. This chapter provides a thorough overview of each stage of the experimental process, from preparation and irradiation to post-treatment analysis, laying the groundwork for understanding the results and subsequent discussions.

3.1 In vitro experimental set up

3.1.1 Cell culture

Two human cholangiocarcinoma (CCA) cell lines, KKU-055 (poorly differentiated adenocarcinoma) and KKU-213A (mixed papillary and non-papillary adenocarcinoma), were extracted from Thai bile duct cancer patients and utilized in this study. The cells were acquired from the Japanese Collection of Research Bioresources Cell Bank. To sustain their growth, the cells were cultured in a monolayer using Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Thermo Fisher Scientific Inc., USA) and 1% Penicillin-Streptomycin (10,000 U/mL; Gibco, USA). The cultures were maintained at a temperature of 37 °C in an atmosphere of 5% CO₂. Every three days, the cells were subcultured in T-25 cell culture flasks (25 cm² with vented caps; Falcon, Corning, USA), each filled with 5 mL of the medium. Following the instructions in the appendix protocol, cells were thoroughly passaged in the T25 flask and trypsinized at 0.25% confluency once 80–100% of the cells were observed.

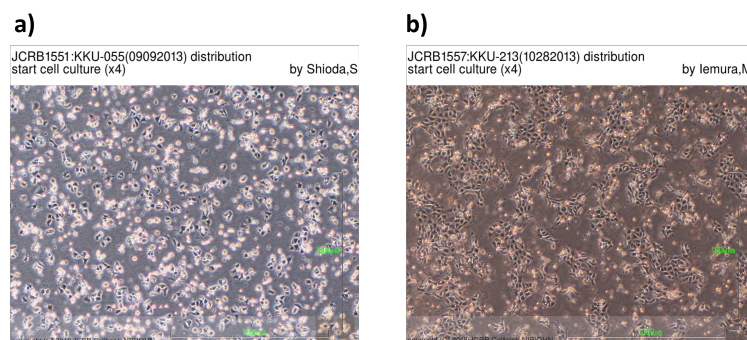


Figure 3.1 Images of KJU-055 cells (a) and KJU-213A cells (b) taken under a 4x magnification microscope. These images highlight the morphological characteristics and density of the two cholangiocarcinoma cell lines. (National Institutes of Biomedical Innovation and Nutrition, 2024)

3.1.2 Preparation of L-BPA-fructose (L-BPA-fr) solution in cell culture medium

4-Borono-L-phenylalanine (L-BPA, BPA, $C_9H_{12}BNO_4$) (17755, Sigma-Aldrich) was the compound of boron utilized. To create the L-BPA-Fr solution stock, 20.9 mg of L-BPA and 27.0 mg of D-fructose were combined in 200 μ L of water purifier. After that, 200 μ L of 1N NaOH was added to dissolve the mixture. Until the experiment was ready, the stock solution was kept at -20°C in the dark.

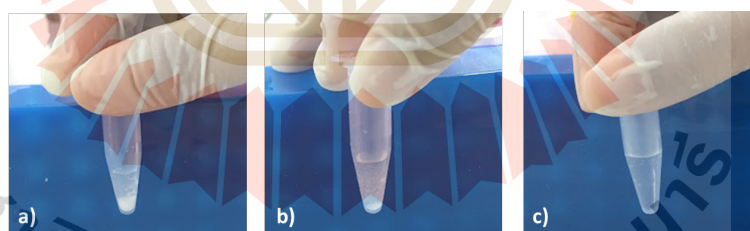


Figure 3.2 The preparation of L-BPA-fr stock working solution: (a) 20.9 mg of L-BPA and 27.0 mg of D-fructose were combined in 200 μ L of purified water. (b) 100 μ L of 1N NaOH was added to the tube. (c) An additional 100 μ L of 1N NaOH was added to ensure complete dissolution of the mixture.

The ^{10}B concentration in cell culture medium (DMEM) is measured. 8 mL of DMEM medium was added to the 50 mL tube, and the medium was gradually removed, resulting in 191.34 μ L of L-BPA-Fr solution. Following thorough mixing, 1N HCl was used to adjust the pH back to 7.2–7.3. The DMEM medium should then be added until the

final volume reaches 10 mL. The concentration of L-BPA in this mixture is 1000 parts per million (ppm) in 10 mL of DMEM medium. It must be sterilized using 0.22 μm filters before use in in vitro experiments.

Before the measurement of ^{10}B concentration using Inductively Coupled Plasma Mass Spectrometry (ICP-MS), microwave digestion of the samples was conducted. This step is essential for the accurate analysis of boron content, as it ensures the complete breakdown of organic compounds and the uniform distribution of boron within the sample.

Preparing the prepared samples for ^{10}B measurement via ICP-MS required a crucial step called microwave digestion, which involved subjecting them to a microwave digestion process. The organic matter was broken down completely using calibrated high-temperature and high-pressure conditions in this process, which resulted in the effective release and even distribution of boron. To get accurate ICP-MS readings, the exact microwave digestion parameters, such as temperature, duration, and the acids or solvents used, had to be carefully optimised to get the most boron out of the sample without changing its chemical state. This step was done by the technical scientist.

3.1.3 Amino acid transporter expression in CCA cells

In this study, two distinct Cholangiocarcinoma (CCA) cell lines, namely KKU-055 and KKU-213A were utilized to determine the expression levels of L-type neutral amino acid transporters such as LAT1, LAT2, LAT3, LAT4, and 4F2hc (CD98), which are implicated in boron uptake. To measure the expression of these transporters, real-time polymerase chain reaction (RT-PCR) was employed, a technique renowned for its sensitivity and quantification capabilities in gene expression analysis.

The experimental process encompassed six essential steps. Initially, the sample preparation involved culturing the CCA cell lines under optimal conditions to harvest RNA. RNA extraction was then performed using the TRIzol reagent (Invitrogen, USA), following the protocols recommended by the manufacturer. The quality and concentration of RNA were quantified with a NanoDrop Spectrophotometer (Thermo Fisher Scientific™). To verify the integrity of the RNA, samples were subjected to agarose gel electrophoresis, a method allowing for the visualization of RNA to confirm its purity.

After validation, the RNA was reverse-transcribed into cDNA using the Sensi-FAST cDNA synthesis kit. This synthesized cDNA served as the template for the RT-PCR, which quantified the expression of the LAT genes using SYBR Premix Ex Taq (Roche 480)

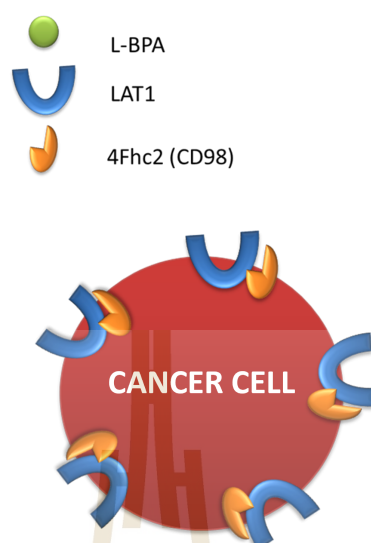


Figure 3.3 Simplified schematic illustrating the relationship between amino acid transporters (LAT1 and 4F2hc) and L-BPA in cancer cells. LAT1 and 4F2hc are overexpressed on the surface of cancer cells, facilitating the uptake of L-BPA.

and specific primers listed in the document. The primers for LAT1, for instance, had the sequences 5'-TTC GTC CAG ATC GGG AAG GGT GA-3' (forward) and 5'-TCC ATA GCC AAA GAC GCC GCT-3' (reverse), with an annealing temperature of 57°C. The internal control for normalization was the β -actin gene, and relative gene expression changes were calculated employing the $2^{-\Delta C_t}$ method. This comprehensive approach, encompassing intricate steps from cell culture to data analysis, was carefully designed to yield precise insights into the L-BPA transport mechanisms in CCA cells, which is critical for the advancement of BNCT as a therapeutic strategy.

3.1.4 CCA cells cytotoxicity of L-BPA

This experimental investigation aimed to identify the optimal concentration of L-BPA that CCA cells can accumulate without inducing cytotoxicity. This threshold is very important for making BNCT work as well as possible while causing as little damage as possible to cells. Cells were seeded into a 96-well plate with medium mixed with various concentrations of L-BPA-fr solution (250, 500, 1000, and 2000 ppm). Both the groups treated with L-BPA-fr and the control group (without L-BPA-fr) were incubated for twenty-four hours. Following this incubation period, the sulforhodamine B (SRB) assay was conducted using a Tecan Plate Reader (Tecan Group Ltd., Switzerland) in

accordance with the protocol detailed in the Appendix A.3. This assay was carried out at specified time intervals to investigate the cytotoxic effects of the L-BPA-fr solution on the CCA cells. Additionally, the resulting data were analyzed using Microsoft Excel.

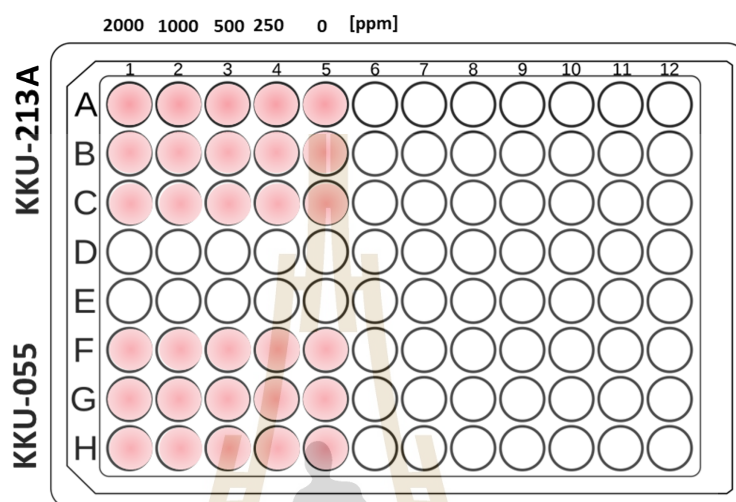


Figure 3.4 Schematic of cell seeding in 96-well plates for testing the cytotoxicity of L-BPA on CCA cells.

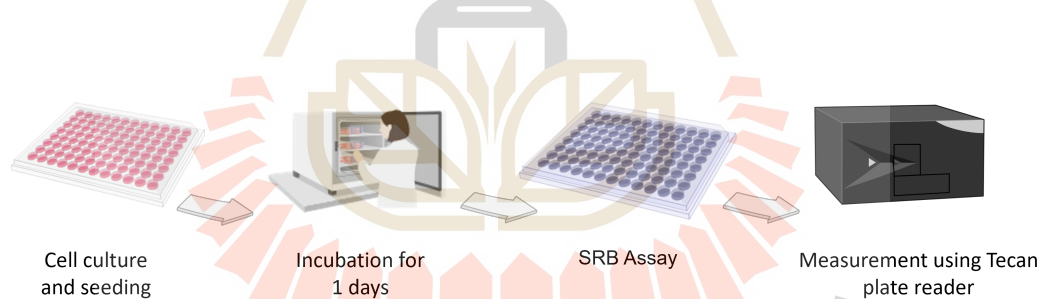


Figure 3.5 Steps of the Sulforhodamine B (SRB) Assay.

3.1.5 Accumulation of boron within CCA cells

The investigation into the uptake of boron by CCA cell lines, specifically KKU-055 and KKU-213, commenced with seeding these cells in T25 culture flasks. Each flask was inoculated with 300,000 cells and bathed in a suitable culture medium. The cells were then allowed a 24-hour incubation period to proliferate, ensuring a sufficient number for the experiments.

After this initial growth phase, the medium was replaced with a fresh batch containing L-BPA-fr at a concentration of 1000 ppm. The cells were incubated for vary-

ing durations, ranging from 0 to 48 hours, to observe the boron uptake over time. After each incubation period, the spent medium was discarded, and the cells were gently rinsed with phosphate-buffered saline (PBS) to remove any traces of non-internalized L-BPA-fr. Subsequently, the cells were detached using a 0.25% Trypsin-EDTA solution as per the established cell culture protocols outlined in the appendix A.1 of the study. After this initial growth phase, the medium was replaced with a fresh batch containing L-BPA-fr at a concentration of 1000 ppm. The cells were incubated for varying durations, ranging from 0 to 48 hours, to observe the boron uptake over time. After each incubation period, the spent medium was discarded, and the cells were gently rinsed with phosphate-buffered saline (PBS) to remove any traces of non-internalized L-BPA-fr. Subsequently, the cells were detached using a 0.25% Trypsin-EDTA solution, following the established cell culture protocols outlined in Appendix A.1 of the study. The detached cells were then subjected to centrifugation twice, ensuring that the resulting cell pellet was clear of any extraneous contaminants. These clean cell pellets were then transferred to 15-mL tubes. For digestion, each pellet was treated with 1 mL of 60% nitric acid (HNO_3), effectively breaking down the cellular components and releasing the incorporated boron

The final step involved the quantification of boron within the digested samples using ICP-MS. This sensitive analytical technique enabled the precise measurement of boron that had been internalized by the CCA cells, providing valuable data for the evaluation of L-BPA-fr as a potential boron carrier in BNCT applications for cancer treatment.

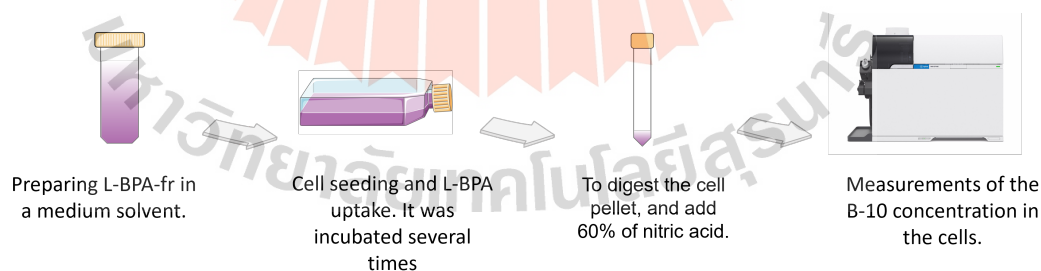


Figure 3.6 Workflow for Boron Accumulation Measurement via ICP-MS.

3.2 Irradiation procedure

3.2.1 X-rays Irradiations

The KKU-055 cells were cultured within a 6-well plate at varying densities of 100, 200, 400, and 1000 cells per well. These densities were chosen to correspond with a range of radiation doses. To allow for cell attachment and stabilization, the cells were incubated for 24 hours before radiation exposure. For the radiation procedure, the plate containing the samples was precisely positioned at the center of a specifically designed container, as illustrated in Figure 3.7.

To mitigate the effects of secondary ionization during the radiation process, the container was filled with a material that simulates human tissue, often referred to as phantom material. This setup aims to mimic the conditions that would be encountered in a clinical scenario. The calculation of the X-ray doses delivered by a medical linear accelerator (LINAC) was executed using the Eclipse clinical treatment planning system, provided by Varian Medical Systems located in Palo Alto, California, USA. This system is a staple in radiation therapy, allowing for precise dose calculations and treatment planning.

The radiation was administered at the King Chulalongkorn Memorial Hospital (KCMH) in Thailand, utilizing the LINAC, which is routinely used for cancer treatment. The parameters for irradiation were meticulously set, including an anterior-posterior/posterior-anterior (AP/PA) beam orientation, which allows for uniform dose distribution across the sample. The field size was established at 15 x 15 cm², and the source-to-sample distance (SSD) was fixed at 100 cm to ensure the correct intensity and coverage.

Samples were exposed to radiation doses of 0 Gy (control), 1 Gy, 3 Gy, and 5 Gy using 6 MV X-rays at a rate of 400 Monitor Units (MU) per minute. This range was selected to study the cells' dose-response relationship. After exposure, the cells were immediately returned to the incubator to allow for colony formation.

After one to two weeks, all plates were kept in the incubator to allow the irradiated cells sufficient time to grow into distinguishable colonies. After this growth period, the cells were stained using a solution of 0.5% crystal violet in 100% methanol for one to two hours, which helps in visualizing the colonies against the background of the well plate. The colonies were then enumerated with the aid of a stereoscope, providing data on the survival and proliferative capacity of the cells post-irradiation. This colony formation assay is a fundamental method in radiobiology, providing insights into the efficacy of radiation doses on cell survival.

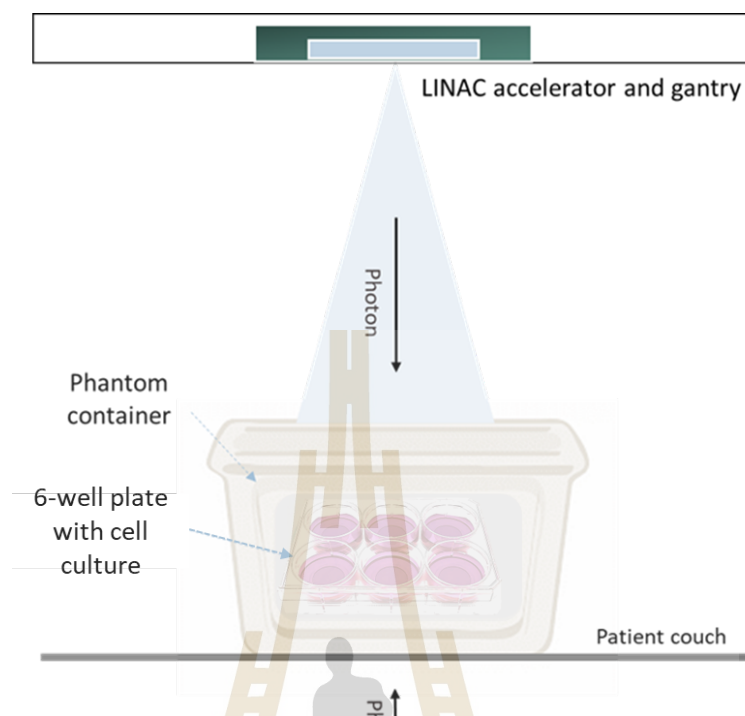


Figure 3.7 Experimental arrangements for photon-based cell irradiations at the King Chulalongkorn Memorial Hospital's medical linear accelerator. The 6 well plate containing a layer of cells are simultaneously irradiated with the same dose.

3.2.2 Neutron irradiation in Thailand

Two days before irradiation, 300,000 cells were cultured in a medium with and without L-BPA-fr inside two T25 cell culture flasks as duplicates. The source of thermal neutron beams used was the Thai Research Reactor-1/Modification 1 (TRR-1/M1), a TRIGA Mark III type at the Thailand Institute of Nuclear Technology (TINT). To moderate fast and epithermal neutrons, the thermal column channel was fully lined with graphite. The depth of the channel was 90.5 cm, and it had a cross-sectional area of $27.5 \times 27.5 \text{ cm}^2$. The thermal neutron flux at the sample's position was measured using the indium activation method. The thermal neutron doses absorbed by the cells were estimated using the Monte Carlo N-Particle (MCNP) simulation program. The samples were modeled as two polystyrene T-25 cell culture flasks on the same plane containing complete DMEM medium with L-BPA-fr solution and the soft tissue elements: hydrogen (H), oxygen (O), carbon (C), and nitrogen (N). The experimental setup is illustrated in Figure 2. The samples were placed at the core of the thermal column of the TRIGA Mark III reactor at TINT, with doses of 0, 1, 3, and 5 Gy administered at 100 kW reactor

power, generating a thermal neutron flux of up to 5.34×10^7 n/cm²/s. The cells were irradiated in a uniform field for durations ranging from 0 to 30 minutes, depending on the dose. Prior to analysis, irradiated samples were checked for radiation levels and contamination. Radioactive samples requiring short-term storage were kept in a lead-shielded temporary storage area adjacent to the reactor pool. Once the sample's dose rate was deemed reasonably low by the reactor staff, it was transferred to the measurement laboratory.

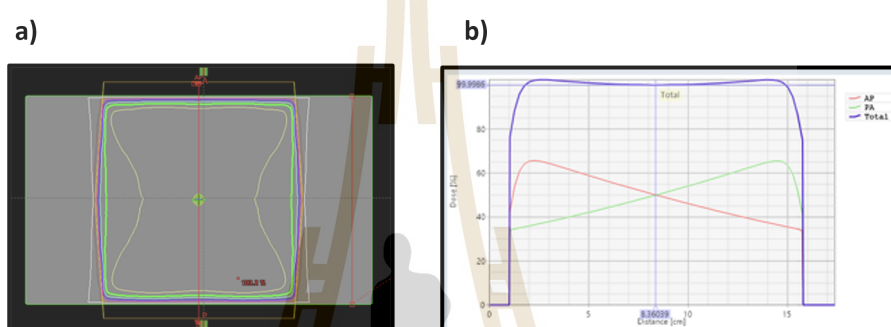


Figure 3.8 (a) Transversal external beam planning for photon irradiation with a dose rate of 400 MU/min and a field size of 15 cm x 15 cm. The color lines represent the percentage of isodose distribution, with the yellow line indicating the 100% isodose level. (b) This isodose line completely covers the cell phantom, ensuring uniform radiation exposure across the entire cell culture area for accurate and consistent experimental results.

3.2.3 Neutron irradiation in Japan

Set-up 1

For three to four days, KKU-055 cells were maintained in a T-25 cell culture flask until there was roughly 70–80% confluence. The preparation of the BPA-fr stock involved dissolving BPA in a solution composed of fructose, sodium hydroxide (NaOH), and deuterium-depleted water (D.D.W.), achieving a final concentration of ¹⁰B close to 1000 ppm. Cells were treated with BPA-fr solution at different ¹⁰B concentrations (0, 5, 10, and 20 ppm) for two hours before irradiation on the beamtime date. After treatment, the medium containing BPA was discarded at predetermined intervals, and the flasks were securely sealed with paraffin to isolate them from the surrounding air outside.

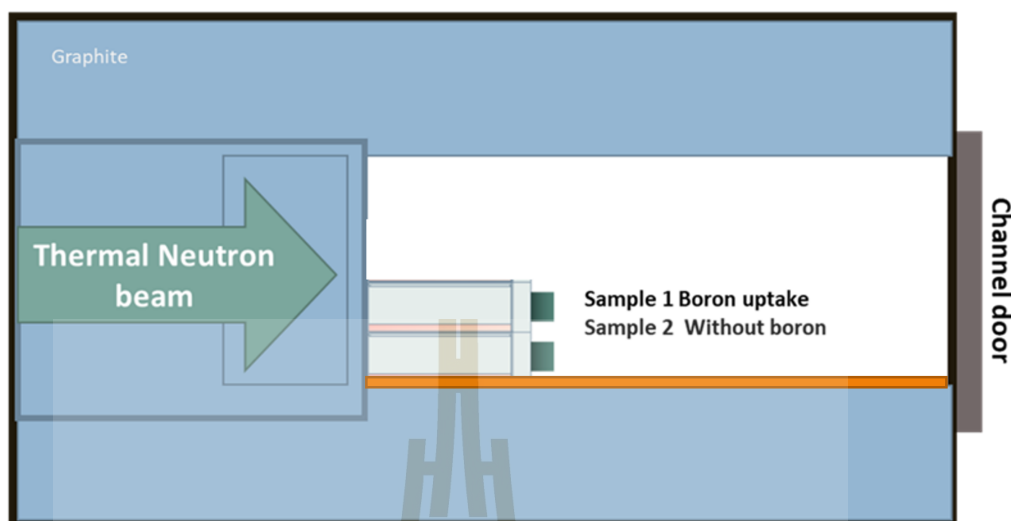


Figure 3.9 The experimental setup for cell irradiations for the BNCT in vitro study at the TRIGA Mark III reactor within the thermal column is shown schematically in a cut view.

The reactor's outline in the Figure 3.10 represents the Heavy Water Neutron Irradiation Facility (HWNIF) of the Kyoto University Research Reactor (KUR), located in Japan, where the neutron irradiations were carried out. In this experiment, the reactor operated in the so-called standard mixed neutron irradiation mode (OO-0000-F), producing a mixed irradiation field of thermal and epithermal neutrons using 1 MW of power. OO-0000-F mode is a facility-side condition, as the name would imply. It regulates various parameters such as the thickness of the middle bismuth layer, the state of the heavy water shutter tanks (full or empty), and the state of the Cadmium and Boral filters. The figure shows a comparison of the neutron energy spectra of each mode.

At the sample site, the thermal neutron flux and accompanying gamma radiation were quantified using thermoluminescent dosimeters (TLD) along with the gold foil activation technique. As detailed in the schematic of the experimental setup in Figure 3.12, the samples, attached to the gold foil and TLDs, were positioned at four distinct locations: top, bottom, left, and right. Subsequently, all samples were subjected to the neutron beam for half an hour at room temperature, and arranged within a holder box specifically designed for this purpose.

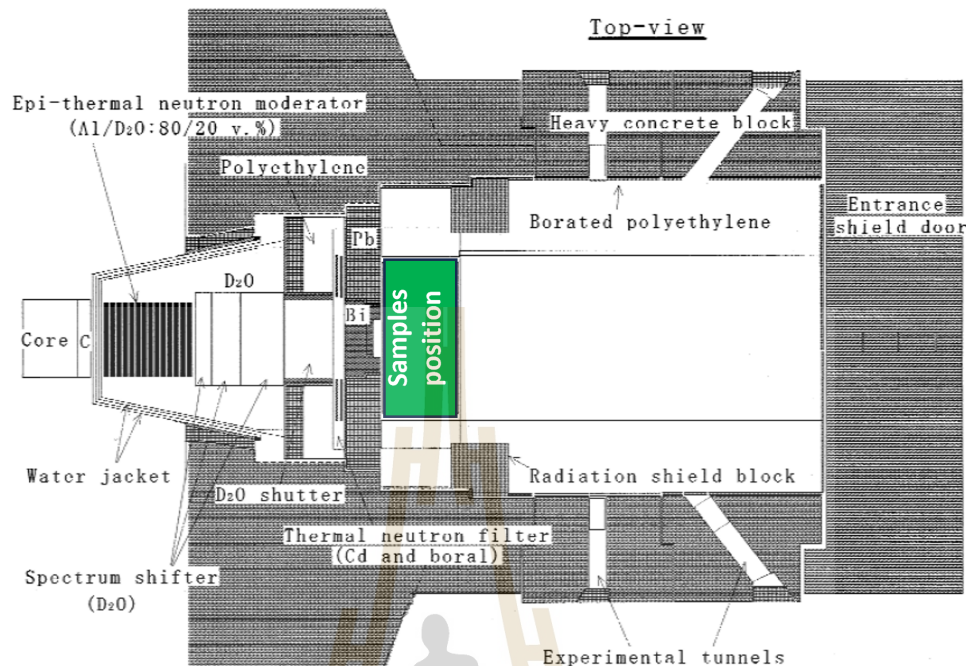


Figure 3.10 Outline of the Heavy Water Neutron Irradiation Facility. The green box indicates the sample position for Set-up 1 of neutron irradiation in Japan. This schematic provides a detailed layout of the facility, highlighting the precise location where samples are placed for neutron exposure, ensuring accurate and reproducible irradiation conditions (Sakurai and Kobayashi, 2004).

Set-up 2

In this experimental arrangement, we maintained conditions nearly identical to those in setup 1, with exceptions primarily concerning the transfer process of the samples to the beam channel and the duration of irradiation. The samples, in this case, were situated in a section marked as the green area in Figure 3.13. Similar to the procedures followed in setup 1, we measured the thermal neutron flux and gamma dose utilizing the gold foil activation method and TLDs, respectively.

For this setup as the Figure 3.14, we placed two samples on a slide holder, ensuring that each was centrally attached to gold foil activation and TLDs for precise and consistent measurements. This strategic placement was integral to the experiment's accuracy. Following this step, the holder with the samples was carefully transported to an irradiation rail device.

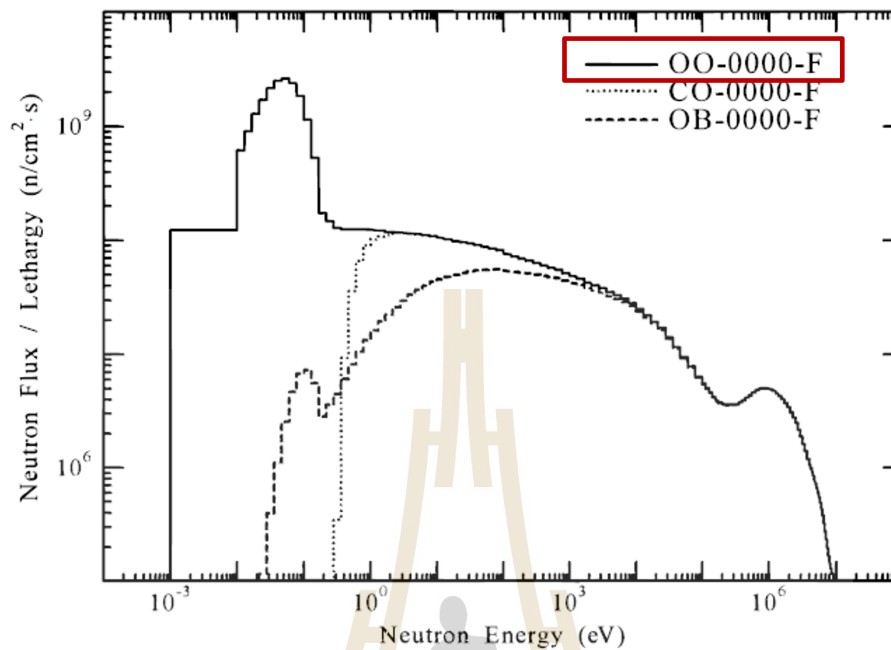


Figure 3.11 Neutron energy spectra at the normal irradiation position for the OO-0000-F, CO-0000-F, and OB-0000-F modes, estimated by the multifoil activation method (Sakurai and Kobayashi, 2004).

After this careful preparation, the samples were exposed to a neutron beam, but unlike the varied irradiation time in setup 1, here the exposure was uniformly set to a duration of 5 minutes. This modification in irradiation time, while maintaining other conditions constant, was a crucial aspect of the study, designed to evaluate the effects of altered exposure times on the samples under the same experimental conditions as the first setup.

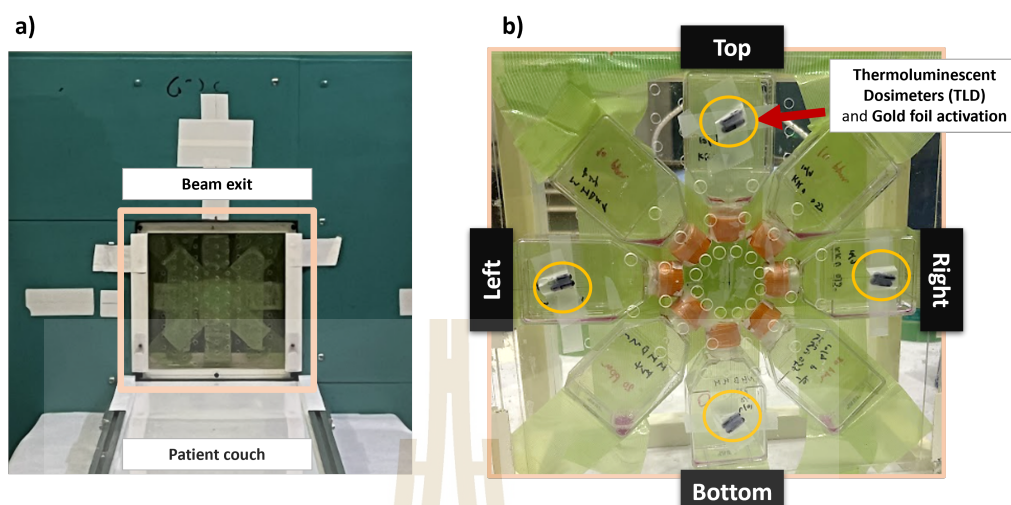


Figure 3.12 Placement of samples in the chamber box at the beam exit position at Kyoto University Research Reactor (KUR). The samples were securely fixed using tape, which also held thermoluminescent dosimeters (TLDs) to measure ionizing radiation, including gamma rays. Additionally, gold foil activations (3 mm diameter) were included to measure neutron fluences. This setup ensures precise measurement of radiation doses received by the samples, facilitating accurate evaluation of their exposure to both gamma rays and neutron radiation at KUR.

3.3 Biological effect after BNCT irradiation

After irradiation, the cells were temporarily housed in a containment box to allow any residual radiation to dissipate. They were then promptly transported to a cell culture laboratory for analysis of the radiation's biological effects. To comprehensively assess these effects, we employed three methodologies: the immunofluorescence assay, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, and the colony formation assay.

The colony formation assay, also known as the clonogenic assay, is the percentage of cell reproductive death after radiation treatment. It is an effective method for evaluating in vitro cell survival, where the ability of a single cell to proliferate into a colony is analyzed. On the other hand, the MTT assay serves as a reliable indicator of cell viability and proliferation after irradiation. This colorimetric assay measures the metabolic activity of the cells, providing insights into their health and viability.

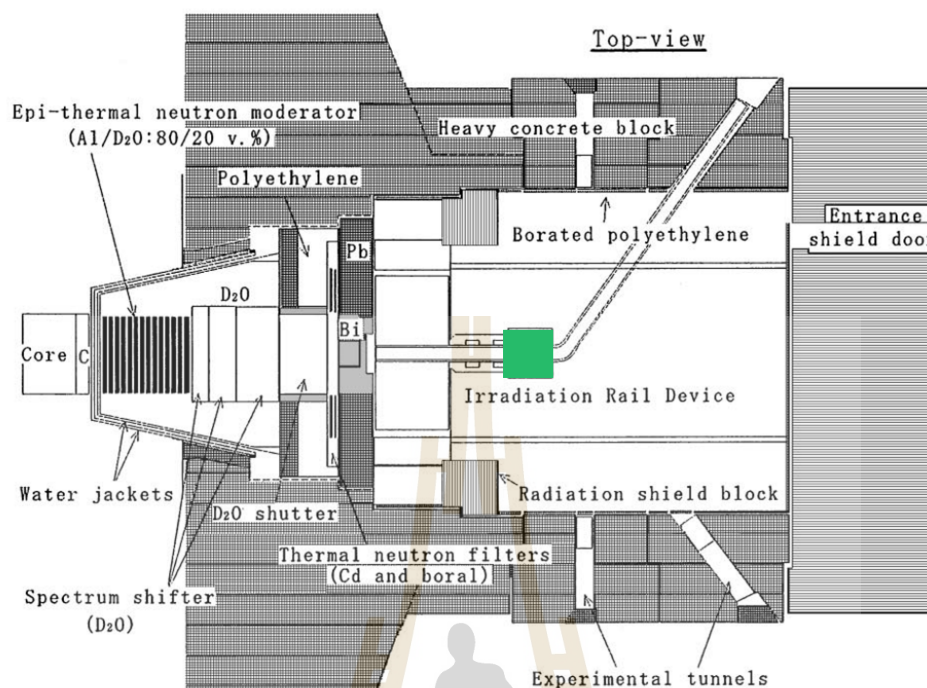


Figure 3.13 Outline of the Heavy Water Neutron Irradiation Facility. The green box indicates the sample position for Set-up 2 of neutron irradiation in Japan. This schematic provides a detailed layout of the facility, highlighting the precise location where samples are placed for neutron exposure, ensuring accurate and reproducible irradiation conditions. (Sakurai and Kobayashi, 2004).

Immunofluorescence assays, a laboratory technique, plays a crucial role in visualizing the expression of DNA damage in cells post-radiation. This technique involves using antigen-specific antibodies to detect specific proteins or antigens in the cells, thereby illustrating the cellular response to radiation at a molecular level. The Figure 3.15 outlines the structural framework and procedural flow of all these assays.

3.3.1 Colony formation assay

After irradiation, cells that were exposed to the X-ray beam were immediately transferred to an incubator to facilitate colony formation over 10–14 days. In contrast, the cells irradiated with a neutron beam at TINT underwent a different process. Clonogenic assays were then carried out after these cells were first separated using 0.25% trypsin-EDTA.

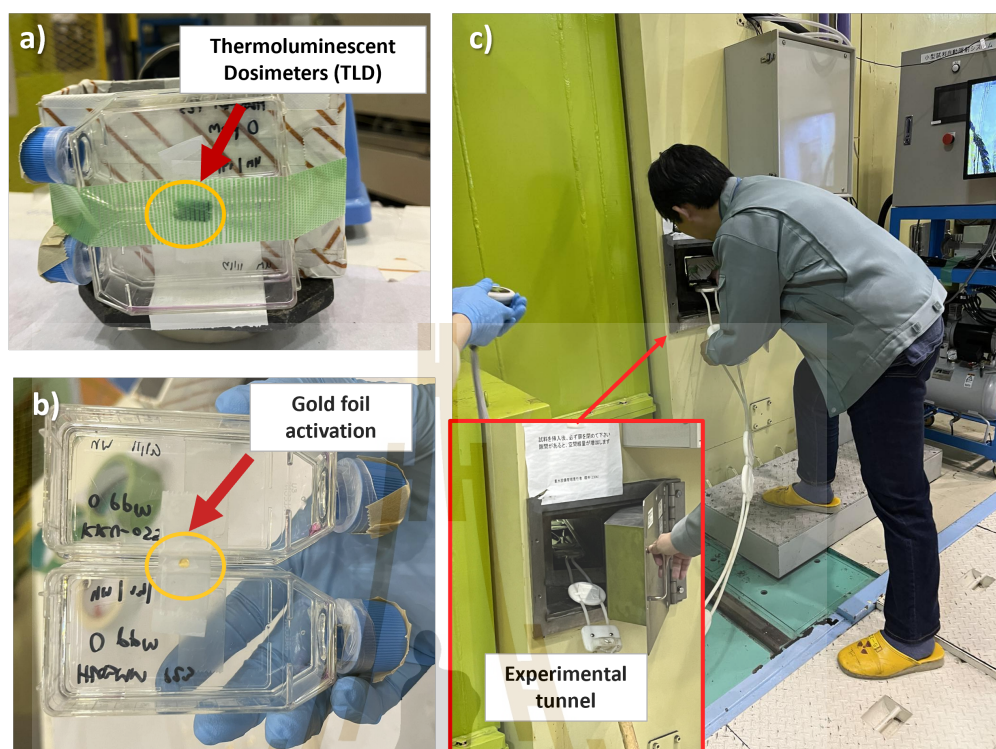


Figure 3.14 Experimental setup for sample exposure: Samples were placed in the channel and fixed with Thermoluminescent Dosimeters (TLDs) and gold foil as per setup 1. The irradiation duration was 5 minutes, conducted under a Thermal Neutron Flux of approximately $1.89 \times 10^9 \text{ n}/(\text{cm}^2 \text{ s})$.

The cells were seeded in 6-well plates in triplicate, with a density of 100 cells per well, for the clonogenic assay. The plates were then incubated for 1-2 weeks, providing sufficient time for the cells to grow into colonies. Subsequently, the cells were fixed using 2 ml of 100% methanol and stained for 30 minutes with a solution comprising 0.5% crystal violet and 100% methanol. The resulting colonies were then enumerated using a stereo microscope.

In the experiments conducted at KUR, the cells subjected to irradiation were detached using 0.25% trypsin-EDTA. Three sets of 400 cells each were seeded into each well of a 6-well plate, which was a minor departure from the previous procedure. These plates were then incubated for a period ranging from 7 to 10 days, a duration specifically chosen to allow for optimal colony growth and development.

After the incubation period, 2 mL of 100% methanol was used to fix the cells. They were subsequently stained with a Giemsa stain solution for 30 minutes. This staining procedure is crucial as it enhances the visibility of cell colonies, making them easier to observe under a microscope.

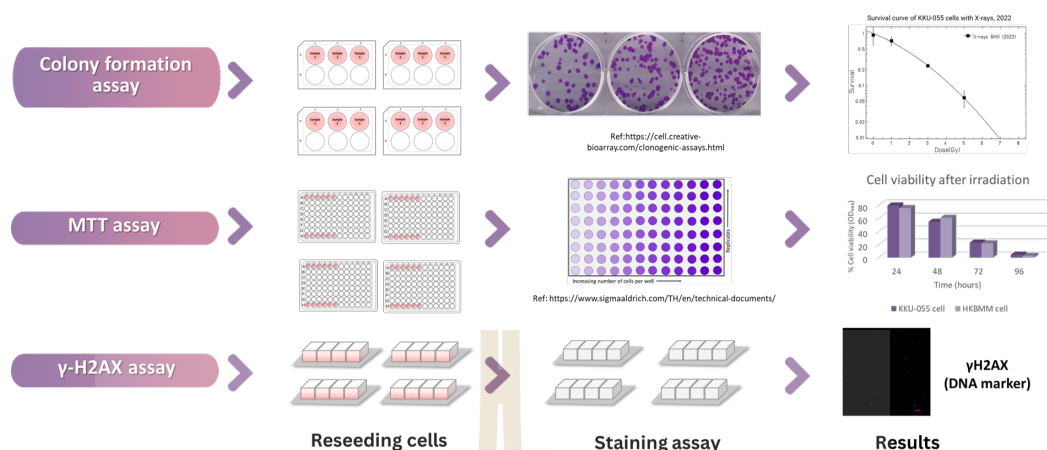


Figure 3.15 The overview of three distinct methodologies conducted to investigate the biological effects after irradiation. These methodologies include: (a) the immunofluorescence assay, used to detect specific proteins and assess DNA damage and repair; (b) the MTT assay, which measures cell viability based on metabolic activity; and (c) the colony formation assay, which evaluates the ability of a single cell to grow into a colony, reflecting its long-term reproductive viability after irradiation.

For quantifying cell survival, any colony that consisted of more than 50 single cells was counted as a single surviving cell, using a stereomicroscope for accurate observation and counting. The number of surviving cell colonies was normalized against the control group, which had not been exposed to radiation, to figure out the relative survival rate of the irradiated cells. From this comparative data, the survival fraction for each irradiated sample was calculated and subsequently analyzed. This analysis provided insights into the cellular response to irradiation, informing the understanding of radiation effects at a cellular level.

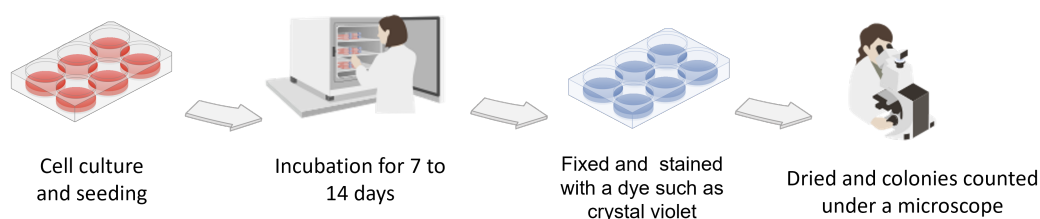


Figure 3.16 The procedure of the colony formation assay.

3.3.2 MTT assays

To assess cell viability following irradiation, the cells underwent irradiation at predetermined dosages to induce varying levels of stress, simulating potential therapeutic exposure. An MTT assay using WST-8 reagent was performed. Initially, irradiated cells were then reseeded into a 96-well plate at a density of 8,000 cells per well. These cells were allowed three days for both attachment and recovery in an incubator.

After this period, 10 μL of WST-8 reagent was added to each well under darkened conditions. This reagent is helpful because it dissolves easily in water and turns into a highly soluble formazan dye when it comes into contact with living cells, which makes the next step easier. The plates were incubated for 4 hours to ensure a complete reaction without causing undue cellular stress, facilitating the reaction between the WST-8. At the end of the incubation period, the absorbance was measured using a Multiskan FC (Thermo Scientific, USA) at OD 450 nm. Higher absorbance values indicate greater cellular viability, as more cells are able to break down WST-8 into formazan. The data obtained from this assay were normalized to the control groups to account for variability and ensure accuracy. Cell viability percentages were calculated, providing insight into the cytotoxic effects of irradiation, and enabling comparison across different radiation dosages or ^{10}B concentrations. This method offers a reliable means to quantify and understand the impact of irradiation on cell survival, which is crucial for evaluating the potential of radiation-based treatments.

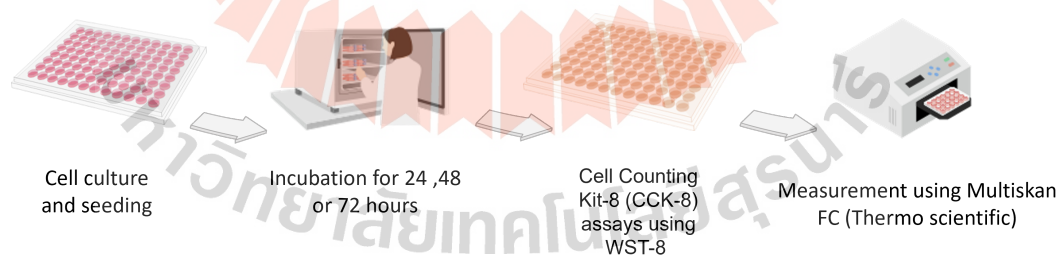


Figure 3.17 The procedure of the MTT assay.

3.3.3 Immunofluorescence assay

To investigate DNA damage, specifically γH2AX expression, an immunofluorescence assay was performed on the cells following radiation exposure. After radiation treatment, cells were reseeded into four-well chamber slides at a concentration

of 50,000 cells per well and incubated for 24 to 48 hours. The subsequent steps for intracellular staining, aimed at confocal analysis, are detailed in Appendix A.4 of the study.

The procedure commenced with the fixation of cells using paraformaldehyde. This was followed by permeabilization with Triton X-100, effectively preparing the cells for staining while ensuring that non-specific binding was minimized through a blocking process. The detection of DNA damage was achieved using a specific mouse monoclonal anti-phospho-Histone H2AX (Ser139) antibody, namely clone JBW301 from Millipore. This primary antibody's role was crucial in targeting and binding to the sites of DNA damage within the fixed cells.

After applying the primary antibodies, the cells were thoroughly washed to remove any unbound antibodies. Next, secondary antibodies, specifically Alexa Fluor 488-conjugated anti-mouse IgG, were added. These secondary antibodies bind to the primary antibodies, facilitating the visualization of DNA damage under a fluorescence microscope.

After staining, the cells were again washed and then mounted using a medium embedded with DAPI. This step was pivotal in staining the cell nuclei, providing a contrasting background to the γ H2AX signal. This dual-staining approach was instrumental in differentiating the specific γ H2AX foci, indicative of DNA damage, from the overall nuclear DNA.

The HS all-in-one fluorescence microscope BZ-9000 (KEYENCE, USA) was used to visualize and analyze the stained cells. This microscope, equipped with appropriate filters for the fluorescent dyes, enabled the efficient acquisition of images. An essential component of the analysis involved measuring the number of γ H2AX foci per nucleus, which is a direct indicator of the extent of DNA damage caused by the radiation.

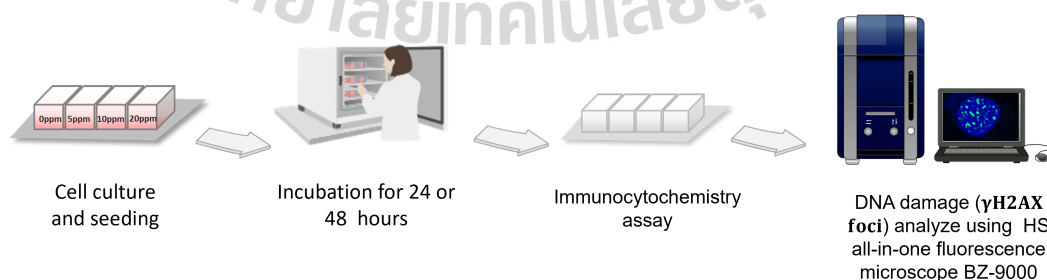


Figure 3.18 The procedure of the immunofluorescence assay.

CHAPTER IV

RESULTS AND DISCUSSIONS

This chapter provides a detailed summary of the experimental results obtained during our study. We first describe the quantification of ^{10}B in DMEM medium, which provides a baseline measurement for the experiments that follow. We next analyze the expression profiles of the L-BPA amino acid transporter and the cytotoxic effects of L-BPA on CCA cells. We also report on the intracellular boron accumulation in CCA cells, which is important for boron neutron capture therapy (BNCT) to work. The plating efficiency of KKU-055 cells after L-BPA treatment is evaluated, offering information about L-BPA's treatment period. Additionally, we outline the characteristics of the X-rays curve, which is necessary for understanding the radiation response. The final section of the chapter IV highlights the potential of BNCT in the treatment of cancer with a comprehensive analysis of the radiobiological parameters and a detailed presentation of the neutron curves from the TINT and KUR experiments.

4.1 Measurement ^{10}B concentration in cell culture medium

The purpose of the study was to measure the amount of ^{10}B in the DMEM medium following the addition of various concentrations of L-BPA-fr. L-BPA-fr was utilized at concentrations of 100 ppm, 500 ppm, 1000 ppm, and 2000 ppm. The medium's boron content increased in proportion to rising L-BPA-fr concentrations as shown in Table 4.1, according to subsequent boron measurements. In particular, a boron measurement of 4.10 ppm with a standard deviation (SD) of 0.4 was obtained at a L-BPA-fr concentration of 100 ppm, suggesting exact control of the experimental conditions and small variance.

As the L-BPA-fr concentration was elevated to 500 ppm, the boron concentration increased to 23.97 ppm, with a slightly higher SD of 2.1. Further elevation to 1000 ppm L-BPA-fr yielded a boron concentration of 51.03 ppm, accompanied by an SD of 7.91, suggesting a greater variability in the boron measurement at this level.

The highest L-BPA-fr concentration tested, 2000 ppm, resulted in the boron concentration peaking at 101.00 ppm. Interestingly, the SD was zero at this concentration, suggesting that this particular data point may represent an anomaly or exceptional

measurement precision. The results show that there is a clear dose-dependent relationship between the resulting boron concentration in the DMEM medium and the L-BPA-fr concentration. This relationship has significance for optimizing the boron content in BNCT in vitro study preparations to achieve the desired effective outcomes.

Table 4.1 Concentration of L-BPA-fr and corresponding boron concentration.

L-BPA-fr concentration (ppm)	DMEM medium solvent (ml)	Average boron concentration (ppm)	Standard deviation
100	10 ml	4.10	0.40
500	10 ml	23.97	2.10
1000	10 ml	51.03	7.91
2000	10 ml	101.00	0.00

4.2 Expression of L-BPA amino acid transporter

The study of amino acid transporters related to L-BPA aimed to identify the optimal expression levels for boron drug uptake in CCA cells. L-BPA, structurally similar to the amino acid phenylalanine (as mentioned in Chapter II), requires these transporters to enter cells. LAT1 is particularly significant because it is highly expressed in many cancer cell types. This similarity to phenylalanine enables L-BPA to be selectively targeted to cancer cells in BNCT applications. The findings demonstrate the expression levels of amino acid transporters LAT1, LAT2, LAT3, LAT4, and 4F2hc (CD98) in two types of CCA cells: KKU-055 and KKU-213A.

Figure 4.1 shows that KKU-213A cells have the highest level of LAT1, a transporter that is known for taking in L-BPA. The higher expression suggests that the cells may be able to take in more L-BPA, which could mean that BNCT works better because LAT1 helps cells take in boron-containing compounds like L-BPA. Figure 4.2 shows relatively low levels of LAT2 across all cell lines, with KKU-213A still displaying the highest expression. LAT2 is also involved in transporting amino acids, but its low expression suggests that it plays a less important role in BNCT taking in L-BPA than LAT1. In Figure 4.3 and Figure 4.4, LAT3 and LAT4 expression levels are observed. KKU-213A cells show significantly higher expression of LAT3 compared to KKU-055. For LAT4, KKU-213A cells also exhibit the highest expression, but the difference is less pronounced compared to LAT3. These transporters may contribute to L-BPA uptake, but their roles are not as well

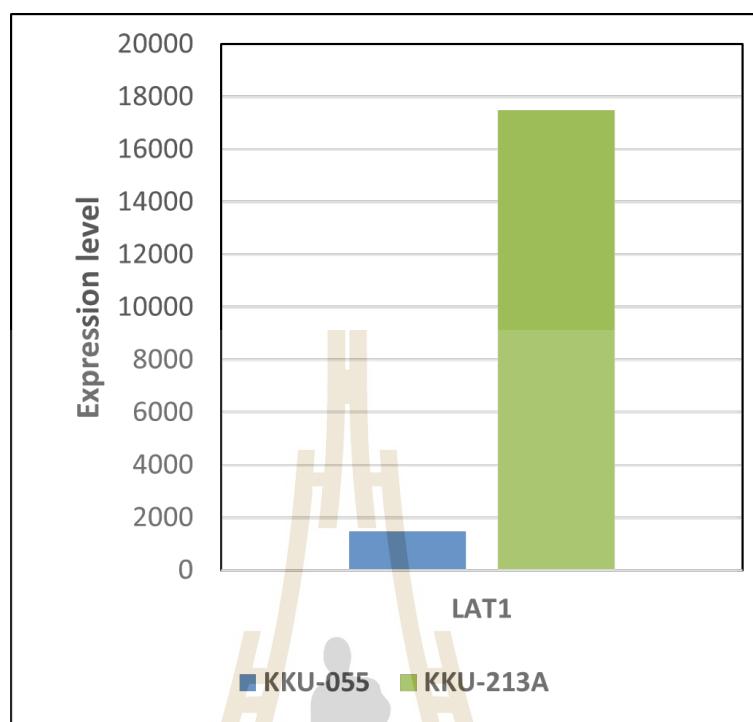


Figure 4.1 The analysis of LAT1 expression levels using RT-PCR in KKU-055 and KKU-213A cells, with the expression levels normalized.

characterized as LAT1. There is a heavy chain of a heterodimeric amino acid transporter shown in Figure 4.5 which is 4F2hc (CD98). It is associated with LAT1 and is essential for its function. KKU-213A cells have a lot more 4F2hc than other cells, which makes it more likely that L-BPA can get into these cells efficiently. In conclusion, KKU-213A cells, with their high expression of LAT1 and 4F2hc, stand out as potentially having the highest uptake of L-BPA. This means that KKU-213A cells might respond better to BNCT since cancer cells need to be able to take in L-BPA efficiently for therapeutic boron concentrations to reach them. The fact that these transporters are not as highly expressed in KKU-055 cells suggests that these lines may not be able to take in L-BPA as well, which could make BNCT less effective. Moving forward, the accumulation of boron within these CCA cells will be evaluated to corroborate these findings and elucidate the relationship further.

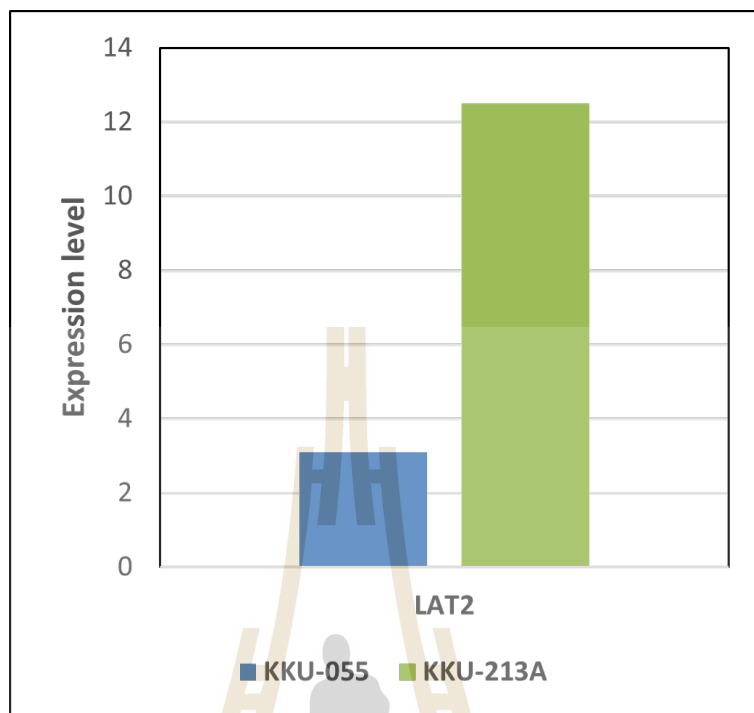


Figure 4.2 The analysis of LAT2 expression levels using RT-PCR in KKU-055 and KKU-213A cells, with the expression levels normalized.

4.3 Cytotoxicity of L-BPA in CCA cells

The viability of two CCA cell lines, KKU-055 and KKU-213A, was quantified after 24-hour treatment with L-BPA-fr at various concentrations. Preliminary data showed in Figure 4.6 that KKU-055 and KKU-213A cells both showed strong viability at the lower range of L-BPA-fr concentrations, 250 ppm and 500 ppm, similar to the untreated control group. On the other hand, when L-BPA-fr was increased to 1000 ppm and 2000 ppm, there was a noticeable decrease in cell viability in both cell lines. However, in comparison to the KKU-055 cells, the KKU-213A cells showed a slightly increased sensitivity to the higher concentrations.

The study outlines the concentration-dependent cytotoxic effects of L-BPA-fr on CCA cells. Consequently, a concentration of 1000 ppm has been chosen for further in vitro experiments, as it maintains over 85% cell viability for the tested cell lines. This decision is underpinned by previous results that indicated a ^{10}B concentration of approximately 51 ppm in the DMEM medium at an L-BPA-fr concentration of 1000 ppm. It is posited that this specific concentration may enhance the cellular uptake of ^{10}B , potentially increasing the efficacy of the BNCT reaction at the cellular level. These

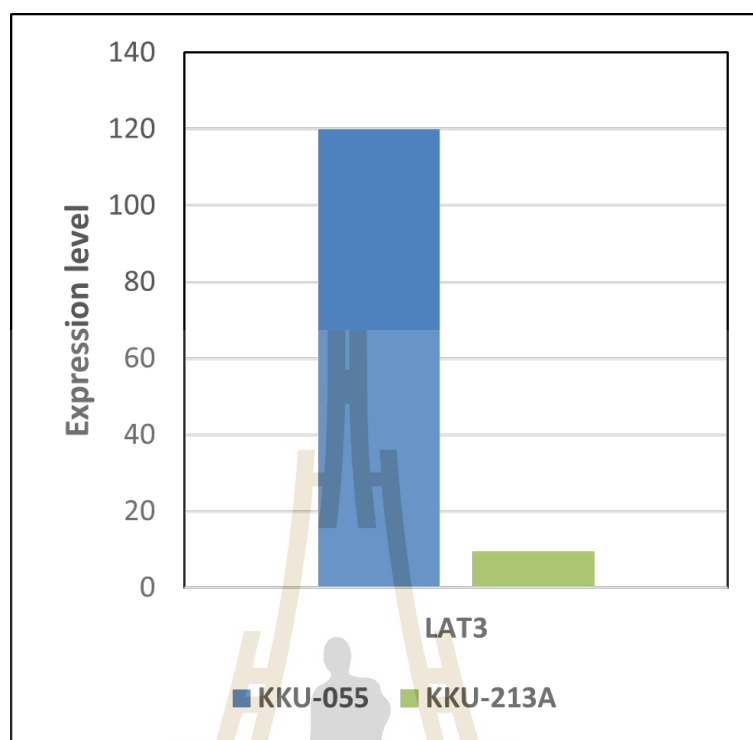


Figure 4.3 The analysis of LAT3 expression levels using RT-PCR in KKU-055 and KKU-213A cells, with the expression levels normalized.

findings are integral to refining boron delivery within BNCT and highlight the critical role of precise concentration management before proceeding to subsequent stages of research.

4.4 Accumulation of ^{10}B within CCA cells

The assay determines the concentration of ^{10}B in KKU-213A and KKU-055, which are cholangiocarcinoma (CCA) cell types, subsequent to the addition of L-BPA. At 24 and 48 hours after treatment, the data are shown for the treated and control groups.

In agreement with previous studies that demonstrated these cells had the highest levels of LAT1 and 4F2hc (CD98) expression, the KKU-213A cells are the ones that take in the most ^{10}B . This shows that the expression levels of particular transporters are correlated with the efficiency of L-BPA uptake, with LAT1 being a key player in this relationship.

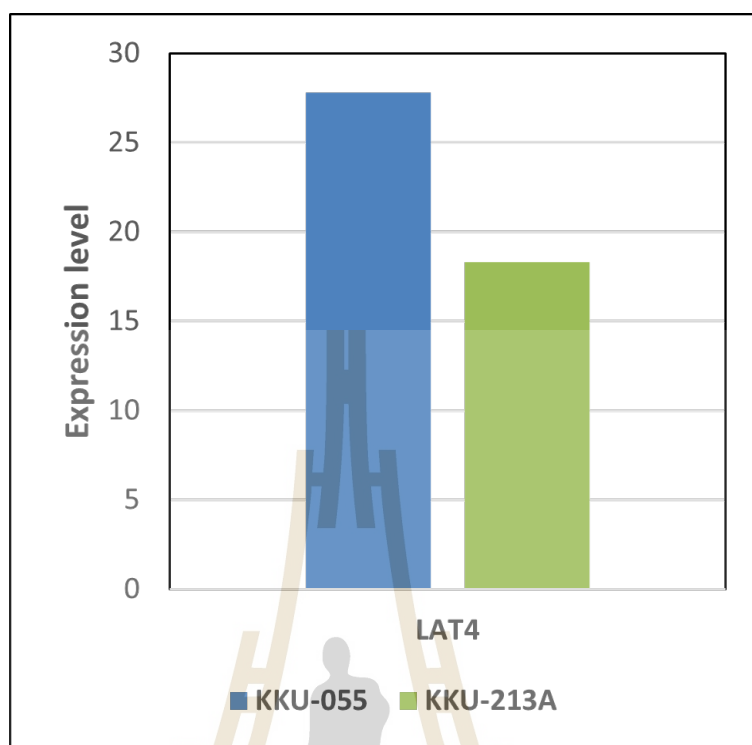


Figure 4.4 The analysis of LAT4 expression levels using RT-PCR in KKU-055 and KKU-213A cells, with the expression levels normalized.

When comparing the treated KKU-213A cells with the control cells, the amount of ^{10}B in the treated cells is significantly higher. This difference is evident after 24 and 48 hours, indicating that transporters are able to absorb L-BPA.

The KKU-055 cells treated with L-BPA accumulated ^{10}B similarly to the KKU-213A cells at the 24-hour time point, but significantly less than the KKU-213A cells at 48 hours. This finding aligns with previous observations of their low LAT1 and 4F2hc levels. Interestingly, even in control cells at both time points, CCA cells contained some ^{10}B , likely produced by cellular mechanisms. However, these levels were insufficient to enhance nuclear reactions and BNCT interactions effectively.

KKU-055 cells were selected for *in vitro* BNCT experiments due to issues with the colony formation assay in KKU-213A cells, which prevented the generation of reliable survival curves. Thus, survival curves derived from KKU-055 cells were expected to provide more accurate and useful data for this study.

The graph in Figure 4.8 illustrates the accumulation of ^{10}B in KKU-055 CCA cells following treatment with L-BPA over different incubation times. The data demonstrate a clear upward trend in ^{10}B concentration with increasing incubation time.

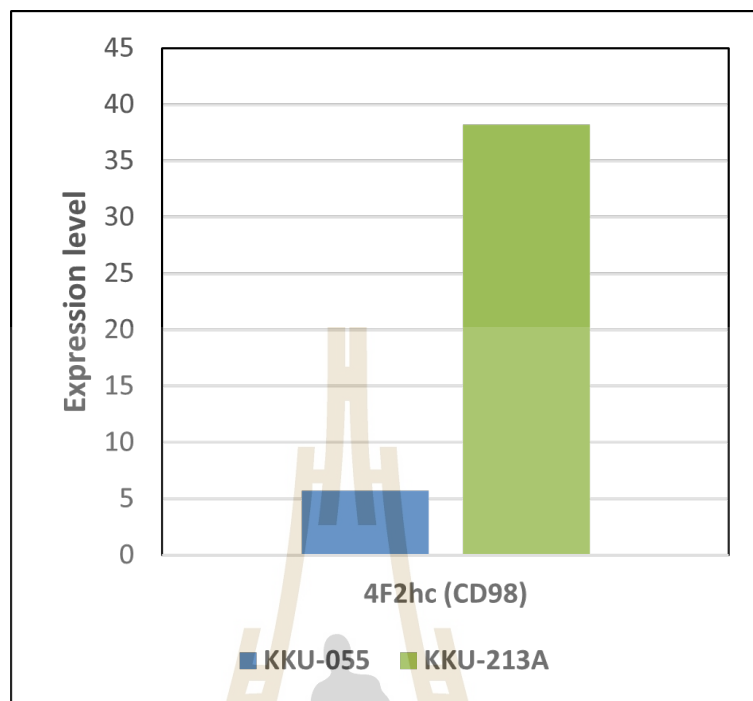


Figure 4.5 The analysis of F2hc (CD98) expression levels using RT-PCR in KKU-055 and KKU-213A cells, with the expression levels normalized.

In the initial hours, the ^{10}B concentration in the cells is relatively low, showing only a slight increase. This suggests that ^{10}B is absorbed gradually during the first few hours. Between 4 and 24 hours, the accumulation of boron increases more noticeably, indicating continued absorption over time. This increase likely reflects ongoing L-BPA treatment, with the cells either enhancing their capacity to absorb boron or approaching their maximum absorption capacity.

At 48 hours, the ^{10}B concentration peaks at $55.67 \text{ ng B}/10^5 \text{ cells}$, more than double the concentration observed at 4 hours. This steady increase suggests that KKU-055 cells can store boron effectively, which is beneficial for BNCT, as higher intracellular boron levels could improve neutron capture and treatment efficacy. Thus, the KKU-055 cell line shows the ability to accumulate ^{10}B over time when treated with L-BPA. This trait is crucial for the success of BNCT, as sufficient intracellular boron levels are necessary for effective therapy. The increasing ^{10}B concentration also suggests that allowing longer incubation times before BNCT could enhance boron uptake in the cells.

To confirm the efficacy of the BNCT experiment conducted in Japan, we assessed the ability of KKU-055 cells to accumulate boron. For this study, the cells were treated with L-BPA-fr containing ^{10}B at concentrations of 0, 5, 10, and $20 \mu\text{g B/}$

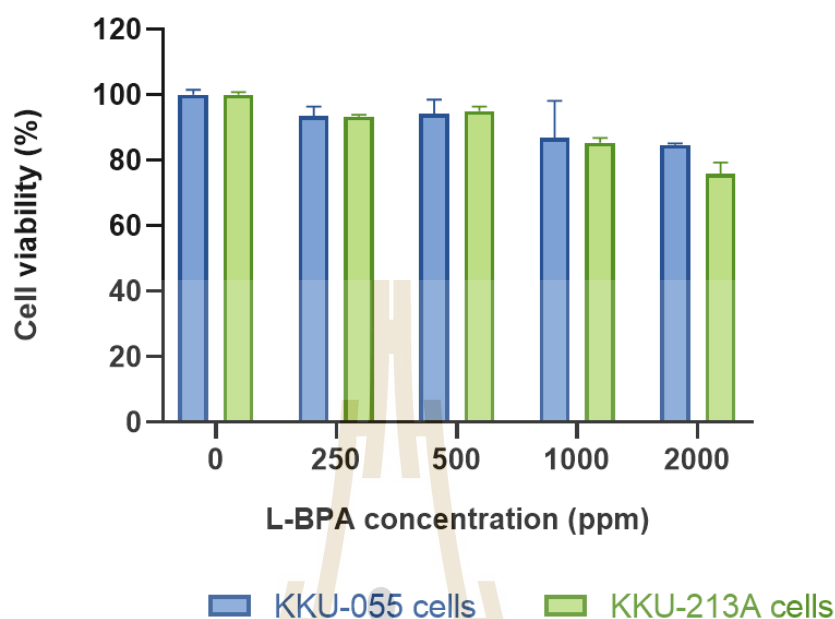


Figure 4.6 Cell viability of KKU-055 and KKU-213A cell lines after 24-hour treatment to varying concentrations of L-BPA-fr. Both cell lines maintained high viability at 250 ppm and 500 ppm, while higher concentrations (1000 ppm and 2000 ppm) resulted in significant cytotoxicity.

mL. The boron concentration within the cells was measured and is presented in the Figure 4.9

At a concentration of 0 $\mu\text{g B/mL}$ (control), the boron concentration within the cells was approximately 100 $\mu\text{g B}/10^9$ cells, establishing a baseline for comparison. When the cells were treated with 5 $\mu\text{g B/mL}$ of B-10, the boron concentration remained similar to the control, indicating minimal uptake at this low concentration. This suggests that the cells do not significantly accumulate boron at lower concentrations of L-BPA-fr.

However, at 10 $\mu\text{g B/mL}$, there was a noticeable increase in boron concentration to approximately 120 $\mu\text{g B}/10^9$ cells. This indicates that the cells begin to uptake more boron at this concentration. The trend continued with the 20 $\mu\text{g B/mL}$ treatment, where the boron concentration further increased to around 140 $\mu\text{g B}/10^9$ cells. This demonstrates a dose-dependent uptake of boron by the cells, with higher concentrations of B-10 leading to higher intracellular boron levels.

Our findings were compared to the previous publication by Huang CY in 2022 (Huang et al., 2022) (Figure 4.10), titled "*Boron Neutron Capture Therapy Eliminates*

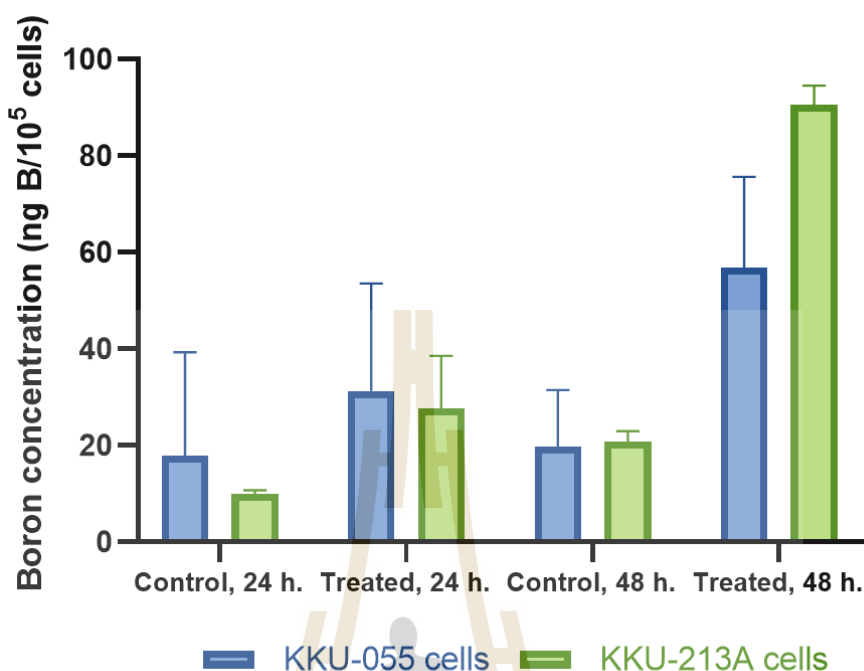


Figure 4.7 The uptake of ^{10}B over 24 and 48 hours in KKU-055 and KKU-213A cells treated with L-BPA. The observed increased ^{10}B concentration within cells over time and the storage of ^{10}B in the control group of both cell types. The error bars representing the standard deviations from three independent experiments

Radioresistant Liver Cancer Cells by Targeting DNA Damage and Repair Responses. According to Huang's study, HepG2 cells, a human hepatocellular carcinoma cell line, were treated with $25 \mu\text{g B/mL}$ for 0, 30, 60, 90, and 120 minutes. At 120 minutes (or 2 hours), the boron concentration in HepG2 cells was about $72 \mu\text{g B}/10^9$ cells. In contrast, our results show that KKU-055 cells accumulated around $140 \mu\text{g B}/10^9$ cells under similar conditions, indicating a significantly higher boron uptake capability.

Nonetheless, the article by Kohei Yoshimura, 2021 (Yoshimura et al., 2021) (Figure 4.11), titled *"Efficacy of Boron Neutron Capture Therapy in Primary Central Nervous System Lymphoma: In Vitro and In Vivo Evaluation,"* examined whether exposure to BPA resulted in boron uptake into the human lymphoma cells, Raji and RL. The cells were cultured in a medium containing 5, 10, and $20 \mu\text{g B/mL}$ of BPA. The results show that the Raji and RL cells have a greater capacity to accumulate boron compared to KKU-055 cells, suggesting variability in boron uptake efficiency among different cell lines.

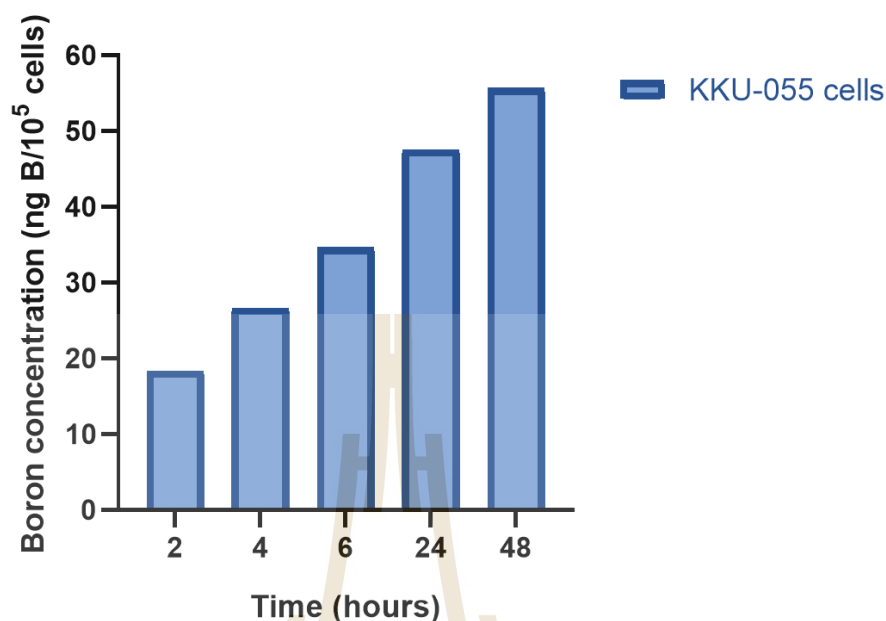


Figure 4.8 The uptake of ^{10}B over time specifically in KKU-055 cells treated with L-BPA. The observation of an increase in ^{10}B concentration within the cells over time with error bars representing the standard deviations from three independent experiments.

In summary, the data show that between 0 and 5 $\mu\text{g B/mL}$, there is minimal change in boron uptake by the KKU-055 cells. However, at higher concentrations of 10 and 20 $\mu\text{g B/mL}$, there is a significant increase in boron accumulation within these cells. This dose-dependent uptake pattern is crucial for optimizing boron concentrations in BNCT experiments, as it suggests that higher concentrations of boron-10 can lead to more effective accumulation within target cells. Comparing these results with previous studies, it is evident that KKU-055 cells have a superior ability to accumulate boron-10 compared to HepG2 cells but not as high as Raji and RL lymphoma cells. This information is vital for improving the efficacy of BNCT in treating cancer cells by ensuring sufficient boron uptake for therapeutic success.

4.5 Plating efficiency of KKU-055 cells after L-BPA uptake

One of the most important metrics in colony formation assays is PE, which measures a single cell's capacity to divide into numerous colonies. This measure is essential for calculating survival curves after treatment. KKU-055 cells were treated with 1000 ppm L-BPA at varying times in the study. Afterward, PE was measured through

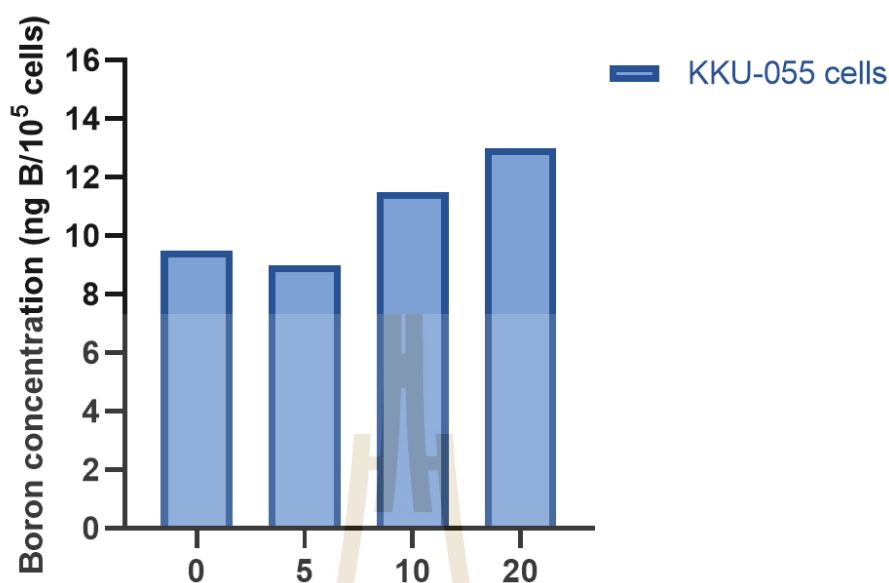


Figure 4.9 Boron concentration in KKU-055 cells treated with varying concentrations of ^{10}B as L-BPA-fr. The cells were exposed to 0, 5, 10, and 20 $\mu\text{g B/mL}$, and the boron concentration within the cells was measured. Results indicate minimal uptake at 5 $\mu\text{g B/mL}$ compared to the control (0 $\mu\text{g B/mL}$), with a significant dose-dependent increase at 10 and 20 $\mu\text{g B/mL}$.

the colony formation assay procedures described in Chapter III. The outcomes of this investigation play an important role in verifying the ability of cells to form colonies, which will allow for the creation of accurate survival curves after BNCT treatment.

The PE values for KKU-055 cell lines after L-BPA treatment over varied incubation times are shown in Table 4.2. According to the data, the PE value reaches a high point of 79% at 0 hours of L-BPA incubation. This suggests that without L-BPA treatment, a significant majority of the cells retain the ability to form colonies. As the cells are incubated with L-BPA, there is an observable decline in plating efficiency at 1 hour to 54%, which slightly recovers at 2 hours to 57% and further to 61% at the repeated 2-hour mark. This variation may be an indication of cellular response mechanisms or adaptation to the boron compound. Nevertheless, PE values drop to 48% at 3 hours and then marginally rise to 51% at 4 hours. These post-treatment variations in PE values indicate that L-BPA damages KKU-055 cells, making it more difficult for them to form colonies, which is required for BNCT for survival. The findings imply that the effects of L-BPA vary with time. Cell viability and colony formation capacity gradually decline with time, but some recovery or resistance to treatment occurs.

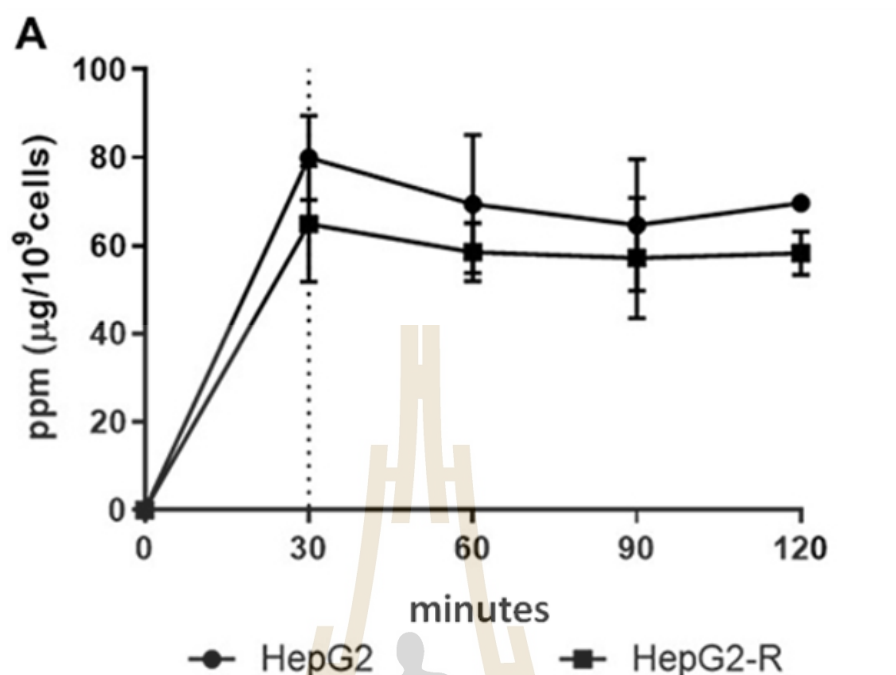


Figure 4.10 Utake of ^{10}B (boric acid) in HepG2 and HepG2-R cells. Cells were treated with $25 \mu\text{g } ^{10}\text{B/mL}$ boric acid (BA). The round dots represent HepG2 cells, and the squares represent HepG2-R cells. (A) Time course of ^{10}B uptake. Cells were treated with $25 \mu\text{g } ^{10}\text{B/mL}$ BA for 0, 30, 60, 90, and 120 minutes (Huang et al., 2022).

In summary, PE values after L-BPA treatment indicate that KKKU-055 cells can form colonies large enough to create a survival curve for BNCT, but this becomes more challenging as the length of the L-BPA incubation period increases. The initial high PE value suggests a time frame of opportunity for BNCT application before an apparent decrease in cell viability takes place. Figuring out how PE changes in response to L-BPA is important for figuring out the best time and amount of BNCT to use to get the desired therapeutic effect while keeping the cells' ability to form colonies.

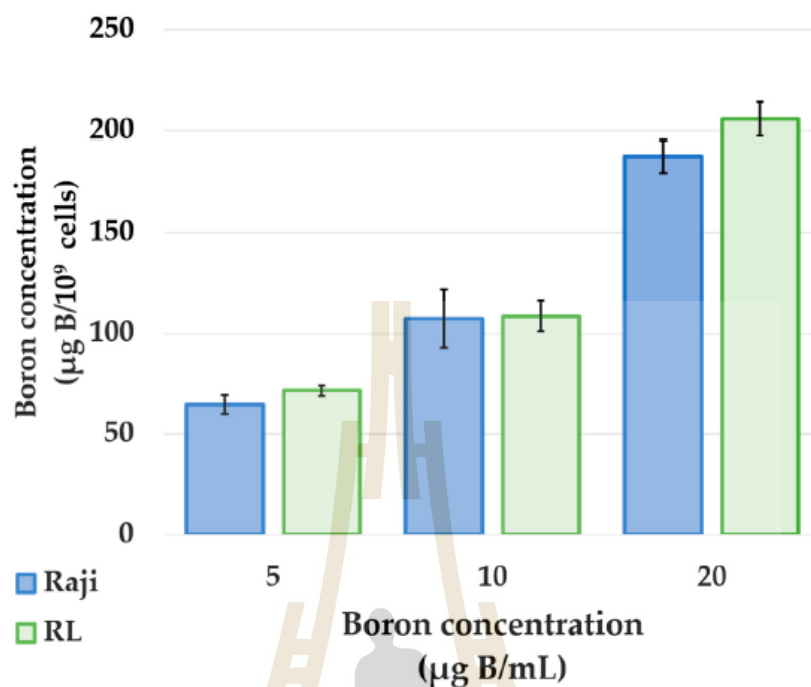


Figure 4.11 Cellular uptake of boron in human lymphoma cells. Boron concentrations of Raji (blue) and RL (green) lymphoma cells in RPMI 1640, using 5, 10, and 20 $\mu\text{g B/mL}$ of BPA for 3 hours. The boron concentration in lymphoma cells was notably high for both Raji and RL (Yoshimura et al., 2021).

Table 4.2 Plating efficiency (PE) of KKV-055 cells after 2 weeks of incubation with L-BPA for various durations.

Boron uptake duration time	PE value
0 h	79%
1 h	54%
2 h	57%
2 h	61%
3 h	48%
4 h	51%

4.6 X-rays curve

In this study, we focused on evaluating the radiosensitivity of the KKU-055 cell line to 6 MV X-rays. Colony formation assays were performed after radiation to determine the number of surviving cell colonies. The survival fractions were then computed using the data from these assays and the linear-quadratic model, a standard radiobiological model that is explained in Chapter II.

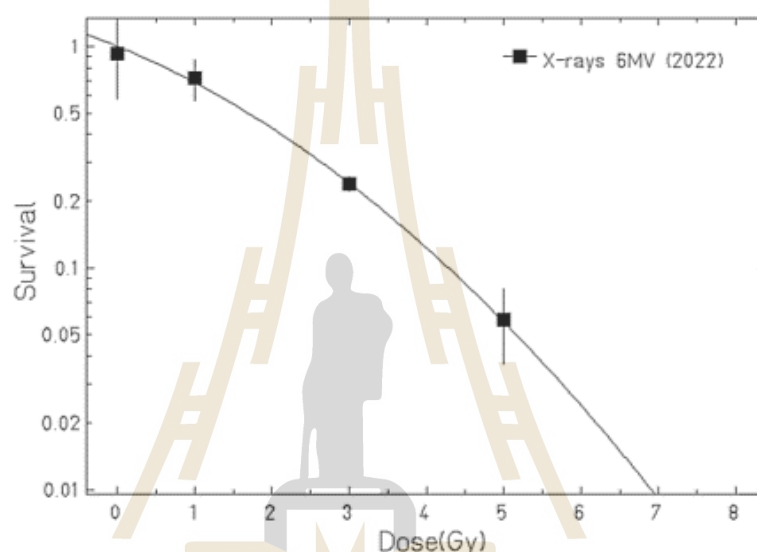


Figure 4.12 Survival curves of KKU-055 cells post-X-ray irradiation are shown, depicting cell survival fraction following exposure to 6 MV X-ray beams. Error bars denote standard deviations, calculated from three sample replicates. This data offers insights into the response of KKU-055 cells to X-ray radiation.

The survival curve, as shown in Figure 4.12 generated using the GD server at GSI, depicts a dose-dependent reduction in the survival of KKU-055 cells. This curve indicates that as X-ray radiation doses increase, the survival rate of KKU-055 cells declines. This pattern is consistent with expected radiobiological trends. The reproducibility and dependability of our experimental results are highlighted by the error bars in the curve.

The significance of this survival curve in our study is fundamental. It provides a comprehensive understanding of the response of KKU-055 cells to 6 MV X-ray exposure and provides a key reference point for comparisons with other radiation treatments in the future, including BNCT.

CHAPTER V

CONCLUSION

CCA is a prominent cause of death in Northeast Thailand, and its early detection poses difficulties, resulting in delayed diagnoses and restricted treatment choices. The combination of radical surgery and radiotherapy is a commonly used method to remove any remaining tumors and prevent the spread of cancer to other parts of the body. However, the current treatment protocols are not very effective. BNCT has emerged as a promising alternative in this context, specifically for the treatment of unresectable tumors. Although there is potential, the complete disclosure of the translation of recent BNCT clinical trials in Thailand has not been provided. This calls for additional research to improve treatment plans and enhance the clinical application.

The objective of this study was to assess the absorption of L-BPA in CCA cells and examine the presence of L-BPA receptors using RT-PCR. In order to collect crucial radiobiological data for BNCT, we conducted a comparison of the biological reactions of CCA cell lines to both X-ray and neutron irradiation. The results of our study demonstrate that the cytotoxicity of L-BPA-fr on CCA cell lines KKU-055 and KKU-213A is dependent on its concentration. At lower concentrations of 250 ppm and 500 ppm, both cell lines exhibited a high level of viability, comparable to the untreated control group. Nevertheless, when the concentrations reached 1000 ppm and 2000 ppm, there was a notable decrease in cell viability, particularly in KKU-213A cells which displayed heightened sensitivity. This indicates that although low levels of L-BPA-fr are not harmful, higher levels can be toxic to cells, highlighting the need to improve the delivery of boron.

After analyzing these observations, a concentration of 1000 ppm L-BPA-fr was chosen for further studies because it ensured that cell viability remained above 85% for both cell lines and resulted in an optimal ^{10}B presence of approximately 51 ppm in the medium. The hypothesis is that this concentration will increase the uptake of ^{10}B inside cells, which is essential for maximizing the effectiveness of BNCT. The study demonstrated that the uptake of L-BPA by CCA cell lines varied depending on the expression of transporters. KKU-213A cells, which exhibit elevated levels of LAT1 and 4F2hc (CD98) transporters, exhibited the greatest capacity for L-BPA uptake, suggesting a

more favorable response to BNCT. On the other hand, K KU-055 cells, which have lower levels of transporter expression, exhibited less effective boron uptake. This indicates that there may be differences in the outcomes of BNCT depending on the levels of transporters.

Our study on the process of boron absorption showed that K KU-055 cells experience a gradual rise in ^{10}B levels over time when exposed to L-BPA, which is essential for the success of BNCT. Nevertheless, the efficiency of their colony formation decreased as the L-BPA incubation time increased, suggesting that there is a specific timeframe during which BNCT application can be optimized to achieve maximum therapeutic effects while maintaining cell viability. The notable augmentation in DNA damage observed in K KU-055 cells treated with L-BPA and exposed to BNCT, especially at higher doses, emphasizes the therapy's potential effectiveness. Prolonged exposure to radiation, along with the use of boron-10 treatment, was discovered to increase the deadly impact of BNCT on K KU-055 cells. This finding offers valuable knowledge for improving the effectiveness of BNCT treatment protocols.

In addition, our study emphasized the specific mechanisms through which X-ray irradiation and BNCT cause cytotoxic effects. While X-ray irradiation directly induces cell death, causing an immediate decrease in survival fraction, BNCT causes a greater extent of DNA damage, which may lead to delayed or indirect cytotoxic effects. Understanding the interaction between these modalities and cells, particularly when a boron carrier such as L-BPA is present, is of utmost importance.

We determined the RBE for different radiation types by using the linear quadratic equation to fit survival curves and calculating the alpha and beta parameters. The RBE for BNCT at the KUR was determined to be 3.466, demonstrating superior effectiveness in comparison to conventional X-rays. The data is crucial for improving BNCT treatment parameters, such as reactor power and irradiation time, in order to achieve the best therapeutic results.

To summarize, this study shows that BNCT can be used effectively for treating CCA and offers important information on how to improve the delivery of boron and the parameters for irradiation. The varying absorption of L-BPA by CCA cell lines, which is affected by the levels of transporter expression, requires the use of specific strategies in the planning of BNCT treatment. These findings highlight the potential of BNCT as a practical treatment method for CCA, which can lead to further research and clinical use. The study's survival curves and radiobiological data will provide valuable references

for current and future research in BNCT, thereby contributing to the progress of cancer therapy.

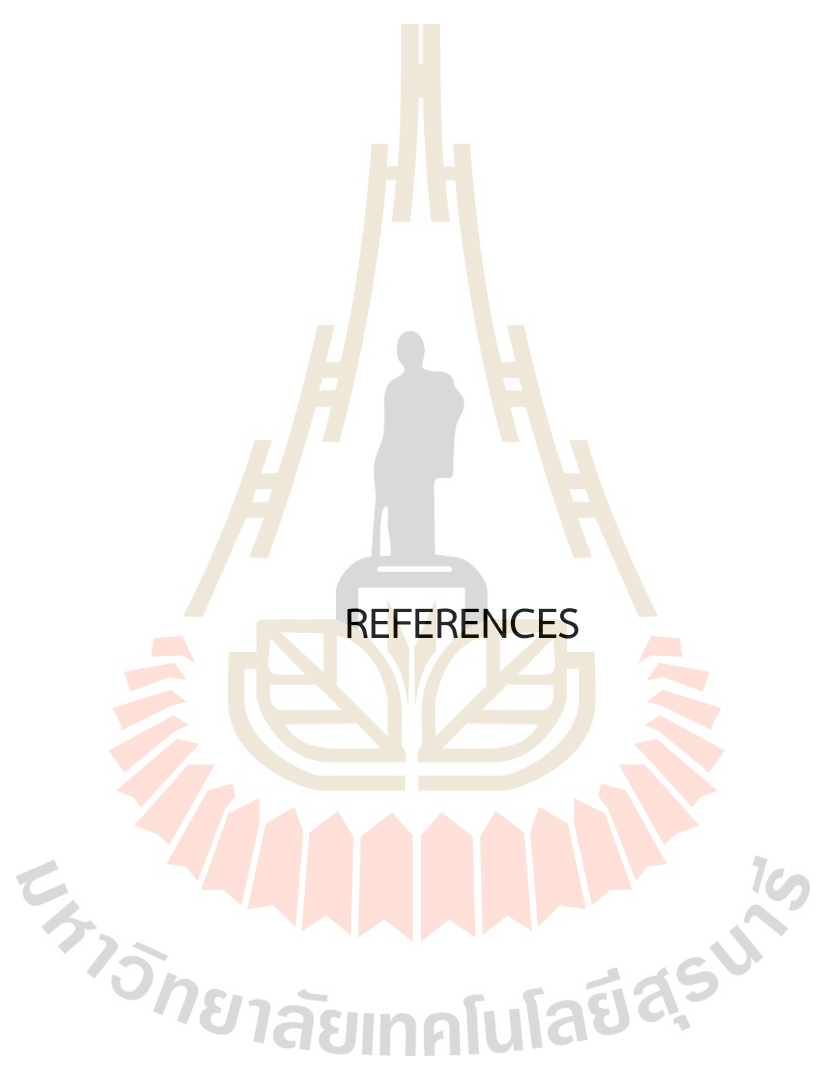
As shown in my report on the RBE of BNCT with CCA cells, BNCT has a lot of potential to damage CCA cells. This supports its use as a clinical treatment for CCA, but there are some issues and suggestions for making the protocol better.

Initially, we suggest performing an identical procedure with a negative control, such as normal bile duct or fibroblast cell lines, followed by a thorough comparison of the outcomes. This comparison is essential to ascertain the specificity and selectivity of BNCT for CCA cells as opposed to normal cells. It will be easier to improve the treatment plan and reduce the possibility of adverse effects on healthy tissues if the differences between the effects on malignant and healthy cells are understood.

Due to the constraints in the quality of the beam, it is essential to accurately simulate the dosage. This entails utilizing computational models to predict the dispersion of dosage and its physiological impacts on the cells. Accurate dose simulation ensures that the intended therapeutic dose is delivered effectively while sparing surrounding healthy tissues, thus guaranteeing successful treatment outcomes.

In our future work, we intend to optimize the irradiation setup to improve the accuracy and effectiveness of BNCT. This optimization may involve modifying the neutron beam parameters, enhancing the delivery mechanism of boron-containing compounds, and refining the irradiation geometry to maximize the therapeutic impact.

Moreover, we aim to advance to the next step by studying a 3D in vitro CCA cell model. This model would offer an improved representation of the tumor microenvironment in contrast to conventional 2D cell cultures. Utilizing a 3D model enables more precise simulation of the in vivo circumstances, yielding more accurate observations regarding the impact of BNCT on tumor development and advancement. This approach also aids in diminishing the dependence on animal models, following ethical considerations and the principles of the 3Rs (Replacement, Reduction, and Refinement) in animal research.

The logo of Sakon Nakhon Rajabhat University is a large, faint watermark in the background. It features a central figure of a person standing on a pedestal, surrounded by a circular arrangement of red and orange geometric shapes. The entire logo is set against a light beige background.

REFERENCES

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REFERENCES

- Axion BioSystems, I. (2023). Clonogenic assay. Accessed: 2023-06-10.
- Barth, R. F., Coderre, J. A., Vicente, M. G. H., and Blue, T. E. (2005). Boron neutron capture therapy of cancer: current status and future prospects. *Clinical Cancer Research*, 11(11), 3987–4002.
- Bokemeyer, A., Matern, P., Bettenworth, D., Cordes, F., Nowacki, T. M., Heinzow, H., Kabar, I., Schmidt, H., Ullerich, H., and Lenze, F. (2019). Endoscopic radiofrequency ablation prolongs survival of patients with unresectable hilar cholangiocellular carcinoma-a case-control study. *Scientific Reports*, 9(1), 1–8.
- Cancer Research UK (2020). Cancer cells. [Online]. Retrieved from <https://www.cancerresearchuk.org/about-cancer/what-is-cancer/how-cancer-starts/cancer-cells>.
- Cente, G. U. H. I. M. (2013). What are heavy ions? Retrieved from <https://heavy-ion.showa.gunma-u.ac.jp/otherlang/page.php?id=8>.
- Chadwick, J. (1932). Possible existence of a neutron. *Nature*, 129(3252), 312–312.
- Coderre, J. A., Glass, J. D., Fairchild, R. G., Micca, P. L., Fand, I., and Joel, D. D. (1990). Selective delivery of boron by the melanin precursor analogue p-boronophenylalanine to tumors other than melanoma. *Cancer research*, 50(1), 138–141.
- Coderre, J. A., Joel, D. D., Micca, P. L., Nawrocky, M. M., and Slatkin, D. N. (1992). Control of intracerebral gliosarcomas in rats by boron neutron capture therapy with p-boronophenylalanine. *Radiation research*, 129(3), 290–296.
- Das, A. and Ferbel, T. (2003). *Introduction to nuclear and particle physics (2nd ed.)*. World Scientific Publishing Co. Pte. Ltd, Singapore.
- Faculty of Medicine Siriraj Hospital, M. U. (2019). Faculty of medicine, 4 institutions join forces to propose "the correct cancer fighting policy to reduce the incidence

reduce disability and death from cancer in thailand ”to scrutinize as the agenda of the national health assembly. Retrieved from <https://www.si.mahidol.ac.th/th/hotnewsdetail>.

Gatto, M., Bragazzi, M. C., Semeraro, R., Napoli, C., Gentile, R., Torrice, A., Gaudio, E., and Alvaro, D. (2010). Cholangiocarcinoma: update and future perspectives. *Digestive and Liver Disease*, 42(4), 253–260.

Hatanaka, H. (1975). A revised boron-neutron capture therapy for malignant brain tumors. *Journal of neurology*, 209(2), 81–94.

Huang, C.-Y., Lai, Z.-Y., Hsu, T.-J., Chou, F.-I., Liu, H.-M., and Chuang, Y.-J. (2022). Boron neutron capture therapy eliminates radioresistant liver cancer cells by targeting dna damage and repair responses. *Journal of Hepatocellular Carcinoma*, pages 1385–1401.

IAEA (2011). Endf/b-vii.1 incident-neutron data. Retrieved from <https://t2.lanl.gov/nis/data/endl/endlvii.1-n.html>.

Im, J. H., Seong, J., Lee, I. J., Park, J. S., Yoon, D. S., Kim, K. S., Lee, W. J., and Park, K. R. (2016). Surgery alone versus surgery followed by chemotherapy and radiotherapy in resected extrahepatic bile duct cancer: treatment outcome analysis of 336 patients. *Cancer Research and Treatment: Official Journal of Korean Cancer Association*, 48(2), 583–595.

Institute, N. C. (2021). What is cancer?. Retrieved from <https://www.cancer.gov/about-cancer/understanding/what-is-cancer>.

Kellert, M. and Hey-Hawkins, E. (2020). Basic requirement of bnct. Retrieved from <https://dgbnct.com/index.php/basic-requirements-of-bnct-bsh-and-bpa/>.

Kim, T.-Y., Yang, S. H., Lee, S. H., Park, Y.-S., Im, Y. H., Kang, W.-K., Ha, S. H., Park, C. I., Heo, D. S., Bang, Y.-J., et al. (2002). A phase iii randomized trial of combined chemoradiotherapy versus radiotherapy alone in locally advanced non–small-cell lung cancer. *American journal of clinical oncology*, 25(3), 238–243.

Kumar, V. and Mazumder, A. (2023). Detection of chemical warfare agents with chemical sensors.

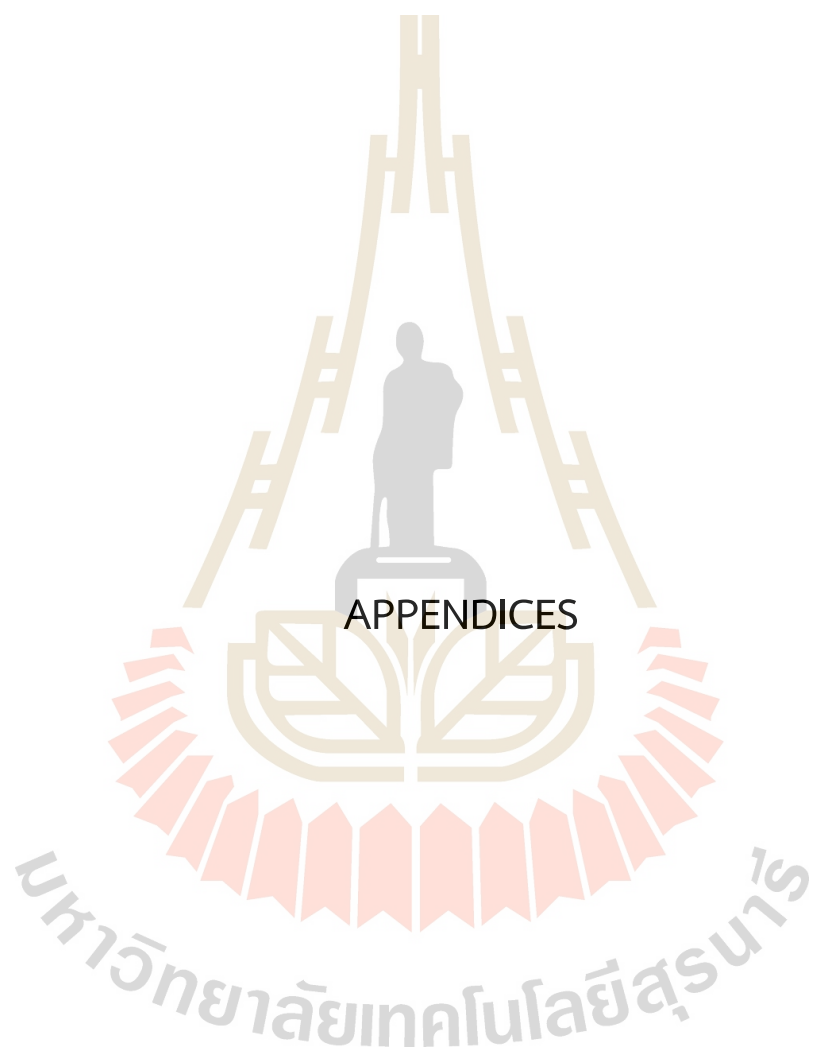
- Linko, S., Revitzer, H., Zilliacus, R., Kortensniemi, M., Kouri, M., and Savolainen, S. (2008). Boron detection from blood samples by icp-aes and icp-ms during boron neutron capture therapy. *Scandinavian journal of clinical and laboratory investigation*, 68(8), 696–702.
- Locher, G. (1936). Ab-x nct. *Am. J. Roentgenol*, 361–13.
- Mahadevan, A., Dagoglu, N., Mancias, J., Raven, K., Khwaja, K., Tseng, J. F., Ng, K., Enzinger, P., Miksad, R., Bullock, A., et al. (2015). Stereotactic body radiotherapy (sbrt) for intrahepatic and hilar cholangiocarcinoma. *Journal of Cancer*, 6(11), 1099.
- Marforio, T. D., Carboni, A., and Calvaresi, M. (2023). In vivo application of carboranes for boron neutron capture therapy (bnct): Structure, formulation and analytical methods for detection. *Cancers*, 15(20), 4944.
- Massachusetts Institute of Technology (2004). Principles of radiation interactions. <https://ocw.mit.edu/courses/22-55j-principles-of-radiation-interactions-fall-2004/pages/lecture-notes/>.
- MORI, Y., SUZUKI, A., YOSHINO, K., and KAKIHANA, H. (1989). Complex formation of p-boronophenylalanine with some monosaccharides. *Pigment Cell Research*, 2(4), 273–77.
- National Institutes of Biomedical Innovation, H. and Nutrition (2024). Jcrb cell bank (japanese collection of research bioresources cell bank). Accessed: 2023-08-03.
- Nedunchezian, K., Aswath, N., Thiruppathy, M., and Thirugnanamurthy, S. (2016). Boron neutron capture therapy-a literature review. *Journal of clinical and diagnostic research: JCDR*, 10(12), ZE01.
- News, T. M. (2019). Japanese researchers discover new biomarker for bile duct cancer. Retrieved from <https://www.thailandmedical.news/news/japanese-researchers-discover-new-biomarker-for-bile-duct-cancer>.
- Nishitani, T., Yoshihashi, S., Tanagami, Y., Tsuchida, K., Honda, S., Yamazaki, A., Watanabe, K., Kiyonagi, Y., and Uritani, A. (2022). Neutronics analyses of the radiation field at the accelerator-based neutron source of nagoya university for the bnct study. *Journal of Nuclear Engineering*, 3(3), 222-232.

- Park, S. H. and Kang, J. O. (2011). Basics of particle therapy i: physics. *Radiation oncology journal*, 29(3), 135.
- Podgorsak, E. e. (2005). *Radiation oncology physics: A handbook for teachers and students*. International Atomic Energy Agency (IAEA), Vienna.
- Puris, E., Gynther, M., Auriola, S., and Huttunen, K. M. (2020). L-type amino acid transporter 1 as a target for drug delivery. *Pharmaceutical Research*, 37, 1–17.
- Record, M. and Databased Cancer Unit, M. D. D. N. C. I. (2021). *HOSPITAL-BASED CANCER REGISTRY 2020*. INTERNATIONAL ATOMIC ENERGY AGENCY, Rama VI Road, Ratchathewi District Bangkok 10400 Tel. +66 2202 6888 ext. 2700, 2416, Fax. +66 2202 6888 ext. 2704.
- Sakurai, Y. and Kobayashi, T. (2004). Spectrum evaluation at the filter-modified neutron irradiation field for neutron capture therapy in kyoto university research reactor. *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment*, 531(3), 585–595.
- Sato, E., Zaboronok, A., Yamamoto, T., Nakai, K., Taskaev, S., Volkova, O., Mechetina, L., Taranin, A., Kanygin, V., Isobe, T., et al. (2018). Radiobiological response of u251mg, cho-k1 and v79 cell lines to accelerator-based boron neutron capture therapy. *Journal of radiation research*, 59(2), 101–107.
- Schardt, D., Elsässer, T., and Schulz-Ertner, D. (2010a). Heavy-ion tumor therapy: Physical and radiobiological benefits. *Reviews of Modern Physics*, 82(1), 383–425.
- Schardt, D., Elsässer, T., and Schulz-Ertner, D. (2010b). Heavy-ion tumor therapy: Physical and radiobiological benefits. *Reviews of Modern Physics*, 82(1), 383–425.
- Skowronek, J. and Zwierzchowski, G. (2017). Brachytherapy in the treatment of bile duct cancer—a tough challenge. *Journal of Contemporary Brachytherapy*, 9(2), 187–195.
- Society, A. C. (2018). What is bile duct cancer?. Retrieved from <https://www.cancer.org/cancer/bile-duct-cancer/about/what-is-bile-duct-cancer.html>.
- Sripa, B. and Pairojkul, C. (2008). Cholangiocarcinoma: lessons from thailand. *Current opinion in gastroenterology*, 24(3), 349.

- Suzuki, M. (2020). Boron neutron capture therapy (bnct): A unique role in radiotherapy with a view to entering the accelerator-based bnct era. *International journal of clinical oncology*, 25(1), 43–50.
- Tanaka, H., Sakurai, Y., Suzuki, M., Masunaga, S.-i., Takamiya, K., Maruhashi, A., and Ono, K. (2014). Development of a simple and rapid method of precisely identifying the position of ^{10}B atoms in tissue: an improvement in standard alpha autoradiography. *Journal of radiation research*, 55(2), 373–380.
- Taylor, H. and Goldhaber, M. (1935). Detection of nuclear disintegration in a photographic emulsion. *Nature*, 135(3409), 341–341.
- Todoroki, T., Kawamoto, T., Koike, N., Fukao, K., Shoda, J., and Takahashi, H. (2001). Treatment strategy for patients with middle and lower third bile duct cancer. *Journal of British Surgery*, 88(3), 364–370.
- Triatmoko, I. M. and Sutjipto, S. (2017). Assessment of analytical instrumentation for boron measurement in bnct system. *Indonesian Journal of Physics and Nuclear Applications*, 2(1), 20–33.
- Ustundag, Y. and Bayraktar, Y. (2008). Cholangiocarcinoma: a compact review of the literature. *World journal of gastroenterology: WJG*, 14(42), 6458.
- Viegas, A. M. D., Postuma, I., Bortolussi, S., Guidi, C., Riback, J. S., Provenzano, L., Marcaccio, B., Rossini, A. E., Ferrari, C., Cansolino, L., et al. (2021). Detailed dosimetry calculation for in-vitro experiments and its impact on on clinical bnct. *Physica Medica*, 89, 282–292.
- Weyrather W. K., Ritter S., S. M. K. G. (1999). Rbe for carbon track-segment irradiation in cell lines of differing repair capacity. *International journal of radiation biology*, 75(11), 1357–1364.
- World Health Organization (2021). Cancer tomorrow. Retrieved from <https://gco.iarc.fr/tomorrow/en>.
- Yoshimura, K., Kawabata, S., Kashiwagi, H., Fukuo, Y., Takeuchi, K., Futamura, G., Hiramatsu, R., Takata, T., Tanaka, H., Watanabe, T., et al. (2021). Efficacy of boron neutron capture therapy in primary central nervous system lymphoma: In vitro and in vivo evaluation. *Cells*, 10(12), 3398.

Yue, A., Anderson, E., Dewey, M., Gilliam, D., Greene, G., Laptev, A., Nico, J., and Snow, W. M. (2018). Precision determination of absolute neutron flux. *Metrologia*, 55(4), 460.





APPENDIX A

CELL CULTURES PROTOCOL

A.1 KKU-055 and KKU-213A Cells culture protocols

The following protocols have been adjusted and modified in some parts according to the GSI Biophysics protocol that was summed up by D Kartini.

A.1.1 Materials

- Medium: DMEM with 10% Fetal Bovine serum (FBS) and 1% penicillin/streptomycin.
- Trypsin: Trypsin-EDTA 0.25%
- Phosphate Buffered Saline (PBS)
- Matrigel (Corning)
- Dispase (Corning)
- Ethanol 70% or 80%
- Tissue culture flask (TCF): T25
- 96-well plate with a flat bottom
- Conical tube: 15 mL and 50 mL
- Microtube: 1.5 mL
- Pipette boy
- Serological pipette: 5 mL, 10 mL, and 25 mL
- Pipette tip: 1000 μ L, 200 μ L, and 20 μ L

A.1.2 Monolayer cell culture

1. Retrieve the vial of cells from storage and let it thaw at room temperature.
2. Prepare one T25 flask by adding 5 mL of culture medium.
3. After the cells have thawed, transfer 1000 μ L of the cell suspension into a 15 mL tube and centrifuge at 1200 rpm for 3 minutes.
4. For resuspending the cells, first discard the old medium and gently dislodge the cell pellet. Then, add 1 mL of fresh medium to the cells.
5. Introduce the entire resuspended cell mixture into the prepared T25 flask.
6. Incubate the flask for 1 to 2 days, keeping an eye out for any floating cells. If floating cells are present, proceed to change the medium.
7. Once the cells have achieved adequate confluency, transfer them to a new T25 flask to continue their growth.

A.2 Plating efficiency (PE) test

1. Prepare 10 T25 flasks and fill each petri dish with 5 mL of appropriate medium.
2. Trypsinize the cells and accurately count the cell number.
3. Seed desired cell numbers (100, 500, or 1000 cells) into each petri dish, adjusting the cell suspension concentration if necessary.
4. Incubate the seeded cells for 9 days without disturbing the petri dishes, maintaining appropriate incubation conditions (temperature, CO₂ levels, etc.).
5. Remove the medium from each petri dish and fix the colonies by adding 100% methanol. Place the dishes in a refrigerator at -4°C for 10 minutes.
6. Remove the methanol and stain the colonies using a solution composed of 0.5% Crystal Violet and 100% Methanol. Freeze the dishes for 30 minutes to enhance staining.
7. Count colonies with more than 50 cells using a stereo microscope, adhering to predefined criteria for colony identification.

A.3 Sulforhodamine B (SRB) assay

Cell viability was determined using a sulforhodamine B (SRB) assay, which measures cell density based on cellular protein content. This assay was performed according to the method described by Voigt, with slight modifications. CCA cell lines were seeded at a density of 50,000 cells/well in 96-well plates for 24 hours, followed by treatment with specified concentrations of L-BPA. After 24 or 48 hours, the media was discarded, and the cells were fixed with 10% TCA (v/v) and stained with SRB dye.

A.3.1 SRB Procedure

1. Gently remove the growth medium by flipping the culture plate (be cautious; medium may contain potentially toxic drugs).
2. Remove any residual medium using a sterile piece of gauze while keeping the culture plate upside down.
3. Fix the cellular protein by adding 100 μL /well of 10% TCA (4°C), and store the plates at 4°C for at least 2 hours or overnight prior to analysis.
4. Rinse the TCA using an automated 96-well plate washer with five washing cycles using deionized water (200 μL /well).
5. Sharply flick the plates over a sink and remove the residual wash solution with a piece of gauze.
6. Pipette 100 μL of SRB solution into each well of the culture plate using a multi-channel pipette. Allow a 30-minute staining period.
7. Remove the SRB from the culture plates using an automated plate washer with five washing cycles using 1% acetic acid (ensure the automated plate washer is prefilled with the appropriate wash solution).
8. Flick the culture plates over a sink and remove the residual wash solution with a piece of gauze.
9. Air-dry the culture plates until no further moisture is visible. (When air-dried, both TCA-fixed and SRB-stained culture plates can be stored indefinitely. Tris-solubilized SRB is also stable for extended periods, provided evaporation does not occur.)

10. Solubilize the protein-bound dye with 100 μL of 10 mM Tris base per well.
11. Shake the plates for at least 10 minutes on a gyratory shaker to homogenize the dye solution.
12. Measure the OD using an automated 96-well plate reader (ELISA reader) at a wavelength of 564 nm.

A.4 Intracellular staining for confocal analysis

1. Cell Seeding:

- CCA cells (3×10^4 cells/ml) were grown on an 8-well slide chamber culture (5×10^4 cells for a 4-well slide).

2. Fixation and Washing:

- After one day of cultivation, the adhering cells were washed once with 1x PBS, fixed with 100 μL of 4% formaldehyde in PBS (pH 7.4) for 15 minutes at room temperature, and washed with 200 μL of PBS once.

3. Permeabilization:

- The fixed cells were treated with 200 μL of permeabilizing buffer (0.1% Triton X-100 in PBS, PBST, pH 7.4) for 10 minutes on ice.

4. Blocking:

- After removing the permeabilizing buffer and washing with 200 μL of PBS three times, the fixed cells were blocked in 100 μL of 2% BSA in 0.1% PBST for 30 minutes on ice.

5. Primary Antibody Staining:

- The cells were stained by adding 100 μL of permeabilizing buffer containing primary antibodies at a final concentration of 20 $\mu\text{g/ml}$, overnight at 4°C.
 - Rabbit polyclonal Anti-Flag tag (1:100)
 - Rabbit monoclonal Anti-TCF4 (1:100)

6. Secondary Antibody Staining:

- After overnight incubation, the cells were washed three times with 200 μ l of 0.1% PBST and then incubated on ice for another 30 minutes with 100 μ l of secondary antibody in 0.1% PBST in a dark area.
 - Alexa Fluor 488-conjugated anti-Rabbit IgG (CST#4412S) 1:500
 - Alexa Fluor 647-conjugated anti-Mouse IgG (CST#4410S) 1:500
- The cells were washed three times with 0.1% PBST.

7. DAPI Staining:

- 100 μ l of 300 nM DAPI staining solution (in PBS) was added to cover the cells and incubated for 3 minutes in the dark. The cells were then washed three times with 0.1% PBST.

8. Mounting:

- After washing, the stained cells on coverslips were mounted with 50% glycerol in PBS (pH 7.2) on glass slides and sealed with nail polish.

9. Confocal Microscopy Analysis:

- The cells were analyzed using a confocal laser scanning microscope.

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